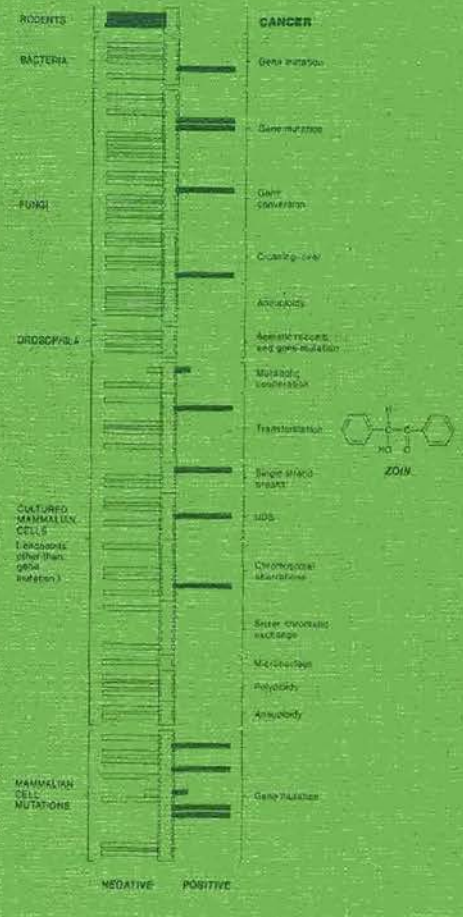
PROGRESS IN MUTATION RESEARCH VOLUME 5

Evaluation of Short-Term Tests for Carcinogens

Report of the International Programme on Chemical Safety's Collaborative Study on In Vitro Assays

Edited by

John Ashby, Frederick J. de Serres, Morrell Draper, Motoi Ishidate Jr., Barry H. Margolin, Bernard E. Matter and Michael D. Shelby



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VOLUME 5

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VOLUME 5

EVALUATION OF SHORT-TERM TESTS FOR CARCINOGENS

REPORT OF THE INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY'S COLLABORATIVE STUDY ON IN VITRO ASSAYS

Published on behalf of the International Programme on Chemical Safety (a collaborative programme of the World Health Organization, the International Labour Organisation and the United Nations Environmental Programme)

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edited by

John Ashby [1], Frederick J. de Serres [2], Morrell Draper [3], Motoi Ishidate Jr. [4], Barry H. Margolin [5], Bernard E. Matter [6] and Michael D. Shelby [7]

[1] Genetic Toxicology Section, Imperial Chemical Industries, Ltd., Alderley Park, Cheshire (Great Britain), [2] Office of the Director, National Institute of Environmental Health Sciences, Research Triangle Park, NC (U.S.A.), [3] International Programme on Chemical Safety, World Health Organization, Geneva (Switzerland), [4] Division of Mutagenesis, National Institute of Hygienic Sciences, Tokyo (Japan), [5] Biometry and Risk Assessment Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC (U.S.A.), [6] Preclinical Research Toxicology, Sandoz Ltd., Basle (Switzerland) and [7] National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC (U.S.A.)

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Preface

The International Programme on Chemical Safety (IPCS) formally came into being in 1980 as a truly cooperative global venture of the United Nations Environment Programme (UNEP), the International Labour Organisation (ILO) and the World Health Organization (WHO). The programme's principal objectives are: to carry out and disseminate evaluations of the risk to human health and the environment from exposure to chemicals based on existing information and data; to encourage the use and improvement, and in areas of controversy the validation of methods for laboratory testing and epidemiological studies that are suitable for health risk evaluations, and propose appropriate methods for assessing health risks, hazards, benefits and exposure; to promote effective international cooperation with respect to emergencies and accidents involving chemicals; and to promote training of the manpower needed for testing and evaluating the health and environmental effects of chemicals, and for the regulating and other control of chemical hazards.

In the fields of genetics and molecular biology from which the science of mutagenesis has evolved and which now have assumed great importance for the understanding of carcinogenesis, scientists have shown great interest and ingenuity in adapting the particular biological systems they use for their researches to assay systems of possible general use. Unfortunately, what can be a powerful and flexible tool in the hands of an experienced researcher cannot easily be transormed into the somewhat inflexible procedure that is necessary for a test system for routine use throughout the world. The plethera of short-term tests, all claiming some promise for revealing mutagenic or carcinogenic potential has posed a considerably problem to legislators and scientists alike in the choice of the most suitable tests. Attempts to resolve this important issue and the considerable resources that have to be mobilized have resulted in the incentive support for international cooperative efforts.

The collaborative study on in vitro assays forms part of a large collaborative study on short-term tests for genotoxicity and carcinogenicity which is one of the most extensive international cooperative attempts to clarify the role of in vitro and in vivo short-term tests in the evaluation of the health hazards of chemicals. It has had a considerable measure of success only because of the remarkable response of scientists throughout the world in volunteering to take part in the study. It thus demonstrates the power of international collaborative efforts to resolve important issues in toxicological matters when the objectives are clearly defined and an international body such as the IPCS can be accepted to be in a position to offer an effective planning and coordinating mechanism.

Many people have helped in this complex project but special thanks are due to the steering committee and in particular to Dr. John Ashby for his outstanding scientific contribution and committee efforts and to Dr. Frederick de Serres as chairman and Dr. Michael Shelby as rapporteur for their considerable efforts and enthusiasm throughout the years it has taken to complete the study.

There is every expectation that the second part of the CSSTT, the in vivo studies , under the same guidance, will be equally successful in adding yet another considerable step to our understanding of the place of short-term tests in the overall evaluation of the risks to human health from the chemicals in the domestic, work and general environments.

Dr. Michel Mercier
Manager, IPCS

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PART I

BACKGROUND AND SUMMARIES

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1

Introduction

Concern that the inadvertent exposure of humans to chemical carcinogens contributes to cancer incidence and that this can be reduced through the identification and control of human carcinogens continues to provide the major impetus for the development and application of short-term tests for chemical carcinogens. By necessity, the carcinogenicity of chemicals must be assessed using data from rodent studies and the results of such studies are accepted as indicative of similar activity in exposed humans. Short-term tests for carcinogenic chemicals were developed largely to detect rodent carcinogens, and short-term test performances are assessed primarily using rodent carcinogenicity test results. During these developments, it has become clear that no single test is capable of detecting all rodent carcinogens as positive. There are a number of possible reasons for these shortcomings, including the specific nature of endpoints of an assay, unique metabolic capability of the organism, compatibility of the assay with the source of exogenous metabolism, and the unique structure or activity of the test chemical. This situation where no single assay can be relied on to detect all carcinogens has led to the proposal or adoption of testing schemes that employ multiple in vitro assays in various configurations such as batteries, tiers, or combinations of the two. The basis for selection and deployment of these multiple tests is, at this time, based almost exclusively on theoretical prudence rather than empirical evidence. That is, a variety of genetic endpoints and organisms representing different phylogenetic levels are selected with the intent of not missing endpoint- or phylum-specific chemical activity.

The objective of the current study was an attempt to generate test results that would contribute to an empirical basis for selecting one or more in vitro short-term tests as complementary to the generally accepted and widely used Salmonella test developed by Ames (Ames et al., 1975).

The rationale for the study was derived to a large extent from the major findings of the "International Collaborative Program for the Evaluation of Short-Term Tests for Carcinogens" (IPESTTC) (de Serres and Ashby, 1981). This study in turn arose as one of a number of major projects set up to evaluate the efficacy of different short-term assays that appeared to have promise for supplementing the traditional long-term bioassay in the rodent. The results clearly confirmed the value of Salmonella reversion assays as a suitable primary test for potential carcinogens and mutagens. However, it was also confirmed that some known rodent carcinogens were either not detected or detected with considerable difficulty by such assays. The study did not succeed in arriving at clearcut conclusions concerning a complementary eukaryotic assay that was capable of giving a positive response for those carcinogens found negative in the standard Salmonella reversion assay. Several assays that might serve in the capacity of a complementary assay were identified, but none were recommended for general adoption at that time because it was considered that the supporting data base was too small.

It was against this background that the Collaborative Study on Short-Term Tests for Genotoxicity and Carcinogenicity (CSSTT) was proposed by the International Program on Chemical Safety (IPCS) * and the National Institute of Environmental Health Sciences of the U.S.A. as a participating institution in that program. The general goals and designs of the study were outlined by an ad hoc Working Group **, who met by

* The International Program on Chemical Safety (IPCS) is a collaborative program of the World Health Organization (WHO), the International Labour Organization (ILO) and the United Nations Environmental Program (UNEP).

** Participants: Dr. J. Ashby, Professor N.P. Bochkov, Dr. B.E. Matter, Professor T. Matsushima, Dr. F.J. de Serres, Dr. M. Shelby and Professor F.H. Sobels.

invitation of the IPCS in Geneva, 30 April–1 May, 1981. The plans were consolidated by an IPCS Working Group *, who met in Geneva, 13–14 November, 1981. The subsequent coordination of the study was the responsibility of a Steering Committee derived primarily from the Working Group (J. Ashby; F. de Serres, Chairman; M. Ishidate Jr.; B. Margolin; B. Matter; M. Shelby; and M.H. Draper, IPCS Secretariat).

The financial burden of the organization of this study has been met largely by the IPCS, together with some of its participating institutions, particularly the National Institute of Environmental Health Sciences in the U.S.A. The funding of the assay work, as occurred in the IPESTTC project (de Serres and Ashby, 1981), was provided in the majority of cases by the individual investigators managing to incorporate the work into their research programs. This could only occur with the goodwill and belief in the project by the senior managements of the approximately fifty involved laboratories from universities, research institutes and industrial research facilities throughout the world. In addition, a number of governments that support the IPCS have provided financial assistance for this study. These include the governments of Belgium, Italy, The Netherlands, and in particular, the United Kingdom.

Some 60 investigators have taken part in the present study, and most of the in vitro eukaryotic tests currently available were represented. Eight organic carcinogens known to be either inactive or difficult to detect as positive in the Salmonella assay were tested (i.e., *o*-toluidine, hexamethylphosphoramide, safrole, acrylonitrile, benzene, diethylhexylphthalate, phenobarbital and diethylstilbestrol) together with two chemicals, caprolactam (Huff, 1982) and benzoin (NTP, 1980), that gave no evidence of carcinogenicity in two-year rodent bioassays.

Current strategies for the detection of potential human carcinogens often rely on the deployment of short-term genotoxicity assays. While this approach appears logical, it must include consideration of other factors that are believed to be of importance in the biological etiology of natural and chemically-induced cancer (Ashby, 1983). Thus, the molecular targets for investigation are no longer dominated by observations of readily discernible changes in the sequence or integrity of nuclear DNA, but now involve considerations of subtle changes in chromosome, gene or oncogene function or expression (Tabin et al., 1982; Weiss, 1982; Klein, 1981; Reddy et al., 1982). It could be advocated that screening efforts will not be really feasible until the conundrum of cancer is solved. However, given that this may not be imminent, it still seems expedient to develop the available screening techniques to the point that they may be usefully employed despite the fact that some of the genetic endpoints monitored for this purpose may ultimately be shown not to relate directly to the critical events in the etiology of some chemically-induced cancers (Cairns, 1981). Internationally, chemical registration and health authorities are assessing genotoxicity test data generated for new chemicals in a wide range of in vitro and in vivo short-term assays, and it is often unclear what relative weight to afford the often discordant test data generated (Taylor and Griffin, 1982).

The present study comprises Part I, in vitro assays, of the full project. It is hoped that this, together with Part II, the in vivo studies, will contribute decisively to these debates and, in so doing, advance significantly the cause of human health.

Criteria for the definition of complementary in vitro assays for the detection of potential carcinogens

The study announcement listed the following criteria by which the study was designed and conducted (Ashby et al., 1983). To qualify as a complementary assay for routine use in conjunction with the Salmonella plate-incorporation assay, a test must fulfill the following requirements:

(1) It should have been successfully employed as a short-term test in a number of laboratories, and should be substantially represented in the literature.

(2) It should have performed well in the detec-

* Participants: Dr. J. Ashby, Dr. G.R. Douglas, Dr. M. Ishidate, Dr. A. Léonard, Dr. N. Loprieno, Dr. B.E. Matter, Professor T. Matsushima, Dr. R. Montesano, Dr. F.J. de Serres, Dr. M. Shelby, Professor F.H. Sobels, Dr. M. Stoltz and Dr. M. Waters.

tion of the present eight carcinogens while concomitantly finding both of the noncarcinogens negative.

(3) Positive responses obtained with the eight carcinogens tested should have been unambiguous, dose-related and reproducible.

(4) Similar qualitative responses should have been observed by the majority of the laboratories using the same assay.

(5) It should be appropriate for routine screening purposes, i.e., not unduly demanding of resources or technical facilities.

Four categories of assay may thus be defined:

(A) Assays suitable for general use in conjunction with the Salmonella assay.

(B) Promising assays; i.e., those that may be capable of fulfilling criteria 1–5 but for which data are not available for all of the test chemicals, or where repeat studies are not available.

(C) Relatively new assays which, while not meeting criterion 1, have performed well in the study, and for which the present ten chemicals form the majority of the available database; these cases would best be handled by the rapid and coordinated acquisition of further information.

(D) Assays that are clearly inappropriate for routine use in testing for potential carcinogens, i.e., that do not meet criteria 2–5.

Assays and endpoints

As discussed above, the design of this study reflected the primary purpose of attempting to identify in vitro eukaryotic assays capable of detecting chemical carcinogens that are not readily detectable using bacterial assays. At the organismal level, four categories of assays were employed: bacteria, yeast, fruit flies and cultured mammalian cells. Within each of these groups of organisms a variety of test endpoints were used. Organisms and endpoints will be described briefly here and are presented in Table 1. Full details are provided in the subsequent assay workgroup reports and individual investigators' reports.

Bacteria

The carcinogens included in this study were selected on the basis of previously published results indicating their lack of activity in routinely conducted Salmonella mutagenicity tests. Five sets of Salmonella data were obtained in this study to confirm those previous results and, specifically, to provide bacterial mutagenicity data on the batches of chemicals used in the current study. Test data are reported for *Salmonella typhimurium* strains TA97, TA98, TA100, TA102 and TA1535 in both pre-incubation and pour-plate protocols and TA1537 and TA1538 in pour-plate only. *S. typhimurium* strain TM677, used to detect azaguanine-resistant forward mutants, was employed using a treat-and-plate method.

Fungi

Fungal systems, which offer the advantages of being both microbial and eukaryotic, were used to evaluate a wide range of genetic endpoints. Test results from *Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Aspergillus nidulans* are reported on the following genetic endpoints: nuclear gene mutation (both forward and reverse), mitochondrial mutation, gene conversion, mitotic crossing-over and aneuploidy.

Drosophila

Three separate laboratories reported test results from three newly developed assays for detecting genetic damage induced in somatic cells of Drosophila. The white–zeste eye mosaicism test detects eye spots resulting from mutations or deletions, while the wing mosaicism and white/white coral eye mosaicism tests detect wing or eye spots resulting from mutations, deletions, chromosome breakage, mitotic recombination or aneuploidy.

Cultured mammalian cells

Five major categories of chemically-induced effects were reported for cultured mammalian cells: inhibition of metabolic cooperation, transformation, DNA damage, cytogenetic effects and gene mutations.

Test data on inhibition of metabolic cooperation, an assay intended to detect promoting agents, as evidenced by increased survival of $HGPRT^-$ V79 cells in the presence of an excess of $HGPRT^+$ cells and 8-azaguanine or 6-thioguanine, were reported by three laboratories.

Six distinct transformation assays were reported

TABLE 1

TEST SYSTEMS EMPLOYED IN THE IPCS COLLABORATIVE STUDY ON SHORT-TERM TESTS FOR MUTAGENS AND CARCINOGENS

I. Bacteria	
Salmonella typhimurium	
TA97, TA98, TA100, TA102, TA1535, TA1537, TA1538	$HIS^- \rightarrow HIS^+$
TM677	$AZA^S \rightarrow AZA^R$
II. Fungi	
Mutation	
Saccharomyces cerevisiae	
XV185-14C	$ARG^- \rightarrow ARG^+$; $TRP^- \rightarrow TRP^+$; $HIS^- \rightarrow HIS^+$; $HOM^- \rightarrow HOM^+$
RM52	$HIS^- \rightarrow HIS^+$
D7	$ILV^- \rightarrow ILV^+$
D6 and D61-M	$ADE^- \rightarrow ADE^+$; $ILV^- \rightarrow ILV^+$
D5	petite colonies due to mitochondrial mutations
Schizosaccharomyces pombe	
P1	red → white colonies (ADE)
Aspergillus nidulans	
35	methionine metabolism mutants
Recombination	
Saccharomyces cerevisiae	
JD1	gene conversion, tryptophan or histidine prototrophy
D7 and D7-144	crossing-over, red and pink colonies — (ADE) gene conversion, tryptophan prototrophy
PV-2 and PV-3	crossing-over, canavanine resistance, gene conversion, lysine protrophy
D6 and D61-M	crossing-over, cycloheximide resistance
Aspergillus nidulans	
P1	crossing over, green → yellow colonies
Aneuploidy	
Saccharomyces cerevisiae	
D6 and D61-M	red, cycloheximide sensitive → white, cycloheximide resistant
Aspergillus nidulans	
P1	Yellow sectors in green colonies
Illegitimate mating	
Saccharomyces cerevisiae	
PV-4a and PV-4b	Mating type $\alpha \rightarrow a$
III. Drosophila	
Somatic cell mutations	
Wing-mosaicism	wing spots from mutations, deletions, chromosome breakage, mitotic recombination or aneuploidy
White–zeste eye mosaicism	eye spots from mutations or deletions
White/white coral eye mosaicism	eye spots (same events as wing spots above)
IV. Cultured mammalian cells	
Metabolic cooperation	
V79	survival of $HGPRT^-$ cells
Transformation	
SHE	colony assay
C3H10T1/2	focus assay
BALB/c 3T3	focus assay
SHE/SA7	viral enhancement of chemical transformation-focus assay

TABLE 1 (continued)

IV. Cultured mammalian cells (continued)	
Transformation (continued)	
RLV/FRE	enhanced survival of Rauscher leukemia virus-infected rat embryo cells
CHO	invasive growth in agar
DNA damage	
Single-strand breaks	
CHO	alkaline sucrose sedimentation
Rat hepatocytes	alkaline elution
Unscheduled DNA synthesis	
HeLa S3	scintillation counting-extracted DNA
Rat hepatocytes	scintillation counting — DNA extracted from isolated nuclei
Rat hepatocytes	autoradiography
Cytogenetic damage	
Chromosomal aberrations	
CHO	structural aberrations; micronuclei
Chinese hamster lung, CHL	structural aberrations; polyploidy
Chinese hamster liver, CH1-L	structural aberrations; polyploidy; aneuploidy
Rat liver, RL4	structural aberrations; polyploidy
Human lymphocytes	structural aberrations
Sister-chromatid exchange	
CHO	
V79	
Rat liver, RL4	
Gene mutations	
L5178Y	$TK^{+/-} \rightarrow TK^{-/-}$ $OUA^S \rightarrow OUA^R$
V79	$HGPRT^+ \rightarrow HGPRT^-$ $OUA^S \rightarrow OUA^R$
CHO	$HGPRT^+ \rightarrow HGPRT^-$
Human lymphoblasts	
TK6	$TK^{+/-} \rightarrow TK^{-}/^{-}$
AHH	$HGPRT^+ \rightarrow HGPRT^-$

including two laboratories using Syrian hamster embryo (SHE) cells, two using C3H10T1/2 mouse cells, and single laboratories using the Syrian hamster embryo/Simian adenovirus-7 and Rauscher leukemia virus-infected rat embryo cell assays. In addition, data for five compounds derived from a new assay in which the endpoint is invasive growth of CHO cells in soft agar were considered. Two investigators who had offered to generate data using the BHK21 transformation assay withdrew from the study due to the lack of adequate time. This was disappointing as they had presented the prospect of a link with the previous study (de Serres and Ashby, 1981) in which the BHK21 assay represented the sole transformation endpoint.

The chemical induction of DNA single-strand breaks was determined by assessing single-strand breaks using alkaline elution or alkaline sucrose sedimentation. Tests for unscheduled DNA synthesis were reported from protocols employing both scintillation counting and autoradiography.

A large body of test data was reported for the two most commonly used cytogenetic endpoints,

structural aberrations and sister-chromatid exchanges. In addition, limited results were reported for the induction of micronuclei, aneuploidy and polyploidy.

Gene mutation induction data were reported for three loci: thymidine kinase (TK), hypoxanthine guanine phosphoribosyl transferase (HGPRT), and Na^+,K^+ ATPase (ouabain resistance) in mouse, Chinese hamster or human cells. These studies included seven sets of test results from the L5178Y $TK^{+/-}$ system.

Protocols

Assays conducted in this study were grouped into seven categories with regard to organism and endpoint, and a coordinator was assigned to each group. Each investigator in the study was asked to submit a protocol to the respective workgroup leader and the central file prior to the initiation of testing. Workgroup chairmen were to review these protocols and, where there were apparent deficiencies, to discuss an improved protocol with the investigator. This was the only attempt to reach agreement on a common protocol among investigators employing similar assays.

As the basic objective of this study was similar to that of IPESTTC (de Serres and Ashby, 1981), i.e., to identify an assay/protocol suitable to detect a selected category of chemicals, a similar philosophy of protocol standardization was followed. Basically, the philosophy is that in the absence of knowledge of an optimum protocol, adherence to a fixed protocol reduces the possibility of identifying advantageous variations in an assay.

Computer database

During the conduct of this study, a decision was made to create, at the National Institute of Environmental Health Sciences, a computerized database of experimental results. This was intended to serve as a permanent repository of test data that would enable future analyses to be made. The existence of this database will, and already has, permitted statistical analyses with the following aims:

(i) to provide an objective assessment of an investigator's own evaluation of his data;

(ii) to apply the same analytical criteria to data sets from an assay conducted in multiple laboratories;

(iii) to discern weakly positive results judged negative by the investigator that actually reinforce other investigators' positive findings for the same assay; and

(iv) to add to the field's understanding of reproducibility of results within and between laboratories for particular assays.

The system selected to create the database (Bolt, Beranek and Newman, 1983), was designed specifically to meet the information handling needs of research scientists. RS/1 stores data in a large two-dimensional table that facilitates processing of subtables, e.g. the collating of all data generated using rat-liver S9 or all SCE data from CHO cells, etc. The database contains approximately 2500 individual dose–response experiments.

Publication of results

This book presents an overview of the study along with the total testing results. Important ancillary information is provided in the Appendices. The bulk of this book is made up of chapters written by the individual investigators that include their protocols, test data and conclusions. Eight workgroup chapters present the summary outcomes of the deliberations and conclusions of the groups of investigators who met to discuss their protocols and results. The overview was prepared by the Steering Committee and represents an attempt to evaluate the entire database with respect to the study objectives and to present clear conclusions regarding the initial goal, i.e., the identification of a robust, sensitive and generally applicable test system with which to complement bacterial mutation tests used to predict chemical carcinogenicity.

A second IPCS study was designed at the same time that the present was conceived (Ashby et al., 1983). The second study aims to generate a database of test results from a variety of short-term in vivo tests and thereby permit an assessment of the relative sensitivities of short-term tests conducted in vitro and in vivo. For this purpose, carcinogen/noncarcinogen pairs (2-acetylaminofluorene, 4-

acetylaminofluorene and benzo[*a*]pyrene, pyrene) are being evaluated using currently available short-term in vivo genotoxicity assays. Results of that study will be described in a future publication.

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2

Summary Report on the Performance of the Bacterial Mutation Assays

Work Group Chairman
Stanley Venitt

Work Group Participants
R. Baker, H. Liber (Absent, but data considered), T. Matsushima (Absent, but data considered), G. Probst and E. Zeiger

Introduction

The object of this part of the CSSTT study was to confirm that the ten chosen chemicals are difficult or impossible to detect in routine mutagenicity assays employing gene mutation in bacteria as the end-point.

These assays determine whether the test compound can induce reverse mutations by frameshifts or base-pair substitutions at the histidine (*his*) operon of *S. typhimurium*. Forward mutation to 8-azaguanine resistance has also been used as an end-point. Both reverse and forward mutation assays employ strains of *S. typhimurium* which carry mutations which confer DNA-repair deficiency, and cell-wall defects which increase permeability to lipophilic molecules. In addition, some strains carry plasmids which confer additional sensitivity to the mutagenic effects of certain classes of chemicals. Routine screening assays are performed with and without the addition of an exogenous source of metabolic activation prepared from the livers of various rodent species (Ames et al., 1975).

This chapter presents a comparative summary of the protocols and results from the five laboratories which participated in the CSSTT Study, and incorporates the views of 4 investigators who attended the meeting at St. Simons Island (October 1983) during which detailed discussions of the data-base took place.

Full descriptions of the methods used and of the results obtained by participating laboratories can be found in the individual Investigators' Reports, together with the criteria used by each laboratory to define positive, negative or questionable results. A brief review of data for four of the test chemicals from an earlier trial is also presented, together with a survey of the literature for all ten compounds.

Methods

Raw data from each participating laboratory were sent to the coordinator, together with the protocol used in the study. In addition each investigator was asked to complete a questionnaire asking for information on the precise formulation of S9 mix and other details of the experimental design. Tables 1 and 2 summarize the protocols used by each laboratory. Of the four laboratories which employed reverse mutation (*his*$^-$ to *his*$^+$), two used the pour-plate technique and two used the pre-incubation method. The fifth laboratory in the study used as a genetic marker forward mutation from 8-azaguanine sensitivity to resistance, employing a 'treat-and-plate' protocol. All five laboratories used exogenous metabolic activation systems prepared from the livers of rats. Four laboratories treated the animals with Aroclor 1254 to induce high levels of oxidative metabolism and one used a mixture of phenobarbitone and 5,6-benzoflavone for this purpose. In addition, one laboratory used S9 prepared from livers of Aroclor-1254-treated Syrian hamsters.

Taking the study as a whole, a very comprehensive range of tester strains was used (including the recently introduced strains TA97 and TA102; Levin et al., 1982a, b) although no single labora-

TABLE 1

SUMMARY OF PROTOCOLS USED IN BACTERIAL MUTATION ASSAYS

	Baker and Bonin	Liber	Matsushima	Zeiger and Haworth	Rexroat and Probst
Species and strain of animal	Sprague–Dawley rat	Sprague–Dawley rat	Sprague–Dawley rat	Sprague–Dawley rat; Syrian hamster (3 separate batches)	Fischer 344 rat
Number in group	8	not given	5	15; 20	not given
Sex	male	male	male	male rat; male hamster	male
Age (weeks)	8	8–10	7	8.3; 8.3–8.4	not given
Weight (g)	250	not given	213	240; 98, 104, 95	not given
Type of diet	rat and mouse cubes (Doust and Raddidge)	not given	Pellet diet (CE-2)	Purina certified rodent chow	not given
Name of inducer	Aroclor 1254	Aroclor 1254	Phenobarbital sodium (PB) and 5,6-benzo-flavone (BF)	Aroclor 1254	Aroclor 1254
Vehicle	Maize oil	Corn oil	0.9% NaCl (PB) Corn oil (BF)	Corn oil	not given
Dose (mg per kg)/route	500 mg/kg i.p.	500 mg/kg i.p.	PB 30 mg/kg x1 60 mg/kg x3 BF 80 mg/kg x1 all i.p.	500 mg/kg i.p.	100 mg/kg i.p.
Days from dose to killing	5	5	PB 4–1 BF 2	5	5
Animals starved?	no	yes	no	no	not given
Method of killing	blow to head, decapitation	not given	cervical dislocation	decapitation	not given

S9 Fresh or frozen?	frozen	frozen	frozen	frozen	frozen
Time-span of storage	6–20 days	9–12 months after purchase from Litton	5–35 days	89–146 days; 50–86 days	6 months
Storage temperature (°C)	−70	−80	−80	−70	−80
Protein concentration of S9 (mg/ml)	not given	36.0	29.8	not given	not given
Composition of S9 mix (μmoles per ml)					
$MgCl_2$	8	3.3	8	8	8
KCl	33	–	33	33	33
G-6-P	2.5	3.38	5	50	4
NADP	2	1.31	8 [a]	4	5
Phosphate buffer	100	–	100	100	250
S9 (ml)	0.1	0.05	0.1	0.1, 0.3	0.1
Amount of S9 per plate (μl)	50	–	50	25, 50, 150	50
Amount of NADP per plate (μmoles per plate)	1	–	2	1, 2, 6	2.5
Salmonella strains	TA97, 98 100, 102	TM677	TA97, 98 100, 102	TA97, 98 100, 1535	TA1535, 1537 1538, 98, 100
Genetic marker	*his*$^-$ to *his*$^+$	Ag^S to Ag^r	*his*$^-$ to *his*$^+$	*his*$^-$ to *his*$^+$	*his*$^-$ to *his*$^+$
Method	pour-plate	treat/plate	pre-incubation 30°, 30 min	pre-incubation 37°, 20 min	ppour-plate
Medium for stock culture	Oxoid NB2	MinE/2% brain heart infusion	Difco NB	Oxoid NB	Oxoid NB2
Growth conditions	Stationary O/N, then 2 h shaking	1.5 h?	Shaking O/N	Shaking O/N	16 h shake, then kept on ice
Incubation time for plates (h)	72	48	48	48	48

[a] NADPH, NADH 1 : 1.

TABLE 2

SUMMARY OF DOSE SCHEDULES AND DESIGN OF EXPERIMENTS

	Bonin and Baker			Liber		
	No. of tests	No. of plates per dose [a]	Doses (μg/plate)	No. of tests [b]	No. of plates per dose	Doses (μg/plate)
HMPA	2	2	320, 1000 3200, 10000	4	6	67, 268, 670: 50, 200, 500
TOL	2	2	320, 1000 3200, 10000	4	6	50, 200, 500
BEN	2	2	320, 1000, 3200, 10000	2	6	50, 100, 500
SAF	2	2	3.2, 10, 32 100	4	6	50, 200, 500
CAP	2	2	32, 100, 320 1000	4	6	30, 100, 300
ACN	3	2	320, 1000, 3200: 2000, 3000 4000, 5000, 10000	4	6	50, 200, 500
DEHP	2	2	320, 1000, 3200, 10000	3	6	50, 200, 500
ZOIN	2	2	32, 100, 320 1000	3	6	30, 100, 300
DES	2	2	3.2, 10, 32, 100	4	6	30, 100, 300
PB	3	2	32, 100, 320 1000: 1000, 2000, 3000, 4000, 5000	4	6	50, 200, 500
Positive controls (μg per plate) B[a]P (5) 2-AAF (100)				4-NQO (0.1/ml) B[a]P (20/ml)		

[a] 3 plates at zero-dose.

[b] 2 tests −S9, 2 tests +S9.

TABLE 2 (continued)

Matsushima			Zeiger and Haworth			Rextroat and Probst		
No. of tests	No. of plates per dose [c]	Doses (μg/plate)	No. of tests [d]	No. of plates per dose	Doses (μg/plate)	No. of tests	No. of plates per dose [a]	Doses (μg/plate)
1	2	100, 200, 500 1000, 2000, 5000	2+	3	100, 333 1000, 3333 10000	1	3	50, 100, 500, 1000 5000
1	2	100, 200, 500 1000, 2000, 5000	2+	3	33, 100, 333, 5000: 1000, 2000, 3333	1	3	50, 100, 500, 1000 5000
1	2	100, 200, 500 1000, 2000, 5000	2+	3	10, 33, 100 333, 1000	1	3	50, 100, 500, 1000 5000
2	2	50, 100, 200 500, 1000: 1, 2.5, 5, 10 25, 50	2+	3	1, 3.3, 10, 33, 67, 100, 200	1	3	50, 100, 500, 1000
1	2	100, 200, 500 1000, 2000, 5000,	2+	3	100, 333, 1000, 3333 10000	1	3	50, 100, 500, 1000 5000
1	2	100, 200, 500, 1000, 2000, 5000	2+	3	100, 333, 1000, 3333, 6666	1	3	50, 100, 500, 1000 5000
1	2	100, 200, 500, 1000, 2000, 5000	2+	3	100, 333, 1000, 3333, 10000	1	3	50, 100, 500, 1000 5000
1	2	100, 200, 500, 1000, 2000, 5000	2+	3	33, 100, 333, 500, 750, 1000, 2000 3333, 10000	1	3	50, 100, 500, 1000 5000
1	2	20, 50, 100, 200, 500, 1000	2+	3	0.03, 0.1, 1, 3.3, 6.6, 10 33, 66	1	3	50, 100, 500, 1000 5000
1	2	100, 200, 500, 1000, 2000, 5000	2+	3	100, 333, 500 1000, 3333, 6666	1	3	50, 100, 500, 1000 5000

AF-2 (0.01, 0.1)	NaN_3 (2.5)	MNNG (0.25, 0.5)
2-AAnthr (0.5)	2-AAnthr (1.5)	9-AAcr (50, 100)
BleoMy (2)	9-AAcr (4)	2-NitFlu (0.5, 5)
B[a]P (1)	4-NOPD (12)	2-AAnthr (2.5, 5)

[c] 4 plates at zero-dose.

[d] variable, depending on results. Separate tests with rat S9 and hamster S9.

TABLE 3

SUMMARY OF BACTERIAL MUTATION DATA

	Bonin and Baker								Liber		Matsushima							
	−S9				+S9				−S9	+S9	−S9				+S9			
	S. typhimurium strain TA																	
	97	98	100	102	97	98	100	102	TM677	TM677	97	98	100	102	97	98	100	102
HMPA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TOL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
BEN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SAF	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CAP	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ACN	–	–	–	?	–	–	–	–	+	–	–	–	–	–	–	–	–	–
DEHP	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ZOIN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DES	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PB	–	–	+w	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

	Zeiger and Haworth								Rexroat and Probst									
	−S9				+S9				−S9					+S9				
	97	98	100	1535	97	98	100	1535	35	37	38	98	100	35	37	38	98	100
HMPA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TOL	–	–	–	–	–	–	+ [a]	+w? [a]	–	–	–	–	–	–	–	–	–	–
BEN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SAF	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CAP	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ACN	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–
DEHP	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ZOIN	–	–	?	+w	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DES	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PB	–	–	–	+w	–	–	–	–	+w [b]	–	–	–	?	–	–	–	–	?

[a] Hamster S9 only.

[b] Single experiment.

tory used more than 5 strains in its routine protocol.

Results

The results of the bacterial assays are summarised in Table 3, each assignment of positive, negative or equivocal being that of the respective group of investigators. The criteria used by each group for defining whether assays were positive, negative or equivocal are given in the reports of the individual investigators. The following discussion, whilst taking into account these assignments, are based on assessment of the data during the discussions by the working party (Dr. R. Baker, Dr. G. Probst, Dr. E. Zeiger and Dr. S. Venitt). *Positive* results were defined by the demonstration of reproducibly dose-related increases in mutant colonies in at least 1 strain of test bacteria. Results were classified as *equivocal* (?) when modest dose-related increases were seen in single experiments, or when such increases in numbers of mutant colonies were not reproducible. Results were classified as *negative* when there was no dose-related increase in the number of mutant colonies in any of the assays of the compound under test.

The following six compounds were classified as giving negative results in all the assays performed in the this trial: hexamethylphosphoramide, benzene, safrole, caprolactam, diethylhexylphthalate and diethylstilboestrol. The remaining four chemicals (*o*-toluidine, benzoin, acrylonitrile and phenobarbitone) gave positive or equivocal results in at least one laboratory. These compounds are discussed below.

o-Toluidine

o-Toluidine gave positive results in TA1535 and TA100, in the dose range 33–3333 μg per plate in the presence of Syrian hamster liver S9 (30% S9 in the S9 mix): negative results were obtained using rat-liver S9 (Zeiger and Haworth, this volume). In a single experiment using S9 from rats induced with phenobarbitone and 5,6-benzoflavone, Matsushima obtained a dose-related increase in *his*$^+$ revertants in TA100, a result classified as negative by the investigator (Matsushima, this volume).

Acrylonitrile

Zeiger and Haworth (this volume) found acrylonitrile to be a convincing mutagen in TA1535 and TA100, with an absolute requirement for S9. However, Baker and Bonin (this volume) obtained a weaker dose-related increase (which was not reproducible) using TA100 in the absence of S9. Liber (this volume) found that acrylonitrile was reproducibly mutagenic in a forward mutation system in the absence of S9. However, this increase in the number of mutants per survivor was obtained only at one dose and even at this dose there was no nett increase over the control in the number of resistant colonies actually scored.

Benzoin

Benzoin gave modest and reproducible dose-related responses at doses between 100 and 2000 μg per plate in TA1535 without S9. These effects were abolished by the addition of S9 (Zeiger and Haworth, this volume).

Phenobarbitone

Modest but consistently positive dose-related increases were seen in 4 laboratories assaying phenobarbitone in reverse-mutation tests employing base-pair-substitution mutants (TA1535 and/or TA100) in the absence of S9.

Zeiger and Haworth (this volume) obtained reproducible dose-response curves at doses ranging from 100 to 1000 μg per plate (Fig. 1) the effect being abolished on addition of S9. In a single experiment these workers obtained a weak dose–response curve for phenobarbitone assayed in TA100.

In replicate experiments Baker and Bonin (this volume) obtained weakly positive dose-related increases at doses ranging from 1000 to 4000 μg per plate in TA100, again in the absence of S9. In a single assay using TA100, Matsushima obtained similar results using a similar dose range, this result being considered negative by the investigator. Again there was no evidence of any effect when S9 was included in the assay. Rexroat and Probst (this volume) obtained positive results for TA1535 treated with phenobarbitone (50–5000 μg per plate) in the absence of S9. Fig. 1 shows the concordance of the results obtained for phenobarbitone assayed in TA1535 without S9 by two different laboratories (Zeiger and Haworth; Rexroat and Probst).

TABLE 4

SUMMARY OF BACTERIAL MUTATION DATA FROM IPESTTC TRIAL: DATA FOR PLATE TESTS USING *S. typhimurium*

de Serres and Ashby (1981)

IPESTTC Lab. No.	Hexamethylphosphoramide												*o*-Toluidine											
	− S9						+ S9						− S9						+ S9					
	S. typhimurium strain TA																							
	35	37	38	92	98	10	35	37	38	92	98	10	35	37	38	92	98	10	35	37	38	92	98	10
1	–	–	–		–	–	–	–	–		–	–	–	–	–		–	–	–	–	–		–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	– –
3		–			–	–		–			–	–		–			–	–		–			–	–
4	–	–			–	–	–	–			–	–	–	–			–	–	–	–			–	–
6		–			–	–		–			–	–		–			–	–		–			–	–
7					–	–					–	–					–	–					–	+
8		–			–	–		–			–	–		–			–	–		+ [a]			+ [a]	+ [a]
9	–	–	–		–	–	–	–	–		–	–	–	–	–		–	–	–	–	–		–	–
10	–	–	–		–	–	–	–	–		–	–	–		–			–	–		–		–	–
11	–	–	–		–	–	–	–	–		–	–	–	–	–		–	–	–	–	–		–	–
12	–	–	–		–	–	–	–	–		–	–	–	–	–		–	–	–	–	–		–	–
13					–	–					+	–					–	–					+	+
16 *	–						–						–						–					

* Forward mutation, AG^{s} to AG^{r}, using a derivative of TA1535.

[a] Positive only in the presence of norharman.

TABLE 4 (continued)

IPESTTC Lab. No.	Safrole												Diethylstilboestrol											
	−S9						+S9						−S9						+S9					
	35	37	38	92	98	10	35	37	38	92	98	10	35	37	38	92	98	10	35	37	38	92	98	10
1	−	−	−		−	−	−	−	−		−	−	−	−	−		−	−	−	−	−		−	−
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3		−			−	−		−			−	−		−			−	−		−			−	−
4	−	−			−	−	−	−			−	−	−	−			−	−	−	−			−	−
6		−			−	−		−			−	−		−			−	−		−			−	−
7					−	−					−	−					−	−					−	−
8		−			−	−		−			−	−		−			−	−		−			−	−
9	−	−	−		−	−	−	−	−		−	−	−	−	−		−	−	−	−	−		−	−
10	−	−	−		−	−	−	−	−		−	−	−	−	−		−	−	−	−	−		−	−
11	−	−	−		−	−	+?	−	−		−	−	−	−	−		−	−	−	−	−		−	−
12	−	−	−		−	−	+?	−	+?		−	−	−	−	−		−	−	−	−	−		−	−
13					−	−					−	−					−	−					−	−
16 *	−						−						−						−					

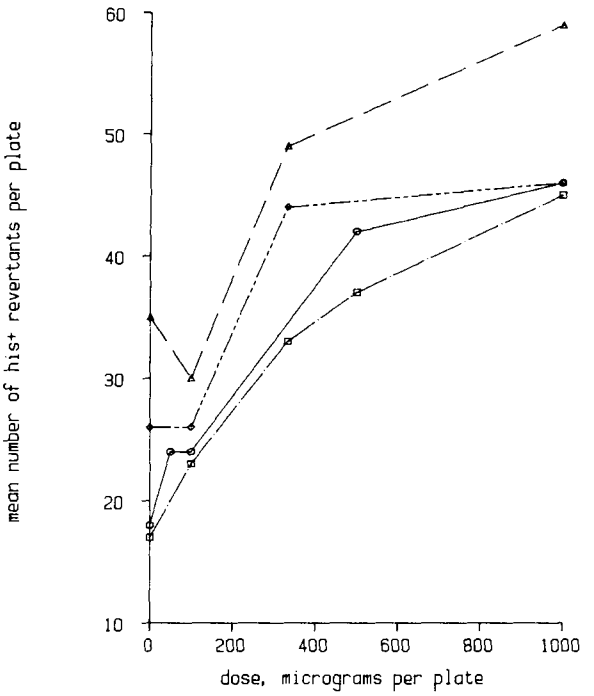

Fig. 1. Dose–response curves for phenobarbitone assayed in the absence of S9 in *S. typhimurium* TA1535. ○, data from a single experiment by Rexroat and Probst (this volume); △, ◇, □, data from 3 separate experiments by Zeiger and Haworth (this volume).

Conclusions drawn from this study

Although 3 carcinogens (phenobarbitone, acrylonitrile and *o*-toluidine) were picked out as mutagens in at least one laboratory, the bulk of the results of from all five laboratories were negative, confirming that in general the test compounds chosen for the CSSTT study were difficult or impossible to detect reliably in routine screening assays using *S. typhimurium*, conventional S9 preparations and conventional protocols. One surprising observation has been the weak but consistently positive data for phenobarbitone, which appears to be a mutagen capable of inducing base-pair substitution mutations without the requirement for an exogenous source of metabolic activation.

Review of data from the International Collaborative Programme for the Evaluation of Short-Term Tests for Carcinogens ('ICPESTC')

The choice of four of the chemicals tested in the present CSSTT study arose from earlier extensive blind testing of these chemicals as part of the ICPESTC Trial (de Serres and Ashby, 1981). In this earlier trial, hexamethylphosphoramide, safrole, diethylstilboestrol and *o*-toluidine were assayed by thirteen laboratories using various versions of the Salmonella/mammalian microsome test, together with other bacterial tests including a forward mutation assay using mutation of *S. typhimurium* from 8-azaguanine sensitivity to resistance. The results of this study are shown in Table 4, the salient features being summarised below:

Diethylstilboestrol was uniformly negative in all thirteen laboratories.

Hexamethylphosphoramide was found to be positive in only 1 laboratory, in TA98 in the presence of S9.

Three laboratories obtained positive results with *o-toluidine*, but there was wide variation among the laboratories in the strains giving positive results. In one laboratory, *o*-toluidine gave positive results only in the presence of S9 supplemented with norharman.

Safrole, in the presence of S9, gave questionably positive results in two laboratories.

It is clear therefore that the largely negative results obtained in the ICPESTC trial for these four chemicals have been confirmed by the findings of the CSSTT study.

Review of published bacterial mutagenicity data

Acrylonitrile

Acrylonitrile was mutagenic to several strains of *E. coli* WP2, inducing reverse mutation (*trp*$^-$ to *trp*$^+$) in the absence of S9 (Venitt et al., 1977). It was also mutagenic to *S. typhimurium*, the strongest effects being seen in base-pair-substitution mutants. The majority of studies indicate that an exogenous source of metabolic activation is necessary to demonstrate the mutagenicity of acrylonitrile to *S. typhimurium* (see, for example, de Meester et al., 1978; Roberfroid et al., 1978; de Meester et al., 1979; Lambotte-Vanderpaer et al.,

1980; Duverger-van Bogaert et al., 1981; Duverger-van Bogaert et al., 1982; Connor et al., 1979; Lijinsky and Andrews, 1980). The fact that acrylonitrile is mutagenic to bacteria with or without the addition of an auxiliary metabolising system is not unexpected, since this compound is an electrophilic $\alpha\beta$-unsaturated compound capable of Michael additions allowing direct cyanoethylation of macromolecules. Acrylonitrile can also undergo oxidative metabolism at the vinylic double bond to an epoxide, 2-cyanoethylene oxide, which binds to macromolecules, including nucleic acids (see, for example, Gieger et al., 1983).

A review of the mutagenicity of acrylonitrile is available (IARC, 1979).

Benzene

Benzene does not appear to be mutagenic to bacteria. It has been tested in a variety of systems, including reverse-mutation assays in several strains of *S. typhimurium* and *E. coli* (for example, Bartsch et al., 1980; Florin et al., 1980; Probst et al., 1981; Shimizu et al., 1983) and forward mutation in *S. typhimurium* (Kaden et al., 1979; Seixas et al., 1982).

A recent review of the mutagenicity of benzene may be found in IARC (1982).

Benzoin

There were no published data for benzoin.

Caprolactam

Negative results were obtained in a single report of tests using several strains of *S. typhimurium* (Greene et al., 1979).

Diethylhexylphthalate

Negative results were reported by two laboratories in tests of diethylhexylphthalate both of which employed only 2 strains of *S. typhimurium* (TA98 and TA100). One laboratory used the microsomal fraction from ram seminal vesicles (Robertson et al., 1983), the other used Aroclor-induced rat-liver S9. Negative results were also obtained in *E. coli* WP2 and WP2uvrA (Yoshikawa et al., 1983).

Diethylstilboestrol

Diethylstilboestrol has not been shown to be mutagenic to bacteria. Consistently negative results have been obtained in several studies employing the Salmonella/mammalian microsome test using several different metabolic activation systems (for example: McCann et al., 1975; Purchase et al., 1978; Glatt et al., 1979; Lang and Redmann, 1979; Dunkel, 1979; Connor et al., 1979; Rudiger et al., 1979; Allaben et al., 1979; Bartsch et al., 1980; Affolter et al., 1983).

Hexamethylphosphoramide

Hexamethylphosphoramide gave questionably positive results in base-pair substitution mutants of *S. typhimurium* in the presence of S9 (Ashby et al., 1977). A second report from this laboratory claimed positive results using TA1535 and TA100 in the presence of S9 (Purchase et al., 1978).

Phenobarbitone

Uniformly negative results have been reported for phenobarbitone assayed in a variety of bacterial mutation tests. For example, it was negative in independent tests using several strains of *S. typhimurium* (McCann et al., 1975; Purchase et al., 1978; King et al., 1979). Multi-locus mutation assays, using *E. coli* K12(343/113), in in vitro tests using liver homogenate, and in host-mediated assays using mice were also negative (King et al., 1979).

Safrole

Attempts to demonstrate the mutagenicity of safrole in bacteria have been largely unsuccessful. Several independent assays of this compound using the Salmonella/mammalian microsome test have proved negative (for example, McCann et al., 1975; Wislocki et al., 1977; Swanson et al., 1979; Rosenkranz and Poirier, 1979; Simmon, 1979; Gocke et al., 1981; Probst et al., 1981; Sekizawa and Shibamoto, 1982). Negative results were also obtained in tests using *E. coli* WP2uvrA (Sekizawa and Shibamoto, 1982). Positive results have been claimed by Purchase et al. (1978) using the Salmonella/mammalian microsome test. In tests using a mouse-liver postmitochondrial fraction, Green and Savage (1978) showed that safrole was mutagenic to *S. typhimurium* strains TA1530 and TA1532. These authors also obtained positive results in a host-mediated assay using mice and *S. typhimurium* strains TA1950 and TA1952.

o-Toluidine

Several independent studies employing a variety of strains of *S. typhimurium* have failed to demonstrate that *o*-toluidine is a bacterial mutagen (see, for example, McCann et al., 1975; Garner and Nutman, 1977; Rosenkranz and Poirier, 1979; Simmon, 1979; Hecht et al., 1979; Zimmer et al., 1980). Nagao et al., (1977) showed that *o*-toluidine was mutagenic to TA98 only when norharman was added to the S9 mix. Tanaka et al. (1980) obtained negative results in in vitro assays using TA100 and TA98, but showed that urine from rats treated with *o*-toluidine was mutagenic to TA98.

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3

Summary Report on the performance of the yeast and Aspergillus assays

Work Group Chairman
James M. Parry

Work Group Participants
P. Arni (Absent, but data considered), T. Brooks, A. Carere, L. Ferguson, J. Heinisch, S. Inge-Vechtomov (Absent, but data considered), N. Loprieno, E. Nestmann and R. von Borstel

Three species of fungi i.e. the budding and fission yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and the filamentous fungus *Aspergillus nidulans* have been used to assay the potential genotoxicity of a range of selected chemicals.

The fungi are amongst the most intensively studied eukaryotic organisms and provide convenient tools for the assay of the induction of a variety of genetic endpoints by environmental chemicals. Their internal structure shows strong similarities to the cells of higher organisms, in that they possess a differentiated nucleus containing a nucleolus. The accurate functioning of cell division is dependent upon the synthesis of a spindle apparatus, however, unlike mammalian cells, the fungi maintain their nuclear membrane during cell division.

For all three of the fungal species used in this study, there is an extensive data base of experiments involving their use in genotoxicity studies of chemicals, which has been reviewed by the U.S. Environmental Protection Agency Gene-Tox Program (Zimmermann et al., 1984; Loprieno et al., 1983; Käfer et al., 1982).

The primary advantages of the use of fungi in genotoxicity studies may be summarized as follows: (1) Eukaryotic chromosome organization. (2) Variety of end-points which may be assayed. (3) Variety of protocols which are available for the optimization of the induction of genetic events. (4) The presence of an endogenous metabolic system. (5) Extensive background of genetic, biochemical, and genotoxicity data. (6) Cost-effective assays requiring limited technical and laboratory facilities using "robust" organisms.

Genetic endpoints

In this study a range of genetic end-points have been used with the intention of providing the maximum information on the biological profiles of the test chemicals. These include: (a) The induction of mutation in both nuclear and mitochondrial genes. (b) The induction of mitotic recombination, both between and within genes. (c) The induction of chromosome aneuploidy during mitotic cell division. (d) The induction of illegitimate mating between cells of the same mating-type resulting from "mating-type" switch events.

Point mutation

In this study the induction of point mutation has been assayed in yeast in the haploid strains *XV185-14C* and *PV-1* and the diploid strains *D7, D7-144, RM52, D6* and *D61-M.*

XV185-14C developed by von Borstel and colleagues has the following genotype:

a, ade 2-1, arg 4-17, lys 1-1, trp 5-48, his 1-7, hom 3-10

The markers *arg 4-17* and *trp 5-48* are ochre nonsense mutations that are reversible by base-substitution mutagens that induce site-specific mu-

tations and ochre suppressor mutations in tRNA loci. The marker *his 1-7* is a missense mutation which is reverted mainly by second site mutations. The *hom 3-10* allele is believed to be a frameshift defect due to its response to a range of diagnostic mutagens.

Pv-1 has the following genotype:

α leu 2-2, pho 1-100, LYS$^+$

Forward mutations resulting in auxotrophy for lysine may be detected by plating on selective medium containing α-aminoadipate instead of ammonium sulphate.

RM52 is a diploid strain of the following genotype:

a/α ade 2-40/ade 2-40, his1-7/his1-7

The strain has been used in this study to score the induction of reverse mutations at the *his1-7* allele.

D7 and *D7-144* carry the mutation *ilv 1-92* in a homozygous condition and have been used used to detect the induction of isoleucine-independent prototrophs. This multipurpose strain can also be used to detect the induction of mitotic recombination (see below).

D6 and *D61-M* carry the mutations *ade2-40* and *ilv1-92* respectively, and have been used for the assay of the induction of adenine and isoleucine-independent prototrophs. Both strains were developed primarily for the detection of induced chromosomal aneuploidy and will be described in full later.

Point mutation in *Aspergillus nidulans* has been assayed in the haploid strain *35* of the following genotype:

anA1, pabaA1, yA2, metG1, S12, nicA2, nicB8

In this strain forward mutations were assayed which result in the induction of suppressors of the defect in methionine metabolism. Such mutations are produced in at least 5 loci.

In *Schizosaccharomyces pombe* forward mutations have been assayed in the genes coding for adenine biosynthesis. The strain *P1* used in this study carries a mutation in the gene *ade-6* which results in the production of red/purple colonies. Forward mutations at any of the five genes which precede the *ade-6* mutation result in the production of white colonies on non-selective medium.

In *Saccharomyces cerevisiae* mitochondrial mutations may be detected by the assay for the frequency of respiration-deficient 'petite' colonies. Such colonies are incapable of aerobic respiration and are characterised by their small size, their inability to grow in non-fermentable carbon sources such as glycerol, and their inability to reduce the dye tetrazolium. Petite colonies may be produced by the induction of both chromosomal and extrachromosomal events. In the diploid culture *D5* used in the study, the petites are predominantly extrachromosomal in origin.

Recombination

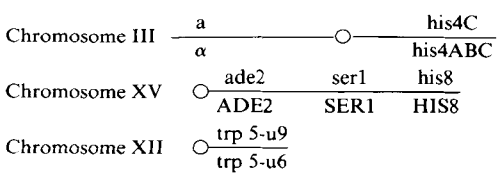
In mammalian cells, genetic exchange of homologous chromosomes are generally confined to the specialized stage of meiotic cell division. However, in fungi, recombinational events may also be detected during vegetative division although the spontaneous mitotic frequency is generally at least 1000 times less than that observed during meiosis.

Mitotic recombination can be detected in fungi both between and within genes. The former event is called mitotic crossing-over and generates reciprocal products, whereas the latter event is most frequently non-reciprocal and is called mitotic gene conversion. Crossing-over is generally assayed by the production of recessive homozygous colonies and sectors, produced in a heterozygous strain, whereas gene conversion is assayed by the production of prototrophic revertants observed in a heteroallelic strain carrying two different defective alleles of the same gene.

The value of the assay of mitotic recombination is that increases are generally produced in a non-specific manner in response to all types of mutagens irrespective of their mode of action (i.e. frameshift or base-substitution).

In this study five strains of yeast have been used for the assay of mitotic gene conversion: they were *JD1, D7, D7-144, PV-2* and *PV-3*.

Strain *JD1* has the following genotype:



his4C, his4ABC, trp5-u9 and *trp5-u6* are heteroalleles at the *HIS4* and *TRP5* loci respectively. They undergo mitotic gene conversion to produce protrophic colonies carrying one wild-type allele which allows for growth on selective medium lacking either tryptophan or histidine.

D7 is a multipurpose tester strain which carries a set of genetic markers which allow the simultaneous assay of mitotic crossing-over, mitotic gene conversion, and point mutation.

The genotype of *D7* is as follows:

Chromosome III $\frac{a}{\alpha}$ Chromosome VII $\frac{\text{trp5-12 cyh}^r\text{-2}}{\text{trp5-27 CYH}^s\text{-2}}$

Chromosome XV $\frac{\text{ade2-40}}{\text{ade2-119}}$ Chromosome V $\frac{\text{ilv1-92}}{\text{ilv1-92}}$

trp5-12 and *trp5-27* are heteroalleles of the *TRP-5* locus and undergo mitotic gene conversion to produce prototrophic colonies carrying one wild-type allele which allows for growth on selective medium lacking tryptophan. *ade2-40* is a completely inactive allele of *ADE2* which produces deep red colonies, whereas *ade2-119* is a leaky allele causing the accumulation of only a small amount of pigment and thus produces pink colonies. In heteroallelic diploids the *ade2-40* and *ade2-119* alleles complement to give rise to white adenine-independent colonies. Mitotic crossing-over in *D7* may give rise to cells homoallelic for the *ade2* mutations and thus lead to the observation of both red and pink reciprocal products. Point mutation in *D7* may be assayed at the *ILV-1* locus by the production of colonies prototrophic for isoleucine.

D7-144 is a derivative of *D7* selected for its inability to undergo sporulation and thus has the advantage of undergoing long periods of incubation with test chemicals without the problem of the induction of meiosis.

Mitotic crossing-over in yeast has also been studied in strains *D6* and *D61-M* using the presence of the single copy of the recessive cycloheximide resistant allele *cyh-2* on Chromosome VII. Crossing-over in such strains between *CYH2* and the centromere of Chromosome VII results in the production of colonies which are capable of growth in medium containing cycloheximide (i.e. cyh^r_2/cyh^r_2).

Induced mitotic crossing-over in *Aspergillus nidulans* has been assayed by the use of diploid strain P1 of genotype:

SuA1, adE20, riboA1, fpaA1, anA1, pabaA1, YA2, adeE20,

biA1, methA1, pyroA4, nicA2, lysB5, nicB8.

In strain *P1*, yellow homozygous (*YA2/YA2*) colonies arising in a heterozygous pale green background indicates the induction of mitotic crossing-over which can be however established only after genetic analysis of several nutritional markers.

Strain *PV-2* has the following genotype:

a/α, his7-1/his7-1, ade2-278/ade2-278, lys2-67/lys2-68,

can^r1/CAN^s1 RAD/rad1-5

Strain *PV-3* is homozygous for *rad1-5* (i.e. *rad1-5/rad1-5*). Mitotic gene conversion in *PV-2* and *PV-3* by the assay of lysine prototrophy produced at the *lysine-2* gene. Mitotic crossing-over in *PV-2* and *PV-3* can be assayed by the production of homozygous canavanine-resistant colonies.

Aneuploidy

Abnormal chromosome segregations leading to the production of numerical chromosome aberrations were assayed by the use of yeast strains *D6* and *D61-M* and *Aspergillus nidulans* strain *P1*.

The diploid yeast strain *D6* has the following genotype:

Chromosome VII $\frac{\text{ade3}}{\text{ADE3}} \bigcirc \frac{\text{leu1 trp5 cyh}^r\text{2 met13}}{\text{LEU1 TRP5 CYH}^s\text{2 MET13}}$

Chromosome XV $\frac{\text{ade2-40}}{\text{ade2-40}}$ Chromosome III $\frac{a}{\alpha}$

The genotype of *D61-M* is as follows:

Chromosome XV $\frac{\text{ade2-40}}{\text{ade2-40}}$

Chromosome VII $\frac{\text{ade6}}{\text{ADE6}} \bigcirc \frac{\text{leu1 TRP5 cyh}^r\text{2}}{\text{LEU1 TRP5 CYH}^s\text{2}}$

Chromosome V $\frac{\text{ilv1-92}}{\text{ilv1-92}}$

Both strains carry a series of recessive marker on Chromosome VII, they produce red colonies on high glucose medium due to the presence of the defective alleles *ade 2-40,* of the gene *adenine-2* on

TABLE 1

FUNGAL TEST PROTOCOLS

Collaborator	Gene conversion			Aneuploidy			Homozygosity			Nuclear mutation			Mitochondrial mutation		
	Strain	Growth phase	S9	Strain	Growth phase	S9	Strain	Growth phase	S9	Strain	Growth phase	S9	Strain	Growth phase	S9
Mehta/von Borstel	D7-144	Log	±							XV185-14C Arg His Trp	Log	±			
										RM52 HIS	Log	±			
Zimmermann/Heinisch				D61-M	Log	–	D61-M	Log	–						
Harrington/ Nestmann										XV-185-14C His Hom Trp	Log	±			
Carere				Asperg. P1	Log	–	Asperg. P1	Log	–	Asperg. 35	Log	–			
Loprieno/Forster										*S. pombe* P1	Log	±			
Ferguson													D5	Log	–
														HG	–
Brooks/Calvert	JD1	Log	±												
		Stat	±												
		HG	–												
Parry	D7	HG	±	D6	HG	–	D7	HG	–	D7	HG	–			
				D61-M	HG	±	D6	HG	–	D6	HG	–			
					Stat	±	D61-M	HG	±	D61-M	HG	±			
								Stat	±		Stat	±			
Arni	D7	Log	±												

Log, cells in exponential growth phase.

Stat, stationary phase cells.

HG, cells grown in high glucose medium, i.e., optimised yeast cytochrome P450.

Chromosome XV, they require adenine for growth and are sensitive to cycloheximide. The loss of a copy of Chromosome VII carrying the wild-type markers results in the production of cells carrying only the recessive and mutant alleles on Chromosome VII. The resulting monosomic cells ($2n-1$) are white in colour due to the presence of the defective allele of the gene *adenine-3*, in the case of *D6*, and *adenine-6* in the case of *D61-M*, which results in a mutant block in the adenine biosynthetic pathways prior to the *adenine-2* gene. This prevents the formation of the red pigment. The expression in monosomic colonies of the recessive form of *cycloheximide-2* results in the production of cells resistant to the presence of cycloheximide.

Mitotic chromosome aneuploidy in *Aspergillus nidulans* was detected in strain *P1* by the analysis of yellow homozygous *YA2 / YA2* and hemizygous (y*A2*) sectors and the classification of such sectors by analysis of their nutritional requirements.

Illegitimate mating

Mating-type "switch" events from the α to the *a* mating-type induced in the presence of the test compounds were assayed by the use of the strains *PV-4a* and *PV-4b* whose genotypes are shown below:

PV-4a:α, his7-1, his4-B26, leu2-1, lys2-A37, metA1

PV4-b:α, ade1-6, ade2-163, leu2-2, thr4-B15, lys2-A12

Both strains carry a range of genetic markers conferring multiple auxotrophic requirements which prevent growth of the strains on selective medium.

Exposure of either strain *PV-4a* or *PV-4b* to agents which induce mating-type switch events results in the conversion of a proportion of the α mating type genes to the *a* mating type. If such an *a* cell is induced in strain *PV-4b* then mating may occur with α cells of strain *PV-4b* to produce a prototrophic diploid capable of growth on minimal selective medium. The frequency of such prototrophic diploids may be used to estimate the frequency of induced mating-type "switch" events.

Treatment protocols

When inoculated into or onto nutrient medium, fungal cells undergo a period of exponential or log-phase vegetative growth until the nutrients are exhausted and the culture enters a resting period called stationary-phase (defined as G_0). The various treatment procedures used in this study are outlined in Table 1. They involve the treatment of stationary-phase cells in an appropriate buffer (STAT), exponential-phase cells (LOG) for various periods of time and the growth of cells in/on nutrient medium (treatments of cells on solid growth medium were only used with Aspergillus, all the yeast experiments involved liquid treatment).

In some of the experiments described here, the use has been made of the considerable endogenous metabolic capacity of yeast and Aspergillus by the use of protocols optimised to produce maximum levels of cytochrome P-450 in the exposed cells (Kelly and Parry, 1983). In Table 1 they are described as high glucose treatments (HG) and were characterised during the study by the resistance of such cultures to the toxic effects of the study chemicals compared to other protocols.

A wide range of different positive control chemicals were used by the collaborators both to verify the sensitivity of their assay and to confirm the activity of their exogenous or endogenous metabolic systems. These chemicals are illustrated in Table 2.

Criteria for the analysis of the data used by the IPCS yeast group

Due to the number of genetic endpoints used in the study and in order for the group to make the analysis as stringent as possible the working group used the following initial criteria to evaluate the results provided:

(a) An increase in plate counts (i.e. yield rather than frequency) of greater than $2\times$ above background at (at least) 3 data points, score = 2 (++).

(b) An increase in plate counts of greater than $2\times$ at 2 data points, score = 1 (+).

(c) No increase above background or an increase at only a single data point, score = 0 (−).

For the final evaluation, additional criteria were later added by the working group to overcome possible inconsistencies between replicate experiments:

(d) If one experiment was clearly positive, the other experiment was also considered positive pro-

vided that there was at least one positive result on the basis of plate counts or frequency; if there was no supportive evidence in a replicate experiment(s) the data were judged as inconsistent and the experiment scored as negative.

(e) When an experiment had clearly been performed under inappropriate dose ranges or experimental conditions this was treated as a "no test" (NT). All chemicals were treated on the same basis and with equal vigour.

Results

The results of all the assays of the various genetic endpoints are illustrated in Table 3(1–10).

(1) Mitotic gene conversion (R3, 163 tests)
(2) Chromosome aneuploidy (CN1, 70 tests)
(3) Mitotic crossing-over producing recessive homozygosis (92 tests)
(4) Nuclear mutation (M1, 301 tests)
(5) Mitochondrial mutation (M2, 39 tests).
Total = 666 tests.

Some data on the test chemicals was available from the laboratory of Professor Inge-Vetchtomov (University of Leningrad) and Dr. P. Arni (Ciba-Geigy) which was considered by the working group but could not be exposed to the same standard of scrutiny in the absence of the experimenters concerned. Such data has not been included in all of the tables but will be discussed in the general evaluation of the data base.

The data considered in this overview was that which was made available at St. Simons Island in October 1983. Subsequent to that meeting some investigators have performed further studies which are reported in their individual papers (Mehta and von Borstel, 1984). All the conclusions outlined in Table 3 have been evaluated using the criteria presented earlier and represent "clear-cut" and unambiguous tests for each chemical in the study using strains capable of detecting the various genetic endpoints.

Table 4 illustrates the lowest effective concentrations (LEC) required to induce individual genetic effects by the test chemicals and the maximum concentrations tested in the case of negative results.

The overall "scores" for the individual test

TABLE 2

LIST OF POSITIVE CONTROL COMPOUNDS USED IN FUNGAL ASSAYS OF IPCS CHEMICALS

2-Acetylaminofluorene	Arni, Inge-Vechtomov
Bavistan	Zimmermann and Heinisch
Benomyl	Carere
Chenodeoxycholic acid	Parry
Chloral hydrate	Carere
Cyclophosphamide	Arni, Brooks and Calvert, Loprieno, Mehta and von Borstel, Parry
Diethylnitrosamine	Carere
Dimethylnitrosamine	Harrington and Nestmann
Ethidium bromide	Ferguson
Ethyl methanesulphonate	Ferguson, Mehta and von Borstel, Harrington and Nestmann
Hycanthone	Zimmermann and Heinisch
ICR-170	Inge-Vechtomov
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine	Brooks and Calvert
Methyl methanesulphonate	Carere, Loprieno
4-Nitroquinoline-*N*-oxide	Arni, Brooks and Calvert
β-Propiolactone	Inge-Vechtomov

TABLE 3b

Test substance: *BENZENE*

Strain	Endpoint	Cell state	S9±	Test 1	Test 2	Test 3	LEC or MAX	Lab
JDI	R3	STAT	−	−	−			
	R3	STAT	+	−	−			
	R3	LOG	−	−	−		MAX 2000 μg/ml	TB/RC
	R3	HG	−	−	NT			
	R3	HG	+	NT	NT			
D7-144	R3	LOG	−	−	−			
	R3	LOG	+	−	−		MAX 4.4 μg/ml	RM/vB
D7	R3	HG	−	−				
	R3	HG	+	−			MAX 2000 μg/ml	JMP
D61-M	CNI	LOG	−	+ +	+ +		LEC 3.9 μl/ml	FZ/JH
D6	CNI	HG	−	+ +				
D61-M	CNI	HG	−	+ +				
	CNI	STAT	−	+			LEC 10 μg/ml	JMP
	CNI	STAT	+	+ +				
	CNI	HG	−	−				
	CNI	HG	+	−				
D61-M	H	L	−	−			MAX 5.96 μl/ml	FZ/JH
D6	H	HG	−	+			LEC 100 μg/ml	
D61-M	H	HG	−	−				
	H	STAT	−	−				
	H	STAT	+	−			MAX 2000 μg/ml	JMP
	H	HG	−	−				
	H	HG	+	−				
D6	MI-ADE	HG	−	−			MAX 2000 μg/ml	JMP
D61-M	MI-ISO	HG	−	−				
	MI-ISO	STAT	−	−				
	MI-ISO	STAT	+	+			LEC 200 μg/ml	JMP
	MI-ISO	HG	−	−				
	MI-ISO	HG	+	+				
RM52	MI-HIS	L	−	−				
	MI-HIS	L	+	−			MAX 1000 μg/ml	RM/vB
D7	MI-ISO	HG	−	+				
	MI-ISO	HG	+	+			LEC 100 μg/ml	JMP
S. pombe								
P1	MI-F	LOG	−	−	−			
	MI-F	LOG	+	−	−		MAX = 1000 μg/ml	NL
Aspergillus								
35	MI-F	LOG	−	−			MAX = 2.197 μg/ml	AC
XV185-14C	MI-ARG	LOG	−	+ +				
	MI-ARG	LOG	+	−			LEC = 275 μg/ml	RM/vB
	MI-HIS	LOG	−	+				
	MI-HIS	LOG	+	−				
	MI-TRP	LOG	−	+				
	MI-TRP	LOG	+	−				
D5	M2F	LOG	−	+	+			
	M2F	HG	−	−	−		LEC = 2500 μg/ml	LF

TABLE 3a

RESULTS OF THE ASSAY OF 10 IPCS CHEMICAL USING 5 GENETIC END POINTS MEASURABLE IN FUNGI

Test substance: *ACRYLONITRILE*

Strain	Endpoint	Cell state	S9 ±	Test 1	Test 2	Test 3	LEC or MAX	Lab.
JDI	R3	STAT	–	–	–			
	R3	STAT	+	+	+			
	R3	LOG	–	–			LEC = 250 μg/ml	TB/RC
	R3	HG	–	NT	+			
D7-144	R3	LOG	–	+	+			
	R3	LOG	+	–	–		LEC = 0.8 μg/ml	RM/vB
D7	R3	HG	–	+ +				
	R3	HG	+	+ +			LEC = 20 μg/ml	JMP
Pv-3	R3	STAT	+	–				
	R3	STAT	–	+			MAX = 1000 μg/ml	I–V
Pv-2	R3	STAT	–	–				
	R3	STAT	+	–			MAX = 1000 μg/ml	I–V
D6	CNI	HG	–	+ +			LEC = 20 μg/ml	JMP
D61-M	CNI	HG	–	+ +				
	CNI	STAT	–	+ +				
	CNI	STAT	+	+ +			LEC = 200 μg/ml	JMP
	CNI	HG	–	–				
	CNI	HG	+	+ +				
D61-M	CNI	LOG	–	–			MAX = 0.99 μg/ml	JH/FZ
D6	H	HG	–	+ +			LEC = 20 μg/ml	JMP
D61-M	H	HG	–	–				
	H	STAT	–	+				
	H	STAT	+	+ +			LEC = 200 μg/ml	JMP
	H	HG	–	+ +				
	H	HG	+	+ +				
Aspergillus P1	H	LOG	–	+			LEC = 806 μg/ml	AC
D7	MI-ISO	HG	–	–				
	MI-ISO	HG	+	–			MAX = 200 μg/ml	JMP
XV185-14C	MI-HIS	LOG	–	+	+			
	MI-HIS	LOG	+	–	–			
	MI-TRP	LOG	–	–	–		LEC = 0.8 μg/ml	RM/vB
	MI-TRP	LOG	+	–	–			
	MI-ARG	LOG	–		+			
	MI-ARG	LOG	+		–			
S. pombe P1	MI-F	LOG	–	–	–		MAX = 250 μg/ml	NL
	MI-F	LOG	+	–	–			
D6	MI-ADE	HG	–	+ +			LEC = 200 μg/ml	JMP
D61-M	MI-ISO	HG	–	–				
	MI-ISO	STAT	–	+ +				
	MI-ISO	STAT	+	+ +			LEC = 200 μg/ml	JMP
	MI-ISO	HG	–	+ +				
	MI-ISO	HG	+	+				
D5	M2F	LOG	–	+	+		LEC = 250 μg/ml	LF
	M2F	HG	–	+	+			

TABLE 3c

Test substance: *BENZOIN*

Strain	Endpoint	Cell State	S9±	Test 1	Test 2	Test 3	LEC or MAX	Lab
JDI	R3	STAT	–	–	–		MAX 2000 μg/ml	TB/RC
	R3	STAT	+	–	–			
	R3	LOG	–	–	NT			
	R3	LOG	+	–	NT			
	R3	HG	–	NT	–			
	R3	HG	+	NT	NT			
D7.144	R3	LOG	–	–	–			
	R3	LOG	+	–	–		MAX 1250 μg/ml	RM/vB
D7	R3	HG	–	–				
	R3	HG	+	–			MAX 5000 μg/ml	JMP
D7	R3	STAT	–	–				
	R3	STAT	+	–			Not stated	PA
D61-M	CNI	LOG	–	NT				FZ/JH
D6	CNI	HG	–	–			MAX 5000 μg/ml	JMP
D61-M	CNI	HG	+	+ +			LEC 500 μg/ml	JMP
	CNI	STAT	–	–				
	CNI	STAT	+	+ +			Suspicion of	
	CNI	HG	–	–			phenotypic	
	CNI	HG	+	–			effects (selection)	
D6	H	HG	–	– i			MAX 5000 μg/ml	JMP
D61-M	H	HG	–	+ +				
	H	STAT	–	–				
	H	STAT	+	–				
	H	HG	–	–			LEC 500 μg/ml	JMP
	H	HG	+	–				
D61-M	H	LOG	–	NT				FZ/JH
D6	MI-ADE	HG	–	–			MAX 5000 μg/ml	JMP
D61-M	MI-ISO	HG	–	+ +				
	MI-ISO	STAT	–	–				
	MI-ISO	STAT	+	–			LEC 500 μg/ml	
	MI-ISO	HG	–	–				
	MI-ISO	HG	+	–				
XV185-14C	MI-ARG	LOG	–	–			MAX 1000 μg/ml	RM/vB
	MI-ARG	LOG	+	–				
	MI-HIS	LOG	–	–	–			
	MI-HIS	LOG	+	–	–			
	MI-TRP	LOG	–	–	–			
	MI-TRP	LOG	+	–	–			
XV185-14C	MI-TRP	LOG	–	–	–			
	MI-TRP	LOG	+	–	–		LEC 25 μg/ml	TH/EN
	MI-HOM	LOG	+	–	–			
	MI-HIS	LOG	–	+	+ +			
	MI-HIS	LOG	+	+	+ +			

TABLE 3c (continued)

Strain	Endpoint	Cell State	S9±	Test 1	Test 2	Test 3	LEC or MAX	Lab
RM52	MI-HIS	LOG	–	–			MAX 1000 μg/ml	RM/vB
	MI-HIS	LOG	+	–				
D7	MI-ISO	HG	–	–				
	MI-ISO	HG	+	–			MAX 5000 μg/ml	JMP
S. pombe	MIF	LOG	–	–	NT			
P1	MIF	LOG	+	–	–		MAX 1900 μg/ml	NL
Aspergillus	MIF	LOG	–	NT				
35	MIF	LOG	+	NT				
D7	MI-ISO	STAT	–	–				
	MI-ISO	STAT	+	–			Not stated	PA
D5	M2F	LOG	–	–				
	M2F	HG	–	–	–		MAX 5000 μg/ml	LF

TABLE 3d

Test substance: *CAPROLACTAM*

Strain	Endpoint	Cell state	S9±	Test 1	Test 2	Test 3	LEC or MAX	Lab
JDI	R3	STAT	–	–	–		MAX 200 μg/ml	TB/RC
	R3	STAT	+	–	–			
	R3	LOG	–	–	NT			
	R3	LOG	+	–	NT			
	R3	HG	–	NT	–			
	R3	HG	+	NT	NT			
D7	R3	LOG	–	–	–			
		LOG	+	–	–		MAX 800 μg/ml	RM/vB
D7	R3	HG	–	–				
		HG	+	–			MAX 2000 μg/ml	JMP
D61-M	CNI	LOG	–	–			MAX 1500 μg/ml	FZ/JH
D6	CNI	HG	–	–			MAX 5000 μg/ml	JMP
D61-M	CNI	HG	–	–				
	CNI	STAT	–	–				
	CNI	STAT	+	–			MAX 5000 μg/ml	JMP
	CNI	HG	–	–				
	CNI	HG	+	–				
D6	H	HG	–	–			MAX 5000 μg/ml	JMP
D61-M	H	HG	–	–			MAX 5000 μg/ml	JMP
	H	STAT	–	–				
	H	STAT	+	–				
	H	HG	–	–				
	H	HG	+	–				
D61-M	H	LOG	–	–			MAX 15000 μg/ml	FZ/JH

TABLE 3d (continued)

Strain	Endpoint	Cell state	S9 ±	Test 1	Test 2	Test 3	LEC or MAX	Lab
D6	MI-ADE	HG	–	–			MAX 2000 μg/ml	JMP
D61-M	MI-ISO	HG	–	–				
	MI-ISO	STAT	–	–			MAX 2000 μg/ml	JMP
	MI-ISO	STAT	+	–				
	MI-ISO	HG	–	–				
	MI-ISO	HG	+	–				
XV185-14C	MI-ARG	LOG	–	+ +				
	MI-ARG	LOG	+	+ +			LEC 100 μg/ml	RM/vB
	MI-HIS	LOG	–	+ +				
	MI-HIS	LOG	+	–				
	MI-TRP	LOG	–	+ +				
XV185-14C	MI-TRP	LOG	–	–	–		LEC 100 μg/ml	TH/EN
	MI-TRP	LOG	+	–	–			
	MI-HOM	LOG	–	–	–			
	MI-HOM	LOG	+	+ +	+			
	MI-HIS	LOG	–	–	–			
	MI-HIS	LOG	+	–	–			
RM52	MI-HIS	LOG	–	–			MAX 800 μg/ml	RM/vB
	MI-HIS	LOG	+	–				
D7	MI-ISO	HG	–	–			MAX 5000 μg/ml	JMP
	MI-ISO	HG	+	–				
S. pombe P1	MIF	LOG	–	–	NT		MAX 1900 μg/ml	NL
		LOG	+	–	–			
Aspergillus 35	MIF	LOG	–	–			MAX 1000 μg/ml	AC
		LOG	+	NT				
D7	MI-ISO	STAT	–	–			Not stated	PA
		STAT	+	–				
D5	M2F	LOG	–	–	–		MAX 5000 μg/ml	LF
		HG	–	–	–			

TABLE 3e

Test substance: *DEHP*

Strain	Endpoint	Cell state	S9 ±	Test 1	Test 2	Test 3	LEC or MAX	Lab
JD1	R3	STAT	–	–	–		MAX 200 μg/ml	TB/RC
	R3	STAT	+	–	–			
	R3	LOG	–	–	NT			
	R3	LOG	+	–	NT			
	R3	HG	–	NT	–			
	R3	HG	+	NT	NT			
D7-144	R3	LOG	–	–	–		MAX 4.9 μg/ml	RM/vB
	R3	LOG	+	–	–			
D7	R3	HG	–	–			MAX 2000 μg/ml	JMP
	R3	HG	+	–				

TABLE 3e (continued)

Strain	Endpoint	Cell state	S9 ±	Test 1	Test 2	Test 3	LEC or MAX	Lab
D7	R3	STAT	–	–				PA
	R3	STAT	+	–				
D61-M	CNI	LOG	–	NT				FZ/JH
D6	CHI	HG	–	+ +			LEC 5 μg/ml	JMP
D61-M	CNI	HG	–	+ +				
	CNI	STAT	–	+ +			LEC 5 μg/ml	JMP
	CNI	STAT	+	+ +				
	CNI	HG	–	–				
	CNI	HG	+	+ +				
D61-M	H	LOG	–	–			MAX 2000 μg/ml	FZ/JH
D6	H	HG	–	–			MAX 2000 μg/ml	JMP
D61-M	H	HG	–	–				
	H	STAT	–	–				
	H	STAT	+	–				
	H	HG	–	–				
	H	HG	+	–				
Aspergillus P1	H	LOG	–	–			LEC 2465 μg/ml	AC
XV185-14C	MI-ARG	LOG	–	–			MAX 9.4 g/ml	RM/vB
	MI-ARG	LOG	+	–				
	MI-HIS	LOG	–	–	–			
	MI-HIS	LOG	+	–	–			
	MI-TRP	LOG	–	–	–			
	MI-TRP	LOG	+	–	–			
D7	MI-ISO	STAT	–	–				PA
	MI-ISO	STAT	+	–				
RM52	MI-HIS	LOG	–	–			MAX 4.9 μg/ml	RM/vB
	MI-HIS	LOG	+	–				
D7	MI-ISO	HG	–	–			MAX 2000 μg/ml	JMP
	MI-ISO	HG	+	–				
S. pombe P1	MIF	LOG	–	–	–		MAX 5870 μg/ml	NL
		LOG	+	–	–			
Aspergillus 35	MIF	LOG	–	NT				AC
	MIF	LOG	+	NT				
D6	MI-ADE	HG	–	–			MAX 5000 μg/ml	JMP
D61-M	MI-ISO	HG	–	–				
	MI-ISO	STAT	–	–				
	MI-ISO	STAT	+	–				
	MI-ISO	HG	–	+ +			LEC 200 μg/ml	JMP
	MI-ISO	HG	+	+				
D5	M2F	LOG	–	–	–		MAX 200000	LF
	M2F	HG	–	–	–			

TABLE 3f

Test substance: *DES*

Strain	Endpoint	Cell state	S9 ±	Test 1	Test 2	Test 3	LEC or MAX	Lab
JD1	R3	STAT	–	–	–		MAX 2000 μg/ml	TB/RC
	R3	STAT	+	–	–			
	R3	LOG	–	–	NT			
	R3	LOG	+	–	NT			
	R3	HG	–	NT	–			
	R3	HG	+	NT	NT			
D7.144	R3	LOG	–	NT				RM/vB
	R3	LOG	+	NT				
D7	R3	HG	–	–			MAX 50 μg/ml	JMP
	R3	HG	+	–				
D61-M	CN1	LOG	–	+ +	+ +		LEC 7.4 μg/ml	FZ/JH
D6	CN1	HG	–	+ +			LEC 5 μg/ml	JMP
D61-M	CN1	HG	–	+				
	CNI	STAT	–	+ +			LEC 5 μg/ml	JMP
	CNI	STAT	+	–				
	CNI	HG	–	+ +				
	CNI	HG	+	+ +				
D61-M	H	LOG	–	–	–		MAX 17.2 μg/ml	FZ/JH
D6	H	HG	–	–				
	H	HG	–	–			MAX 50 μg/ml	JMP
	H	STAT	–	–				
	H	STAT	+	–				
	H	HG	–	–				
	H	HG	+	–				
D6	MIR-ADE	HG	–	–			MAX 50 μg/ml	JMP
D61-M	MIR-ISO	HG	–	–				
	MIR-ISO	STAT	–	–			MAX 50 μg/ml	JMP
	MIR-ISO	STAT	+	–				
	MIR-ISO	HG	–	–				
	MIR-ISO	HG	+	–				
XV185-14C	MI-ARG	LOG	–	NT				RM/vB
	MI-ARG	LOG	+	NT				
	MI-HIS	LOG	–	NT				
	MI-HIS	LOG	+	NT				
	MI-TRP	LOG	–	NT				
	MI-TRP	LOG	+	NT				
RM52	MI-HIS	LOG	–	NT				RM/vB
	MI-HIS	LOG	+	NT				
D7	MI-ISO	HG	–	–			MAX 50 μg/ml	JMP
S. pombe P1	MIF	LOG	–	–	–		MAX 50 μg/ml	NL
		LOG	+	–	–			
Aspergillus 35	MIF	LOG	–	NT				AC
DS	M2F	LOG	–	–	–		MAX 500 μg/ml	LF
		HG	–	–	–			

TABLE 3g

Test substance: *HMPA*

Strain	Endpoint	Cell state	S9±	Test 1	Test 2	Test 3	LEC or MAX	Lab
JD1	R3	STAT	–	–	–		MAX 2000 μg/ml	TB/RC
		STAT	+	–	–			
		LOG	–	–	NT			
		HG	–	NT	–			
		HG	+	NT	NT			
D7-144	R3	LOG	–	–	–		MAX 41.1 μg/ml	RM/vB
		LOG	+	–	–			
D7	R3	HG	–	–			MAX 500 μg/ml	JMP
		HG	+	–				
D61-M	CNI	LOG	–	NT				FZ/JH
D6	CNI	HG	–	+ +			LEC 200 μg/ml	JMP
D61-M	CNI	HG	–	+ +			LEC 200 μg/ml	JMP
	CNI	STAT	–	+ +				
	CNI	STAT	+	+ +				
	CNI	HG	–	+ +				
	CNI	HG	+	+ +				
D61-M	H	LOG	–	NT				FZ/JH
D6	H	HG	–	–			LEC 50 μg/ml	JMP
	H	STAT	–	–				
	H	STAT	+	–				
	H	HG	–	–				
	H	HG	+	–				
RM52	MI-HIS	LOG	–	+ +			LEC 5.1 μg/ml	RM/VB
	MI-HIS	LOG	+	+ +				
D7	MI-ISO	HG	–	–				JMP
	MI-ISO	HG	+	–			MAX 500 μg/ml	
S. pombe P1	MIF	LOG	–	+	NT		LEC 20 μg/ml	NL
		LOG	+	–	–			
XV185-14C	MI-HIS	LOG	–	–	–		LEC 174 μg/ml	TH/EN
	MI-HIS	LOG	+	+	+			
	MI-HOM	LOG	–	–	–		MAX 3490 μg/ml	TH/EN
	MI-HOM	LOG	–	–	–			
	MI-TRP	LOG	–	–	–			
	MI-TRP	LOG	+	–	–			
	MI-ARG	LOG	–	+ +			LEC 5140 μg/ml	RM/vB
	MI-ARG	LOG	+	+				
	MI-HIS	LOG	–	+ +	+ +			
	MI-HIS	LOG	+	–	–			
	MI-TRP	LOG	–	NT	+ +			
			–	–				
D6	MI-ADE	HG	–	–			MAX 2000 μg/ml	JMP

TABLE 3g (continued)

Strain	Endpoint	Cell state	S9 ±	Test 1	Test 2	Test 3	LEC or MAX	Lab
D61-M	MI-ISO	HG	–	–			LEC 200 μg/ml	JMP
	MI-ISO	STAT	–	+ +				
	MI-ISO	STAT	+	+ +				
	MI-ISO	HG	–	+ +				
	MI-ISO	HG	+	+				
D5	M2F	LOG	–	–	–		MAX 100000 μg/ml	LF
		HG	–	–	–			

TABLE 3h

Test substance: *PHENOBARBITAL*

Strain	Endpoint	Cell state	S9 ±	Test 1	Test 2	Test 3	LEC or MAX	Lab
JD1	R3	STAT	–	–	–		MAX 2000 μg/ml	TB/RC
	R3	STAT	+	–	–			
	R3	LOG	–	–	NT			
	R3	LOG	+	–	NT			
	R3	HG	–	NT	–			
	R3	HG	+	NT	NT			
D7-144	R3	LOG	–	–	–		MAX 2000 μg/ml	RM/vB
		LOG	+	–	–			
D7	R3	HG	–	–			MAX 5000 μg/ml	JMP
		HG	+	–				
D7	R3	STAT	–	NT				PA
		STAT	+	NT				
D61-M	CNI	LOG	–	NT				FZ/JH
D6	CNI	HG	–	+ +			LEC 1000 μg/ml	JMP
D61-M	CNI	HG	–	+				
	CNI	STAT	–	–			LEC 1000 μg/ml	JMP
	CNI	STAT	+	+ +				
	CNI	HG	–	–				
	CNI	HG	+	+				
D6	H	HG	–	–			MAX 5000 μg/ml	JMP
D61-M	H	HG	–	+			LEC 3000 μg/ml	JMP
	H	STAT	–	–				
	H	STAT	+	–				
	H	HG	–	+				
	H	HG	+	–				
D61-M	H	LOG	–	NT				FZ/JH
D6	MI-ADE	HG	–	–			MAX 5000 μg/ml	JMP

TABLE 3h (continued)

Strain	Endpoint	Cell state	S9±	Test 1	Test 2	Test 3	LEC or MAX	Lab
D61-M	MI-ISO	HG	–	–				JMP
	MI-ISO	STAT	–	–				
	MI-ISO	STAT	+	–				
	MI-ISO	HG	–	+ +				
	MI-ISO	HG	+	–				
XV185-14C	MI-ARG	LOG	–	+ +			LEC 250 μg/ml	RM/vB
	MI-ARG	LOG	+	–				
	MI-HIS	LOG	–	+ +	NT			
	MI-HIS	LOG	+	–	NT			
	MI-TRP	LOG	–	+	NT			
	MI-TRP	LOG	+	+ +	NT			
RM52	MI-HIS	LOG	–	–			MAX 2000 μg/ml	RM/vB
	MI-HIS	LOG	+	–				
D7	MI-ISO	HG	–	–			MAX 5000 μg/ml	JMP
	MI-ISO	HG	+	–				
S. pombe P1	MIF	LOG	–	–	–		MAX 40 μg/ml	NL
		LOG	+	–	–			
Aspergillus 35	MIF	LOG	–	NT				AC
		LOG	+	NT				
D7	MI-ISO	STAT	–	–				PA
	MI-ISO	STAT	+	–				
D5	M2F	LOG	–	–	–		MAX 5000 μg/ml	LF
		HG	–	–	–			

TABLE 3i

Test substance: *SAFROLE*

Strain	Endpoint	Cell state	S9±	Test 1	Test 2	Test 3	LEC or MAX	Lab
JD1	R3	STAT	–	–	–			
	R3	STAT	+	–	–			TB/RC
	R3	LOG	–	–			MAX 2000 μg/ml	
	R3	LOG	+	–				
	R3	HG	–	–				
	R3	HG	+	NT				
D7-144	R3	LOG	–	–	–		MAX 0.08768 μg/ml	RM/vB
D7	R3	HG	–	+ +			LEC 10 g/ml	JMP
	R3	HG	+	+				
D61-M	CNI	LOG	–	–			MAX 0.06 μg/ml	FZ/JH
D6	CNI	HG	–	+ +			LEC 10 μg/ml	JMP

TABLE 3i (continued)

Strain	Endpoint	Cell state	S9 ±	Test 1	Test 2	Test 3	LEC or MAX	Lab
D61	CNI	HG	–	+				
	CNI	STAT	–	+ +			LEC 10 μg/ml	JMP
	CNI	STAT	+	+ +				
	CNI	HG	–	+				
	CNI	HG	+	+ +				
Asp. P1	CNI	LOG	–	+			LEC 5480 μg/ml	AC
D61-M	H	LOG	–	NT				FZ/JH
D6	H	HG	–	–			MAX 1000 μg/ml	JMP
D61-M	H	HG	–	–				
	H	STAT	–	–			MAX 1000 μg/ml	JMP
	H	STAT	+	–				
	H	HG	–	–				
	H	HG	+	–				
D6	MI-ADE	HG	–	+ +			LEC 100 μg/ml	JMP
D61-M	MI-ISO	HG	–	–				
	MI-ISO	STAT	–	+ +				
	MI-ISO	STAT	+	+ +			LEC 50 μg/ml	JMP
	MI-ISO	HG	–	–				
	MI-ISO	HG	+	+ +				
RM52	MI-ISO	LOG	–	–			MAX 0.08768 μg/ml	RM/vB
D7	MI-ISO	HG	–	–				
	MI-ISO	HG	+	–			MAX 200 μg/ml	JMP
S. pombe	MI-F	LOG	–	+	NT		LEC 7 μg/ml	NL
P1	MI-F	LOG	+	–	–			
Aspergillus	MI-F	LOG	–	–			MAX 5480 μg/ml	AC
	MI-F	LOG	+	NT				
XV185-14C	MI-ARG	LOG	–	+				
	MI-ARG	LOG	+	–			LEC 0.0109 μg/ml	RM/vB
	MI-HIS	LOG	–	NT	+			
	MI-HIS	LOG	+	NT	–			
	MI-TRP	LOG	–	NT	+			
	MI-TRP	LOG	+	NT	–			
XV185-14C	MI-HIS	LOG	–	–	–			
	MI-HIS	LOG	+	+	+ +		LEC 3.7 μg/ml	TH/EN
	MI-HOM	LOG	–	–	–			
	MI-HOM	LOG	+	–	–			
	MI-TRP	LOG	–	–	–			
	MI-TRP	LOG	+	–	–			
D5	M2F	LOG	–	+ +	+ +		LEC 312 μg/ml	
		HG	–	–	–		MAX 25000 μg/ml	LF

TABLE 3j

Test substance: *o-TOLUIDINE*

Strain	Endpoint	Cell state	S9 ±	Test 1	Test 2	Test 3	LEC or MAX	Lab
JDI	R3	STAT	–	–	–		MAX 2000 μg/ml	TB/RC
		STAT	+	–	–			
		LOG	–	–	NT			
		LOG	+	–	NT			
		HG	–	NT	–			
		HG	+	NT	NT			
D7-144	R3	LOG	–	–	–		MAX 3.0 μg/ml	RM/vB
		LOG	+	–	–			
D7	R3	HG	–	–				
		HG	+	–			MAX 500 μg/ml	JMP
D61-M	CNI	LOG	–	–	–		MAX 2.4 μg/ml	FZ/JH
D6	CNI	HG	–	+ +			LEC 600 μg/ml	JMP
D61-M	CNI	HG	–	+ +			LEC 600 μg/ml	JMP
	CNI	STAT	–	+ +				
	CNI	STAT	+	+ +				
	CNI	HG	–	+				
	CNI	HG	+	+				
D61-M	H	LOG	–	–	–		MAX 259 μg/ml	FZ/JH
D6	H	HG	–	–			MAX 2000 μg/ml	JMP
D61-M	H	HG	–	+ +				
	H	STAT	–	–			LEC 50 μg/ml	JMP
	H	STAT	+	–				
	H	HG	–	–				
	H	HG	+	–				
S. pombe								
P1	MIF	LOG	–	–			MAX 1920 μg/ml	NL
		LOG	+	–				
XV185-14C	MI-ARG	LOG	–	–				
	MI-ARG	LOG	+	–			MAX 3 μg/ml	RM/vB
	MI-HIS	LOG	–	–	–			
	MI-HIS	LOG	+	–	–			
	MI-TRP	LOG	–	–	–			
	MI-TRP	LOG	+	–	–			
RM52	MI-HIS	LOG	–	+			LEC 378 μg/ml	RM/vB
	MI-HIS	LOG	+	–				
D7	MI-ISO	HG	–	–			MAX 500 μg/ml	JMP
	MI-ISO	HG	+	–				
XV185-14C	MI-HIS	LOG	–	–	–	NT	MAX 21.2 μg/ml	TH/EN
		LOG	+	–	–	–		
	MI-ARG	LOG	+	–	–	–		
	MI-TRP	LOG	–	–	–	NT		
	MI-TRP	LOG	+	–	–	–		
D6	MI-ADE	HG	–	–			MAX 2400 μg/ml	JMP

TABLE 3j (continued)

Strain	Endpoint	Cell state	S9 ±	Test 1	Test 2	Test 3	LEC or MAX	Lab
D61-M	MI-ISO	HG	–	+				
	MI-ISO	STAT	–	+				
	MI-ISO	STAT	+	–			LEC 100 μg/ml	JMP
	MI-ISO	HG	–	–				
	MI-ISO	HG	+	–				
D5	M2F	LOG	–	+	NT		LEC 8000 μg/ml	LF
		HG	–	–	–		MAX 25000 μg/ml	

Footnote to Table 3

R3	Mitotic gene conversion
CN_1	Mitotic aneuploidy
H	Mitotic segregation leading to homozygosity
M1	Nuclear point mutation
M2	Mitochondrial mutation
Stat	Stationary phase culture
Log	Exponential phase culture
HG	High glucose treatment
NT	compound not tested or test inadequately performed
MI-ISO	reverse mutation producing isoleucine$^+$ prototrophs
MI-HIS	reverse mutation producing histidine$^+$ prototrophs
MI-TRP	reverse mutation producing tryptophan$^+$ prototrophs
MI-ARG	reverse mutation producing arginine$^+$ prototrophs
MI-ADE	reverse mutation producing adenine$^+$ prototrophs
Mi-HOM	forward mutation

TABLE 4

LOWEST EFFECTIVE CONCENTRATIONS (LEC) FOR DIFFERENT GENETIC ENDPOINTS (μg/ml) USED TO ASSAY THE GENOTOXICITY OF THE IPCS TEST COMPOUNDS USING FUNGI

Chemical	R3	CNI	H	MI	M2
BEN	–	3.9–10	100–200		8000
DEHP	–	5	2465	200–1000	–
ZOIN	–	500	500	25–500	–
SAF	10	10–5480	–	10–100	312
TOL	–	600	50	100	8000
HMPA	–	200	50	174	–
DES	–	7.4	–	–	–
PB	–	1000	3000	250	–
CAP	–	–	–	100	–
ACN	0.8–250	200	200–806	0.8–200	250

R3 Mitotic gene conversion
CN1 Chromosome aneuploidy
H Homozygosis produced by mitotic crossing-over
M1 Point mutation in nuclear genes
M2 Mutation in cycloplasmic genes

TABLE 5

SUMMARY OF THE RESULTS OF THE FUNGAL DATA OBTAINED IN THE IPCS STUDY

Scores as indicated in text.

		R3	CN1	H	M1	M2
BEN	Positives	0	11	1	10	2
	Tests	16	8	7	20	4
DEHP	Positives	1	10	0	2	0
	Tests	16	6	7	24	4
ZOIN	Positives	4	4	4	8	0
	Tests	21	6	8	39	4
SAF	Positives	3	11	0	13	4
	Tests	17	8	10	40	4
TOL	Positives	0	10	2	3	1
	Tests	15	8	10	40	3
HMPA	Positives	0	12	3	27	0
	Tests	16	6	9	37	4
DES	Positives	0	14	1	0	0
	Tests	12	9	12	14	4
PB	Positives	0	6	3	9	0
	Tests	13	6	8	22	4
CAP	Positives	0	0	0	11	0
	Tests	15	7	9	38	4
ACN	Positives	10	10	10	12	4
	Tests	22	7	12	27	4

R3 Mitotic gene conversion
CN1 Chromosome aneuploidy
H Homozygosis produced by mitotic crossing-over
M1 Point mutation of nuclear genes
M2 Mutation of mitochondrial genes

chemicals for each of the genetic endpoints in fungal cells are summarised in Table 5.

Review of the responses of the individual chemicals in the yeast assays

BEN

Benzene was an inducer of mitotic aneuploidy and "petite" mutants in yeast. For both endpoints, negative results were found in cultures with high P450 levels. Base-substitution mutations were induced in experiments performed in the absence of exogenous S9 mix. Benzene was negative in assays for mitotic gene conversion.

DEHP

DEHP was negative in all assays with the exception of its potency in the induction of mitotic chromosome aneuploidy. Further evidence to support the induction of chromosome aneuploidy was provided by the Soviet observations of induced mitotic segregation by this compound.

ZOIN

This chemical generated an essentially negative genetic profile in yeast. A number of positive results were generated under conditions in which there was supporting evidence for a possible selective effect (such as growth advantage). A signifi-

cant contribution to the positive score came from a single unreplicated experiment for which there was evidence of selective effects occurring.

SAF

Safrole was an inducer of mitotic aneuploidy and mitochondrial mutations. Where nuclear mutations were detected they were in strains capable of inducing transitions leading to the reversion of missense alleles. There was some evidence for the induction of mitotic gene conversion under high glucose conditions.

TOL

Toluidine was a potent inducer of mitotic aneuploidy and also showed mitochondrial activity in cultures with low levels of endogenous cytochrome P450. The chemical was essentially negative for the induction of mitotic gene conversion and point mutation. For this chemical, there was some variation observed with regard to its toxicity in different experiments.

HMPA

HMPA was a potent inducer of point mutations and mitotic aneuploidy in yeast. Those mutation experiments generating negative data were shown in re-examination to have been performed at inappropriate dose levels. There was no evidence presented that HMPA was capable of inducing mitotic gene conversion or mitochondrial mutations.

DES

The results for DES dramatically illustrate the value of a range of genetic endpoints in genotoxicity studies. This chemical only showed activity for the induction of mitotic aneuploidy in fungi in the work associated with the IPCS programme.

PB

Phenobarbital was shown to be an inducer of base-substitution mutations at high doses of chemicals. PB also induced mitotic aneuploidy in 3 experiments with supporting evidence for these positive results in three other experiments.

CAP

CAP shows no activity in the assays for gene conversion, mitotic crossing-over, aneuploidy or mitochondrial mutations. However, caprolactam showed some activity in point mutation assays capable of detecting both base-substitution and frameshift events.

ACN

ACN was a potent inducer of mitotic gene conversion, nuclear and mitochondrial mutations and mitotic chromosome aneuploidy in the presence of the appropriate metabolic activation systems.

Conclusions

(1) Assays for the detection of induced forward mutation in *S. pombe* and mitotic gene conversion in *S. cerevisiae* were shown to be capable of detecting potent eukaryotic mutagens. The pre-existing data base for these two genetic endpoints is comprehensive and both assays have proven their value in genotoxicity studies.

(2) In this study the induction of cytoplasmic "petite" mutations was observed for 4 of the 8 carcinogens whereas no induction was observed with either of the non-carcinogens. Although petite mutagenesis has been reported for a large number of chemical types, its use as a screening test for carcinogens has primarily been advocated by only a simple laboratory (Wilkie and Evans, 1982). The results presented here which involved a relatively single and rapid protocol indicate that some expansion of the use and validation of the test would be justified at the present time.

(3) The point mutation assays in fungi have an extensive data base and the available strains are characterised by the variety of mutational events which may be identified. The available strains show high sensitivity as illustrated by their ability to detect a number of weak mutagens in this study.

(4) Assays for the detection of induced mitotic aneuploidy when used with the appropriate metabolic and protocol conditions were shown to detect the genetic activity of a wide range of the chemicals in this study. These assays have the advantage of being capable of detecting chemicals whose cellular targets are other than the DNA of the chromosomes. However much of the data generated in this study was produced by a single laboratory and the results require more extensive

duplication. A more extensive discussion of three aneuploidy assays used to study the 10 IPCS chemicals can be seen elsewhere (Parry et al., 1984).

(5) The results obtained by the plate incorporation assay for the detection of somatic segregation in Aspergillus deserve further comment. The induction of somatic segregation (particularly aneuploidy induction) by chemicals is usually observed when colonies are exposed to highly cytotoxic or inhibiting concentrations. Such conditions could not be obtained with the chemicals used in this study. For this reason most of the negative results reported here should be regarded as provisional. Further experiments are being performed to clarify the result using other protocols such as the liquid incubation assay.

(6) The unambiguous demonstration of mitotic crossing-over in fungi requires the use of strains that carry an adequate range of genetic markers and an appropriate experimental design. For example the demonstration of homozygosity of a single genetic marker does not represent definitive proof of the induction of reciprocal recombination between genes. Studies of the induction of recessive homozygosis using a single heterozygous marker are therefore inappropriate for screening purposes.

(7) The results of this study illustrate the utility of a chemical screening programme which includes a number of strains capable of detecting a variety of genetic endpoints and capable of protocol variation. Such a package might include strains capable of detecting: (a) induced point mutation, (b) induced mitotic gene conversion, and (c) induced mitotic aneuploidy.

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4

Summary Report on the performance of the Drosophila assays

Work Group Chairman
Ekkehart W. Vogel

Work Group Participants
H. Frei (Absent, but data considered), K. Fujikawa, U. Graf (Absent, but data considered), S. Kondo (Absent, but data considered), H. Ryo (Absent, but data considered) and F.E. Würgler

Rationale for the use of Drosophila mitotic recombination and mutation assays

The Drosophila recessive lethal assay is, when compared with the fast systems utilizing bacteria or lower eukaryotes, a relatively tedious and time-consuming method. The flexibility one would wish to reach in theory in routine testing is, in practice, applicable only for a small number of chemicals. Moreover, the possibility of strong elimination during meiosis of cells carrying a mutation should be taken into consideration as a potential disadvantage when screening chemicals for the induction of X-linked recessive lethals in Drosophila larvae. In view of these intrinsic limitations of the X-linked recessive lethal method as a means of screening a large number of chemicals, several investigators have recently drawn attention to the potential use of somatic recombination and mutation assays (Graf et al., 1983; Rasmuson et al., 1978; Vogel et al., 1980; Vogel and Ramel, 1980). These methods use cells heterozygous for recessive alleles. Induced genetic events will lead to homo-, hetero- or hemi-zygous daughter cells. Their progeny (spot, marked clone) will exhibit the marker trait. These genetic alterations, caused by mitotic recombination or somatic mutation, can occur spontaneously or be induced by genotoxic agents.

Test description and evaluation criteria

(1) Wing mosaicism

One system using wing tissue is based on the markers *mwh* (multiple wing hairs) and *flr* (flare). Larvae trans-heterozygous for these two markers are obtained from crosses between virgin *y; mwh jv* females and males from the stock *y; Dp (1; 3) sc^{J4}, flr / TM1, Me ri sbd^{2}*. (For marker descriptions, see Lindsley and Grell, 1968, and Garcia-Bellido and Dapena, 1974.) In the F_1, the flies carrying *mwh + / + flr* can be distinguished by their wild-type body color from their *mwh / TM1* sibs (yellow) which are not routinely used in screening tests (Graf et al., 1983, 1984). Since the two marker genes are both located on the left arm of chromosome 3, they can be used to score for recombinogenic effects. Any recombination event between two non-sister chromatids taking place on the left arm of chromosome 3 between *flr* and the centromere results in a twin spot, one part showing *mwh* and the other *flr*. Single spots which are either *mwh* or *flr* may originate from mutations or deletions at the wildtype locus of *mwh* or *flr* (Graf et al., 1983, 1984). They may also result from aneuploidy or chromosome loss. Single *mwh* spots also arise from recombination between the *mwh* and *flr* loci.

Test protocol

One of the three procedures used with the wing system was acute treatment of 48 or 72 h old larvae up to 6 h (method A, Table 1). In all cases, chronic treatment (method B1) was performed. Larvae were continuously fed until pupation (duration of feeding 48, 72 or 96 h depending on the start of treatment during larval development).

TABLE 1

DROSOPHILA SOMATIC MUTATION AND RECOMBINATION ASSAY

Investigator	Würgler, Graf and Frei	Fujikawa, Ryo and Kondo	Vogel
Key reference	Graf et al., Mutation Research, 120 (1983) 237–239; Environ. Mutagen., 6 (1984) 153–188	Rasmuson et al., Mutation Res., 54 (1978) 33–38	Becker, Current Topics Develop. Biol., 1 (1966) 155–171
Stocks			
Females	*y; mwh jv*	–	w^{co} *sn*^{2}*; se h*
Males	*y; Dp(1;3)sc*^{J4}*, flr/ TM1, Me ri sbd*^{2}	–	*w + ; se h*
exr^{-}			
Females	*y; mei-9*^{L1} *cv; mwh jv*	*C (1) DX, yf*	–
Males	*flr*^{3}*/TM2, Ser*	*sc z w mei-9*^{a}	–
Treatments	(A) Acute treatment; feeding of 48 h or 72 h old larvae		
	(B1) Continuous feeding of larvae for 48, 72 or 96 h	(B2) Continuous feeding; treatment of 48 h old cultures; egg-laying period 24 h	(B3) Continuous feeding; test substances mixed into food; egg-laying period 4 days
	(C) Exposure of 48 or 72 h old larvae to gas-chemical for 0.5 or 1 h		
Toxicity measurements	Reduced number of progeny	% Lethality	Reduced number of progeny
Analysis			
Repair-profic.	Males and females	–	Females
Exr-defective	Males	Males	–
Detection of genotoxic action			
Single spots	Multiple wing hairs (*mwh*) or flare (*flr*)	Red spots	Light spots (loss of w^{co})
Twin spots	*mwh* and *flr* spots	–	w^{co} and *w* spots
Statistical technique	Selby–Olson analysis Kolmogorow–Smirnov test	Fisher exact test	Fisher exact test

In another type of treatment, 48 or 72 h old larvae were exposed to gaseous chemicals for 0.5 or 1.0 h (method C). With every experiment, the frequencies of three types of spots were analyzed separately: small single spots (1–2 cells affected), large single spots (more than 2 cells) and twin spots.

For each spot type, the frequency of wings bearing such spots in the treated group was compared with the frequency observed in a matching control using the Selby–Olson statistical analysis (Selby and Olson, 1981). To test for a negative result, a 5-fold increase had to be excluded for the twin spots and the large single spots (low spontaneous frequencies) and a 2-fold increase in the case of the small single spots (high spontaneous frequencies). In addition, the spot density distribution of the control and the treated group were compared using a chi-square test and the Kolmogorov–Smirnov test (Lindgren, 1976). All tests were applied at the 5% level. Each experiment leads to nine statistical decisions. The interpretation of these results was worked out chemical-by-chemical. No detailed rules have yet been fixed because (i) the test is still under validation and (ii) from the large number of statistical decisions that

can be obtained for a compound (about 100 in the case of ACN), the individual 'false positive cases' which are due to chance variations (e.g., one in the case of DEHP) have to be eliminated in the decision-making process.

Surviving adults were dissected and the mounted wings were analyzed at 400 × magnification, whereby spots showing a *mwh* or *flr* phenotype of the trichomes (hairs on the wing blade) were registered with respect to the location on the wing and the number of cells affected (clone size). For feeding experiments, Tween-80 and ethanol (5% + 5% v/v) in aqueous solution was used as vehicle in practically all experiments. Water was used for CAP and 5% ethanol for TOL. In the very first experiment with HMPA a 2% DMSO solution was employed but was replaced later by the Tween + ethanol standard.

(2) The white–zeste eye mosaic system

A system developed by Rasmuson (Rasmuson et al., 1978) is based on the scoring of somatic mutations in an unstable *white* locus, leading to red sectors against a yellow eye background. The instability is caused by the insertion of a transposable element into the regulatory part of the *white* locus. A series of chemical mutagens, including both direct-acting agents and procarcinogens, gave positive results with this system (Fahmy and Fahmy, 1980; Nylander et al., 1978). This unstable strain shows a resolving power for AF-2 and tryptophan pyrolysates which is considerably higher than that for X-linked recessive lethals (Fujikawa, 1982; Fujikawa et al., 1983).

Test protocol

Newly hatched larvae from the cross between *C(1)DX, yf* females and *sc z* w^+ *mei-9*^{a} males were used. In all experiments, the chemical was given as 1-ml test solution pippeted onto the surface of 48-h-old cultures where 0–24 h old larvae were developing (method B2 in Table 1). The vehicles used in the preparations were distilled water, ethanol, or a mixture of Tween 60, ethanol and distilled water (1 : 1 : 2). Among the emerging flies, males were scored for pigmented sectors (red spots; size range 4–80 ommatidia) in the *zeste* compound eyes, and the total number of males with sectors (or the total number of sectors, if there is more than one sector per male) provides the frequency of somatic mutations.

A chemical which caused a significant ($P < 0.05$) increase of the mutation frequency over the control level in a linear or non-linear fashion with increasing doses was registered as positive. A chemical was regarded as marginally positive when the mutation frequency was significantly increased over the control level but showed a shift which did not follow a simple dose–response relation. A chemical which produced no significant increase of the mutation frequency at any dose was considered negative.

(3) The white / white-coral eye mosaic system

The third method chosen for the Collaborative Study was the eye imaginal disk of heterozygous w/w^{co} female larvae (Becker, 1957, 1966). After mitotic recombination, one of the two segregation possibilities leads to a coral-white twin spot ($w^{co}//w$). Unequal recombination between sister-strands or between homologous strands can also give rise to mosaic twin spots (TS), whereas forward mutations and deletions are believed to

TABLE 2

DROSOPHILA ASSAY SYSTEMS EMPLOYED IN THE IPCS-COLLABORATIVE STUDY

Genetic endpoint	Gene mutations	Deletions	Chromosome-breakage	Mitotic recombination	Aneuploidy
(1) Wing-mosaicism	+	+	+	+	+
(2) White-zeste eye mosaicism	+	+ (mostly)	?	n.d. [a]	n.d. [a]
(3) White/white-coral eye-mosaicism	+	+	+	+	+

[a] n.d., not detectable.

REFERENCE MUTAGENS POSITIVE IN CALIBRATION STUDIES

Investigator:	Würgler et al. [a]			Fujikawa et al.			Vogel et al. [b]		
	P	Conc. (mM)	Activity	P	Conc. (mM)	Activity	P	Conc. (mM)	Activity
COMPOUND									
I. *N*-Nitroso compounds									
MNU, *N*-methyl-*N*-nitrosourea	B1	0.05	+				B3	1.0/2.0	+
ENU, *N*-ethyl-*N*-nitrosourea	B1	0.5–1.0	+				B3	0.1/1.0	+
DMN, dimethylnitrosamine	A	10.0–135.6	+				B3	1.0	+
DEN, diethylnitrosamine	A	20.0	+				B3	1.0	+
	B1	6.0	+						
DPN, dipropylnitrosamine							B3	1.0	+
MEN, methylethylnitrosamine							B3	1.0	+
DNPT, dinitrosopentamethylene-tetramine							B3	1.6	+
MNNG, *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine	A	6.8–34.0	+	B2	2, 4, 8	+	B3	5.0	+
ENNG, *N*-ethyl-*N'*-nitro-*N*-nitroso-guanidine				B2	2, 4, 8	+	B3	10.0	+
N-nitroso-piperidine	B1	0.10	+						
II. Aryldialkyltriazenes									
1-(2,4,6-Trichloro)-3,3-dimethyl-triazene							B3	0.15	+
Phenyldimethyl triazene (PDMT)	A	25	+						
III. Pyrrolizidine alkaloids									
Seneciphylline	B1	0.05	+						
Senkirkine	B1	0.05	+						
IV. Hydrazine									
1,2-Dimethyl-hydrazine	A	100	M						
Hydralazine	A	200	+						
Hydrazine	B1	4.12	+						
Isoniazid	B1	20	+						
Methylhydrazine	A	19.0	+						
Procarbazine	A	300	+						
	B1	1.0	+				B3	2.0	+
V. Benzodioxole									
Safrole	B1	0.68	+				B3	0.1/1.0	M
VI. Acridine, aziridine									
Acridine orange	A	3.3–33.6	+						
	B1	0.0033	+						
5-Amino-acridine	A	20.1–40.2	+						
Triethylenemelamine (TEM)	A	5–10	+						
Thio TEPA	B1	0.1–0.5	+						
Trenimon	A	0.3	+						

VII. Oxirane, thiirane									
Diepoxybutane	A	13	+						
1,2;7,8-Diepoxyoctane	A	138	+						
	B1	6.9–34.5	+						
Epichlorohydrin	C	2–4 μl/1150 ml	+						
VIII. Lactone									
β-propiolactone	A	100–200	+						
IX. Heteroaromatic									
Aflatoxin B_1	A	0.16	+						
Hycanthone	A	111.0	M						
	B1	11.1–22.2	+						
X. Haloalkanes, haloolefins									
DBE, 1,2-dibromoethane	C	20 ml/1150 ml	+				B3	0.35	+
2,3-Dibromo-1-propanol							B3	0.75	+
1,2-Dibromo-3-chloropropane							B3	0.50	+
1,2,3-Tribromopropane							B3	0.25	+
Tris(2,3-dibromopropyl)phosphate							B3	0.75	+
XI. Sulfate, Sulfonate, Sultone									
MMS, methylmethanesulfonate	A	0.118–117.5	+	B2	0.6–2.4	+	B3	5.0; 2.5	+
EMS, ethylmethanesulfonate	A	46.1	+						
	B1	46.1	+	B2	5, 10, 20	+	B3	20.0	+
Busulfan (myleran)	B1	20	+						
XII. Aromatic amines									
NA, 2-naphthylamine							B3	1.5	+
2-AAF, 2-acetylaminofluorene							B3	0.5	M
Benzidine							B3	0.6/5.0	M
4-DAB, 4-dimethylaminoazobenzene							B3	1.0/20.0	M
2-Aminofluorene	B1	0.6	M						
XIII. Polycyclic hydrocarbons									
3-MC, 3-methylcholanthrene							B3	2.0	+
DA, 9,10-dimethylanthracene							B3	1.0	+
DMBA, 7,12-dimethylbenzanthracene							B3	1.0	+
BP, benzo[*a*]pyrene							B3	1/10	−, M
XIV. N-, S- or O-Mustard									
Cyclophosphamide	A	100	+				B3	0.5	M
ICR-170	A	20.9	M						
Mitomycin C	A	0.625	+						
Nitrogen mustard (HN2)	A	5.2–10.4	+						

TABLE 3 (continued)

Investigator:	Würgler et al. [a]			Fujikawa et al.			Vogel et al. [b]		
	P	Conc. (mM)	Activity	P	Conc. (mM)	Activity	P	Conc. (mM)	Activity
XV. Antimetabolites, DNA-synthesis inhibitors									
Vinblastine	A	0.1	+						
	B1	0.005	+						
Bleomycin	B1	0.01%	+				B3	0.005%	+
								0.01%	+
Adriamycin	B1	1	+				B3	0.05%	+
Daunomycin							B3	0.05%	+
Bromodeoxy uridine	A	50	M						
cis-Platinum diamine dichloride (cis-PDD)	B1	0.1	+						
Cytosine arabinoside (ara-C)	B1	0.5–10.0	+				B3	0.1%	+
5-Fluorouracil	A	5	+						
	B1	0.1	M						
6-Mercaptopurine	B1	25	M						
Methotrexate	B1	0.0002–0.002	+						
XVI. Miscellaneous									
HMPA, hexamethylphosphoramide	A	0.6	+				B3	0.6	+
Auramine							B3	0.6/1.3	+
Aristolochic acid	A	0.05	M						
	B1	0.005–0.05	+						
Emodin	A	37.0	M						
Ethidium bromide	A	12.7	M						
Formaldehyde	B1	10–50	+						
Moxnidazol	B1	5–10	M						
p-Chloroaniline	A	7.8–15.7	+						
Hydroxylamine	B1	45	M						
Malondialdehyde	B1	85	+						
Rutin (water-soluble)	B1	7.1–28.5	M						

P, test protocol used; +, mutagenic; M, marginally-positive; −, negative

[a] This list includes published data (Graf et al., 1983, 1984; Szabad, 1983; Würgler et al., 1983a, b) as well as unpublished data of U. Graf, H. Frei, A.T. Katz, H. Juon, and F.E. Würgler. It is important to note that most of the unpublished chemicals classified as marginally positive (M) are still under test, mostly using additional application methods.

[b] Vogel, 1984, and unpublished results of C.A. Raaymakers-Jansen Verplanke, and E.W. Vogel.

cause mosaic single spots (ML), i.e., reflecting loss of gene function.

Test protocol

Females to be tested for induction of mitotic recombination and somatic mutation came from a cross in which w^{co} sn^2; *se h* virgin females were mated to *w; se h* males. The females were allowed to oviposit for 4 days on standard medium containing the test compound. The substances were dissolved either in distilled water or in ethanol/Tween 60 (3 : 1) and then mixed into the food. This chronic treatment (method B3, Table 1) allows for the detection of mosaicism induced in first or second instar larvae.

The eyes of females which had developed from treated and control larvae were checked for $w^{co}//w$ twin spots (TS), as well as *w* single (light) mosaic spots (ML). Scoring was conducted in an ethanol/water/Tween 60 or Tween 80 mixture (90 : 9 : 1) at a magnification of 75 ×.

The decision criteria applied were as follows: the compound was scored as positive if the sum of TS + ML events was elevated above the 99% confidence limit of the accumulated control value (0.40%). A test chemical was not considered to be a satisfactory negative ($P > 0.05$) until 2000 eyes (40000 to 400000 primordial pigment cells) had been screened for spots; and it had to be repeatedly ineffective in at least three separate experiments. Results were scored as marginal-positives if small but reproducible effects were found in at least three separate experiments, with $0.01 < P < 0.05$ (Fisher exact test).

Results and discussion

Comparison of test protocols

The three Drosophila systems described in this report represent a new development in the sense that none had been used before for screening large numbers of chemicals. Therefore, the members of the working group had made no effort to employ standardized test procedures but rather developed their own protocol on the basis of results with reference mutagens (Table 3).

It can be seen from Table 1 that five different application procedures have been tried in the collaborative study, i.e., acute feeding (A), chronic exposure of developing cultures (B1–B3), and gaseous treatment (C). Regarding the kinds of genetic alterations measured, it seems quite clear from calibration studies with reference mutagens that both the wing and the *white–coral* eye mosaic system can detect a wide spectrum of genetic alterations, including mitotic recombination, chromosomal aberrations, aneuploidy, gene mutations and deletions (Table 2). The unstable *zeste–white* system identified genetic damage due to gene mutations and, most likely, small deletions. There is yet no indication that this technique can detect two-break chromosome aberrations.

In view of the limited data base developed for the three new systems, the group members agreed to include in the report results from calibration studies with known reference mutagens/carcinogens. The data compiled in Table 3, at this stage of the evaluation process, only represent positive findings with 74 chemicals (16 different classes) produced by one of the five test protocols, in order to document the principle performance of the assay under consideration.

The list of compounds positive in calibration studies not only covers the established strong reference mutagens but also includes some of those chemicals that are difficult or impossible to detect in other assays, such as the Drosophila X-linked recessive lethal test or the Salmonella assay.

Criteria for classification

For reasons which will be discussed later, the group considered it necessary to discriminate between the following three categories of results:

+	mutagenic
M	marginal genotoxic activity (needs further testing)
–	non-mutagenic

Comparison of results

Hexamethylphosphoramide. The mutagenic effects of HMPA in Drosophila have been well documented in earlier studies. Also in these investigations, HMPA was found to be an effective mutagen under all test conditions (Table 4).

o-Toluidine. With TOL, clear-cut positive effects were obtained at different dose levels in both the wing and the w–w^{co} eye mosaic system. The negative response recorded for the *zeste–white* technique, in the opinion of the group, either

TABLE 4

DROSOPHILA SOMATIC RECOMBINATION AND MUTATION

Investigator:	Würgler et al.			Fujikawa et al.	Vogel
Method:	A	B1	C [a]	B2	B3
HMPA					
Dose (range) tested μg/ml (or mg/ml *)	0.1–1.79 *	358		0.6–2.2 *	100
Dose response (range + ve)	0.1–1.79 *	358		0.6–2.2 *	100
Lethal dose (LD_x)	n.d.	896		2.2 * (LD_{30})	800
Lowest positive dose	0.1 *	358		0.6 *	100
Highest negative dose	–	–		–	–
Separate experiments/dose	1	1		2	2
TOL					
Dose (range) tested μg/ml (or mg/ml *)	1–5 *	0.1–0.5 *		1–10.7 *	107–114
Dose response (range + ve)	1–5 *			–	107–114
Lethal dose (LD_x)		0.5–1.0 *[b]		10.7 * (LD_{50})	418 (LD_{100})
Lowest positive dose	5 *	0.1 *		–	107
Highest negative dose	–	–		10.7 *	–
Separate experiments/dose	1	1		1–2	2–3
BEN					
Dose (range) tested μg/ml (or mg/ml *)	0.9–4.4 *	4.4 *	38.2–229	5–20 *	156
Dose response (range + ve)		4.4 *	38.2–229	–	–
Lethal dose (LD_x)	n.d.	8.8 *	n.d.	20 * (LD_{50})	No test
Lowest positive dose	0.9 *	4.4 *	38.2	–	156
Highest negative dose	–	–	15.3	20 *	–
Separate experiments/dose	1	1	1–2	1–2	4
SAF					
Dose (range) tested μg/ml	1096	55–110		81–324	81
Dose response (range + ve)	1096	55–110		–	81
Lethal dose (LD_x)	n.d.	550 (LD_{100})		324 (LD_{70})	324 (LD_{100})
Lowest positive dose	1096	55		–	81
Highest negative dose	–	–		324	–
Separate experiments/dose	1–2	1–2		2–3	4
CAP					
Dose (range) tested μg/ml (or mg/ml *)	50–200 *	1–5 *		23–91 *	566
Dose response (range + ve)	50–200 *	1 *		45–91 *	566
Lethal dose (LD_x)	n.d.	10 * (LD_{90})		91 *	n.d.
Lowest positive dose	50 *	1 *		45 *	566
Highest negative dose	–	–		–	–
Separate experiments/dose	1–2	1		1–3	3
ACN					
Dose (range) tested μg/ml	806–4030	81	0.35–0.70	53–425	266
Dose response (range + ve)	–	81	0.70	425	266
Lethal dose (LD_x)	n.d.	403	n.d.	n.d.	532 (LD_{50})
Lowest positive dose	–	81	0.70	425	266
Highest negative dose	4030	–		212	–
Separate experiments/dose	1	1	1–2	1–2	3

TABLE 4 (continued)

Investigator:	Würgler et al.			Fujikawa et al.	Vogel
Method:	A	B1	C [a]	B2	B3
DEHP					
Dose (range) tested μg/ml (or mg/ml *)		78.1 *		3.9–124.8 *	781
Dose response (range + ve)		–		7.8 *	781
Lethal dose (LD_x)		n.d.		n.d.	n.d.
Lowest positive dose		–		7.8 *	781
Highest negative dose		78.1 *		124.8 *	–
Separate experiments/dose		1		1–2	3
ZOIN					
Dose (range) tested μg/ml (or mg/ml *)		42.4 *		10–80 *	424
Dose response (range + ve)		–		–	–
Lethal dose (LD_x)		n.d.		n.d.	n.d.
Lowest positive dose		–		–	–
Highest negative dose		42.4 *		80 *	424
Separate experiments/dose		1		1–2	3
DES					
Dose (range) tested μg/ml (or mg/ml *)		1–5 *		6.3–50 *	376–537
Dose response (range + ve)		–		–	537
Lethal dose (LD_x)		10 *		50 * (LD_{80})	n.d.
Lowest positive dose		–		–	537
Highest negative dose		5 *		50 *	–
Separate experiments/dose		1		2	1–3
PB					
Dose (range) tested μg/ml *		4.6 *		2.5–10 *	186
Dose response (range + ve)		–		–	–
Lethal dose (LD_x)		46 *		10 * (LD_{50})	744 (LD_{30})
Lowest positive dose		–		–	–
Highest negative dose		4.6 *		10 *	186
Separate experiments/dose		1		1–2	3

[a] μg in ml air.
[b] depending on duration of treatment.
n.d., not determined = not toxic in the dose range tested.

stemmed from repellent effects (high concentrations of test solution were dropped on the surface of the cultures; Method B2) or from the genetic technique employed.

Benzene. BEN may be regarded as a typical border-line case, showing low genetic activity at rather high exposures in the wing-mosaic system, and showing marginal activity with Method B3. In the *zeste–white* system, the frequency of red spots was not elevated above background level.

Safrole. SAF gave reproducible effects in both the wing and eye mosaic assay. The clone size produced by SAF was generally small, pointing to an induction late during larval development (second or third instar).

Caprolactam. This compound which has been classified as a non-carcinogen was uniformly active in all three assays, indicating a positive response under different treatment conditions. Data on eye mosaicism (w/w^{co}) from three separate experiments were pooled, showing a reproducible clone induction at 566 μg/ml. Caprolactam at doses ranging from 1 to 200 mg/ml also produced highly significant elevations in wing-clone

TABLE 5

7.2 DROSOPHILA SOMATIC RECOMBINATION AND MUTATION: OVERALL ASSESSMENT OF RESULTS

Investigator:	Würgler, Graf and Frei			Fujikawa, Ryo and Kondo	Vogel	Consensus
Method:	A	B1	C	B2	B3	
Chemical						
HMPA	+	+		+	+	+
TOL	+	M		−	+	+
BEN	M	M	+	−	M	M
SAF	M	+		−	+	+
CAP	+	M		+	+	+
ACN	−	M	M	+	+	+
DEHP		−		M	M	M
ZOIN		−		−	−	−
DES		−		−	M	−
PB		−		−	−	−
Correct result/ compounds tested		5/10		3/10	5/10	5/10

Assessment: +, positive; M, marginally positive ($P < 0.05$); −, negative (sample size 'sufficiently' large; replicate experiments).

frequency and eye-colour mutations (*zeste–white*).

Acrylonitrile. ACN represents another case for which mutagenic activity was noted in all three assays.

Diethylhexylphthalate. In the unstable eye mosaic test, DEHP appeared to be active only at a dose of 6.1 mg/ml, in two separate experiments, whereas lower but also higher concentrations did not show an effect. Testing a narrower dose range around the positive doses would be desirable. Chronic exposure of growing cultures to 611 μg/ml DEHP also produced white-spots in w/w^{co} females. No activity, however, was noted with the wing system, at the single dose of 6.1 mg/ml. Clearly, more experimental data is needed to clarify the situation regarding a possible genotoxic effect of this carcinogen in Drosophila. For the time being, DEHP results may be interpreted to show marginal-positive responses.

Benzoin. There was no indication of mutagenic activity in the Collaborative Study for ZOIN.

Diethylstilbestrol. Based on protocols B1 (wing mosaicism) and B2 (*zeste–white*), there was no indication of mutagenesis by DES. A small but significant elevation in the frequency of mosaic spots was noticed for w/w^{co} females treated with 533 or 761 μl/ml DES. However, this result needs re-examination because in this set of experiments, DES was mixed into the food at a temperature of about 50 °C. In view of the uncertainty regarding the preparation procedure (possibility of oxidation), DES has been classified as a 'negative'.

Phenobarbitone. In none of the three assays was there any indication of genotoxic activity of PB.

General conclusions and summary

Three laboratories reported results on all 10 compounds from Drosophila, utilizing mitotic recombination and somatic mutation assays. In summary, the range of doses tested and the treatment procedures varied considerably. Taking into account these procedural variations, the data generated are surprisingly consistent. In cases where interlaboratory variations were noticed, the presumable cause could be identified.

As the present study progressed, it became apparent that the feeding procedure used with method B2 should not be used in further genetic toxicology testing because 'false negatives', even with well-established reference mutagens, may result from 'repellent effects'. Another question raised with regard to the performance of the unstable system with *zeste–white* concerns the recovery of genetic changes induced late during larval devel-

opment. An additional limitation intrinsic to this system is that chromosome-breakage events (aberrations) may not be detectable. All these considerations, taken together, have led to the following consensus: the four carcinogens HMPA, TOL, SAF and ACN, as well as the non-carcinogen CAP, are positive in the Drosophila assays, whereas ZOIN, DES and PB are considered to be non-mutagenic. No clear-cut decisions could be made in the case of BEN and DEHP. More experimental data are needed for a final classification of these two compounds. Further efforts should also deal with the types of genetic alterations caused by the five positive compounds. By using inversion-heterozygous and compound chromosomes, one could trace the presence and extent of chromosomal breakage events and aneuploidy contributing to mosaic spot-induction.

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5

Summary Report on the performance of the assays for DNA damage

Work Group Chairman
Gary M. Williams

Work Group Participants
R. Barratt, M. Bradley, G. Douglas, H. Glauert, T. Lakhanisky, C. Martin and G. Probst

I. Introduction

Chemical damage to DNA can affect cell function and proliferation and result in permanent alterations such as mutation, chromosomal aberrations and neoplastic conversion. Thus, tests for effects on DNA are important components of the safety assessment of chemicals.

Interactions of chemicals with DNA can be measured by a variety of techniques (IARC, 1980). Some methods are specific for a particular chemical and others are too demanding for routine screening purposes. Two approaches that are broadly applicable to screening are the measurement of DNA strand breaks and the measurement of DNA repair, which is a specific response to DNA damage.

The 10 chemicals selected for the IPCS Collaborative Study were tested in eight laboratories of which three performed DNA damage assays and five performed DNA repair assays.

II. Assays

The details of the assays used in this study are given in Table 1. These differ in several characteristics, particularly metabolism, the endpoint measured and the specific assay for the endpoint.

1. Metabolism

The work group noted that, broadly speaking, two distinct metabolising systems were employed in the measurement of DNA damage. These were (a) an S9 fraction from the liver of Aroclor-1254-induced rats, to which cofactors (primarily NADP and G-6-P had been added (as used by Barrett, Douglas, Lakhanisky and Martin) and (b) the endogenous metabolic capability of intact hepatocytes which were derived from the livers of individual rats (as used by Bradley, Glauert, Probst and Williams). Rat-liver S9 was used to supplement the metabolic capability of the target cells which were established cell line cultures, whereas rat hepatocytes were themselves the biological target. It was noted that differences in the metabolic capability of the two systems were likely. In the case of rat hepatocytes, the cellular compartmentalisation of the various xenobiotic metabolising enzyme systems are maintained and thus presumably the balance of activating/detoxifying reactions more closely resembles the in vivo situation. This, coupled with the near normal activity of the NADPH-generating system in early cultures, would be expected to lead to a system in which detoxification of a given xenobiotic remains a quantitatively important mechanism. In the case of induced rat-liver S9 plus cofactors, loss of cellular compartmentalisation coupled with enzyme induction and the addition of excess NADPH-generating capability, might well lead to an alteration in the balance of activating/detoxifying reactions. The conditions would be expected to favour activation. Thus it might be that both systems have advantages for specific applications. On the one hand, it might be expected that the hepatocyte assay would be specific, i.e., that it would detect

TABLE 1

CHARACTERISTICS OF DNA DAMAGE AND REPAIR ASSAYS

	Bradley	Douglas	Lakhanisky
Endpoint	DNA single-strand breaks	DNA single-strand breaks	DNA single-strand breaks
Assay type	Alkaline elution/ rat hepatocyte	Alkaline sucrose gradient sedimentation (ASG)	ASG
Cell	Male rat hepatocytes (Fischer 344)	CHO (wild-type)	CHO
Medium	Leibowitz L-15 without phenol red	Eagles MEM with NEAA and Na pyruvate	Hams F10
Serum (conc.)	10% fetal bovine	7.5% fetal bovine	15% newborn calf
Proliferative status	Non-replicating	Replicating	Replicating
Metabolism system (concentration)	Intrinsic	Rat-liver S9 (4%)	Rat-liver S9
Inducing agent	None	Aroclor 1254	Aroclor 1254
Toxicity measurement	Trypan blue dye exclusion	Growth inhibition	Survival
Length of exposure	3 h	1 h	1 h
Duration of TdR availability (activity)	NA	NA	NA
Duration of cell lysis	45 min	10.5 h	1 h
Positive −S9 controls +S9	Dimethylnitrosoamine	MMS DMN	MMS DEN
Definition of positive result	A 3-fold or greater increase in the elution slope of the treated samples compared to the concurrent negative controls	Dose-related increase in induced damage	Number of ssb > $5 \cdot 10^9$ dalton DNA + dose-related increase in induced damage
	Barrett	**Glauert**	**Martin**
Endpoint	UDS	UDS	UDS
Assay type	Scintillation counting of extracted DNA	Nuclear isolation followed by scintillation counting of extracted DNA	Scintillation counting of extracted DNA
Cell	HeLa S3	Male rat hepatocyte (Holtzman)	HeLa S3
Medium	(i) EMEM + Hepes (ii) Arginine-free EMEM with Hepes. + 10 mM HU	L-15 + 10 mM hydroxyurea added 1 h before exposure to test agent	(i) Eagles MEM (ii) Arginine-free Eagles MEM + 10 mM HU

TABLE 1 (continued)

	Barrett	Glauert	Martin
Serum (conc.)	(i) 15% fetal bovine (ii) 2.5% dialysed FBS	5% newborn calf	(i) 10% bovine (ii) 2.5% dialysed fetal calf
Proliferative status	Proliferation inhibited	Non-replicating culture	Proliferation-inhibited
Metabolism system (conc.)	Rat-liver S9 (2% final conc.)+cofactors	Intrinsic	Rat-liver S9 (2% final conc.)+cofactors
Inducing agent	Aroclor 1254	None	Aroclor 1254
Toxicity measurement	Estimated from DNA content of flask	Light microscopy and decrease in UDS and DNA content	Not measured except for extreme cases where DNA content is reduced
Length of exposure	2.5 h	18 h	2.5 h
Duration of TdR availability (activity)	2.5 h (3.33 μCi/ml)	18 h (1 μCi/ml)	2.5 h (5 μCi/ml)
Duration of cell lysis	NA	NA	NA
Positive −S9 controls +S9	MNNG or 4NQO B(a)P, aflatoxin B_1 or 2-AAF	UV	4NQO 3,3′-dichlorobenzidine
Definition of positive result	Statistically significant (F-test) dose-related increase in ^{3}H-TdR incorporation	Means at maximum effective dose compared by a 1-sided Student's t-test (for equal variance) or Lohrding's test (in one case where samples had unequal variance, but equal coefficients of variation). A sample mean which was greater than a control mean with > 95% confidence was considered positive	Student's t-test for equal variance. $p \leq 0.01$ and at last one adjacent dose giving $p \leq 0.05$.

	Probst	Williams
Endpoint	UDS	UDS
Assay type	Autoradiography	Autoradiography
Cell	Primary rat hepatocyte from male Fischer 344	Male rat hepatocyte (Fischer F344)
Medium	Williams' Medium E	Williams' Medium E
Serum (conc.)	10% fetal bovine at time of plating only	10% calf serum at time of plating only
Proliferative status	Non-replicating	Non-replicating
Metabolism system (conc.)	Intrinsic	Intrinsic

TABLE 1 (continued)

	Probst	Williams
Inducing agent	None	None
Toxicity measurement	Morphological at assay termination	Morphological
Length of exposure	18 h	18 h
Duration of TdR availability (activity)	18 h (10 μCi/ml)	18 h (10 μCi/ml)
Duration of cell lysis	None	None
Positive −S9 controls +S9	MNNG and 2AAF	2AAF
Definition of positive result	Dose response, with values to exceed control by 3 standard deviations of the control	Significant increase in net nuclear grains; must be greater than 5/nucleus

compounds which, despite the presence of detoxifying enzymes, would still undergo a significant degree of activation. The disadvantage may be that it is, for this reason, less sensitive and, therefore, cannot detect those compounds which undergo only low extents of activation in the presence of detoxifying enzymes and yet, nevertheless, are carcinogenic. The advantage of the use of induced rat-liver S9 would seem to be that if a compound can be activated to a DNA-binding species, then it has a higher probability of detection in assays employing this metabolising system. However, with this added sensitivity may come a reduction in specificity. Thus it is possible to envisage that in certain circumstances a compound which at non-toxic doses could be completely detoxified by the liver in vivo may undergo some activation by rat-liver S9 in vitro.

2. Endpoints

DNA single-strand breaks (SSBs) and unscheduled DNA synthesis (UDS) were the two endpoints measured by the investigators in the work group. SSBs were measured by either alkaline elution (Bradley) in rat hepatocytes or by alkaline sucrose gradients in CHO cells with and without S9 metabolic activation (Douglas; Lakhanisky).

A number of different mechanisms and lesion types will induce the endpoint of DNA SSBs in eucaryotic cells or in the alkaline solutions in which they are measured. These include: (1) base and nucleotide excision repair, (2) direct scissions of the DNA backbone by chemical, oxygen radical or other radical attack, (3) DNA adducts that form SSBs in alkali, (4) endonuclease and other enzymatic scissions, (5) scissions of the DNA backbone induced by intercalating agents. (6) double-strand DNA breaks of the sort that induce chromosome damage, (7) SSBs induced by cellular DNA hydrolases such as those found in lysosomes and that may be associated with certain types of cytotoxicity. Thus, almost any chemical attack on DNA will result in SSBs in these assays.

UDS was measured in rat hepatocytes by both autoradiography (Probst and Williams) and by scintillation counting (Glauert) and in HeLa cells by scintillation counting (Barrett and Martin). UDS occurs when cells in G_1, G_2, or M excise a patch of one DNA strand that is 3′ from a DNA adduct. This excision between 1 and 80 nucleotides leaves a gap in one DNA strand near where other DNA adduct had been. This gap is resynthesized by DNA polymerase using [^{3}H]thymidine. This "unscheduled" incorporation (i.e., non-S-phase incorporation) is what is measured by

either autoradiographic methods or scintillation counting methods. Obviously, those adducts that produce the longest excising gaps will be the easiest to detect with UDS because more ^{3}H-TdR will be incorporated into the longer patch.

The types of DNA lesions that produce UDS that can be measured by these techniques include: (1) the bulky lesions produced by ultraviolet right and chemicals such as 4-nitroquinoline oxide and *N*-acetoxyacetylaminofluorene. Smaller lesions produced by a variety of different agents may also be detected.

There are certain types of DNA lesions that will not be detected by UDS. These include; (1) direct single- and double-strand scissions of DNA, (2) alkali-labile lesions, (3) certain chemical adducts that do not induce excision repair. (4) pure intercalator induced damage, and (5) adducts removed by a demethylase type reaction. Whether oxygen radicals and free radicals produce UDS is not known. In general, most compounds induce a variety of different lesions some of which are likely to be removed by excision repair and thus detectable by UDS.

3. Assays

(a) Single-strand breaks

The two assays were used in this study to detect the endpoint of DNA damage. These assays have in common the measurement of single-strand discontinuities (single-strand breaks) under alkaline conditions. In these assays, a spectrum of lesions, described above, are expressed finally as single-strand breaks. Following a period of cell lysis involving DNA denaturation and unwinding, single-strand breaks are detected either by changes in the rate of sedimentation in alkaline sucrose gradients or by alterations in the rate of elution through filters.

Although these assays detect fundamentally similar types of DNA damage, the spectrum of specific DNA lesions revealed may be different. These differences cannot be easily characterized. In addition, two alkaline sucrose-gradient assays were employed. Differences between the protocol of these assays, such as composition of gradients and length of lysis times, may lead to further varieties in the nature of the lesions detected. In light of these differences, discrepancies in the results of these single-strand break assays are not unexpected.

(b) DNA repair

DNA repair can be measured in a variety of ways. The laboratories participating in this study have measured [^{3}H]thymidine incorporation during repair synthesis by two techniques. Probst and Williams used autoradiography, while Barrett, Glauert and Martin used liquid scintillation counting (Table 1). Autoradiography was considered to offer the advantages of simplicity, precise distinction between replicative and repair synthesis, and capability to quantify the fraction of responding cells. Liquid scintillation counting was considered to be rapid and potentially more sensitive than autoradiography.

A potential pitfall of autoradiography is confusion between cells in repair synthesis and impaired S-phase cells. This was not a significant factor in the two autographic assays used here since they both employed hepatocyte cultures in which less than 0.1% of cells are replicative. In autoradiographic techniques, it is conventional to subtract the cytoplasmic background grains from the nuclear grains to obtain net counts. Although this is necessary for practical purposes, it does raise questions because the two sources may be of a different nature. Moreover, a high background could obscure a low level response. This may be a particular problem with hepatocyte systems since they often have a high and variable background.

In liquid scintillation counting, the background from control cultures must be considered in determining the amount of repair synthesis. To reduce the background, Glauert isolated nuclei followed by DNA extraction while Barrett and Martin isolated DNA from whole cells. However, an agent that suppresses residual replicative synthesis in the treated cultures could lower their background such that induced repair synthesis would not exceed the incorporation in controls. Liquid scintillation counting cannot distinguish between replicative and repair synthesis. Consequently, increases in thymidine incorporation, due to increased DNA synthesis for a variety of reasons, including release of the hydroxyurea block used by the present investigators, may be mistaken for repair synthesis.

TABLE 2

RESULTS IN DNA DAMAGE TESTS

Endpoint/ laboratory	Chemical and result									
	HMPA	TOL	BEN	SAF	CAP	ACN	DEHP	ZOIN	DES	PB
Single-strand breaks										
Bradley	–	+	–	+	–	+	–	+	+	–
Douglas	–	+	–	–	–	+	–	–	+	–
Lakhanisky	–	+	+	+	–	–	–	–	+	ND
DNA repair										
Barrett	I	+	–	+	–	ND	ND	ND	ND	ND
Glauert	+	+	+	+	–	+	+	+	–	–
Martin	+	+	–	+	–	–	–	–	–	–
Probst	–	–	–	–	–	–	–	–	–	–
Williams	+	–	–	–	–	–	–	–	–	–

+, positive.
–, negative.
I, indeterminate, positive, but not completely convincing results in one test that was not confirmed.
ND, not done.

TABLE 3

	HMPA		TOL		BEN		SAF		CAP	
	Lowest positive dose [a]	Highest dose	Lowest positive dose	Highest dose	Lowest positive dose	Highest dose	Lowest positive dose	Highest dose	Lowest positive dose	Highest dose
Bradley	None	5218	319	3190(T) [b]	None	270	148	444(T)	None	3390
Douglas	None	17900	4280	7500(T)	None	7800(T)	None	320(T)	None	11300
Lakhanisky	None	8960	2140	13200	1320	1320(T)	162	162	None	11300(T)
Barrett	I	2570	50	1000	None	660	6	110	None	1130
Glauert	180	1790	54	2140(T)	78	3900	8	1620(T)	None	1130
Martin	2.5	250	25	250	None	250	13	250(T)	None	250
Probst	None	1790	None	107(T)	None	780(T)	None	160(T)	None	1130
Williams	10000	10000		180(T)				10(T)		10000

	ACN		DEHP		ZOIN		DES		PB	
	Lowest positive dose	Highest dose	Lowest positive dose	Highest dose	Lowest positive dose	Highest dose	Lowest positive dose	Highest dose	Lowest positive dose	Highest dose
Bradley	66	660(T)	None	3900	640(T)	640(T)	81(T)	805(T)	None	1060
Douglas	3710	5300	None	39000	None	6360	28	280	None	11600
Lakhanisky	None	5300(T)	None	3900	None	2120(T)	3	30	ND	ND
Barrett	ND		ND		ND		ND		ND	
Glauert	53	530(T)	195	3900	212	2120	None	1415(T)	None	2320(T)
Martin	None	250(T)	None	250	None	250(T)	None	250(T)	None	250
Probst	None	530	None	3900	None	210(T)	None	270(T)	None	2540(T)
Williams	None	1000(T)	None	10000	None	100(T)	None	10(T)	None	100(T)

a, all doses are in μg/ml.
b, T = toxic.

III. Results

The results from each laboratory are tabulated in Table 2.

For the results given in Table 2, the lowest effective concentrations and highest dose tested are given in Table 3.

IV. Assay performance

The work group appraised the overall performance of assays for either single-strand breaks or DNA repair. A consensus was reached on the effects of all compounds in all assays, except for benzoin in single strand break assays (Table 4).

Although a high degree of consensus was achieved on overall results, for all chemicals except CAP and PB there were differences in findings between laboratories (Table 2). Thus, the agreements given in Table 4 required a rationalization of certain data for 8 chemicals. The thinking behind the decisions for these compounds was as follows:

TABLE 4

OVERALL PERFORMANCE OF ASSAYS

Chemical	Single-strand break assays	DNA-repair assays
HMPA	−	+
TOL	+	+
BEN	+	−
SAF	+	+
CAP	−	−
ACN	+	−
DEHP	−	−
ZOIN	?	−
DES	+	−
PB	−	−

+, consensus positive.
−, consensus negative
?, mixed results; no concensus.

HMPA

In the single-strand break assays, HMPA was uniformly negative, while it was positive in most DNA-repair assays. Since DNA repair is a response to DNA damage, some types of which are manifested as single-strand breaks, this difference represents a paradox.

Among the DNA-repair assays, 3 out of 5 were positive, but in some cases, only at high levels of exposure. There are indications that HMPA may be activation-dependent. The negative results in some systems may be due to metabolic detoxification.

o-Toluidine

Using single-strand breaks as the endpoint, TOL has been classified as positive: two of the three investigators found it to be positive, whereas the third obtained indeterminate results.

Using unscheduled DNA synthesis as the endpoint, TOL was judged to be positive. Two investigators using an autoradiographic analysis in rat-hepatocyte culture found it to be negative, whereas two investigators using a liquid scintillation method in HeLa cells (with S9 activation), and one investigator using a nuclear isolation–liquid scintillation method in rat-hepatocyte culture, found it to be positive. The discrepancy in rat hepatocytes may result from differences in culture technique. The autoradiographic assays used hepatocytes cultured during the first 18 h in culture, whereas the liquid-scintillation method used hepatocytes after 1 day in culture. Since change in biotransformation capability occurs during culture, the one-day culture period may have altered the metabolic capability of the cells. Another possibility is that the nuclear isolation-liquid scintillation analysis may be more sensitive than the autoradiographic method.

Benzene

No clear-cut conclusion could be drawn from the data on benzene. In the single-strand breaks assays, one laboratory obtained positive results, though under toxic conditions for survival (Lakhinisky). Bradley screened lower concentrations under which no breaks might have produced or detected. Douglas discounted his positive results because of cytotoxicity.

In the UDS assay, one test showed positive results for benzene (Glauert); as their response was only slightly above the control, the consensus for benzene in the UDS group was to consider it as negative.

Safrole

Safrole induced DNA single-strand breaks (SSBs) in all three of these assays. In the alkaline-elution/rat-hepatocyte assay, safrole produced SSBs at sub-toxic doses, whereas in the two ASG/CHO/S9 assays, the SSBs were associated with some apparent toxicity. Whether toxicity or direct chemical attack on DNA produced the SSBs cannot be determined from these data. Nevertheless, there is no evidence that supports discarding positive SSB results that may be associated with toxicity.

In the DNA-repair assays, the group consensus was that safrole induced significant UDS. This result was apparent in the scintillation counting assays but not in the autoradiography/hepatocyte assays. Williams commented that his group had previously found safrole positive in mouse and hamster hepatocytes but not in rat hepatocytes. One possible explanation for the discrepancy between the autoradiography and scintillation UDS results is the latter's theoretically greater sensitivity due to the greater number of cells sampled ($2–5 \times 10^7$ versus 50) and the statistical reliability of scintillation counting.

ACN

This compound was positive in two assays for single-strand breaks and negative in one. The negative assay used a short lysis time such that alkali sites may not have been revealed. In the DNA-repair assays, it was negative in two of three.

DEHP

This compound was found negative by all three investigators measuring single-strand breaks, thus the consensus was that DEHP did not induce single-strand breaks as measured by both alkaline elution and density-gradient centrifugation. One out of the four tests measuring DNA repair found this compound to be positive. However, this result was not dose-related and was only statistically significant at one dose. The three negative results showed no response to this compound which was tested at doses high enough to produce significant toxicity. Therefore, the consensus was that DEHP was negative for UDS.

Benzoin

In assays for the detection of induced DNA single-strand breaks, two tests utilising CHO cells with an exogenous metabolising system (S9) and an alkaline sucrose-sedimentation technique gave negative responses for this compound. However, in one test using an alkaline-elution technique with primary cultures of rat hepatocytes, a positive response was recorded at a single dose level which was associated with cytotoxicity. It is possible that the difference in metabolic profile between the two assay systems may explain the differing responses. However, in the absence of a dose response for the positive effect, it was not possible to achieve a definite consensus for this compound in assays for strand breakage; ZOIN is therefore regarded as having given a questionable response for this endpoint.

In assays for detection of DNA-repair activity, two tests using an autoradiographic assessment of repair in primary cultures of rat hepatocytes gave negative responses; in addition, a negative result was also recorded in a test with HeLa cells using the scintillation counting method of repair measurement. However, one test utilising primary rat hepatocytes cultured for one day in conjunction with nuclear isolation and a scintillation counting technique gave a positive responsive. The consensus of the group was that ZOIN was negative in assays for DNA repair.

DES

DES was found to be positive in all three single-strand break assays but was negative in the five assays for UDS. These discrepancies may be attributable to differences in the types of DNA lesions detected by the two classes of assays. For example, it is possible that DNA lesions resulting from treatment of cells with DES may not be repaired by a pathway utilizing re-synthesis of excised nucleotides or that DES may inhibit DNA-repair processes attempting to repair DES lesions. The specific nature of DNA lesions leading to the detection of single strand breaks cannot be determined. It is possible that these lesions may not be formed from DES adducts directly, but from the formation of free radicals.

BEN, ACN, DEHP, ZOIN

The investigator who found the chemical to be positive wishes to issue a minority report that the chemical in question is positive in his assay.

V. Assay categorization

Assay	Categorization	Reason
Glauert	C	Positive 6 of 8 carcinogens. However, one of the non-carcinogens scored positive.
Probst and Williams	D	Did not meet criterion 2. Detected only 1 of 8 carcinogens.
Barrett and Martin	B	Did not fully meet (2); detected only 3 of 8, while scoring no false positives.
Bradley	C	Did not meet (2); detected 4 of 8 carcinogens; 1 non-carcinogen positive.
Lakhanisky	B	Detected 4 of 7 carcinogens tested.

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Summary Report on the performance of cytogenetic assays in cultured mammalian cells

Work Group Chairman
Brian J. Dean

Work Group Participants
N. Danford, G. Douglas, D. Gulati, M. Ishidate Jr., F. Palitti, B. Phillips, C. Richardson and G. van Went

Introduction

This paper presents the results of cultured mammalian cell studies for structural chromosome aberrations, numerical chromosome changes including polyploidy and aneuploidy, and sister-chromatid exchanges (SCE). Initially, chromosome assays and SCEs are considered in separate sections, though the value of combining the data from these two parameters is also discussed. For convenience, the activity of the chemicals in the different assays is presented first, followed by the overall performance of the individual assays An attempt is made to define the critical steps in the protocols that appear to explain differences in responses among investigators. Details of all the chemicals are given elsewhere in this volume and the approved abbreviations are used in this paper.

The criteria used by each investigator to judge positive and negative results were discussed at length and accepted by the Working Group. It was not possible to identify a statistical technique that was generally acceptable for use with either aberration or SCE data. Four categories of results were agreed upon. These were: negative (−), positive (+), weak positive (+w) and equivocal (±), i.e. further testing is required.

Structural and numerical chromosome aberrations

Eight workers contributed data to this group and five cell types were represented. Four workers presented data or structural aberrations in CHO cells, although one, Douglas, used the enumeration of micronuclei as a measure of chromosome breakage. The other cell types comprised the human peripheral blood lymphocyte system, a Chinese hamster lung (CHL) fibroblast, a cell strain derived from Chinese hamster liver and the rat-liver (RL_4) line. Ishidate and Dean recorded polyploid cells and Danford included data on both polyploidy and aneuploidy.

Protocols

Technical details of protocols used by each experimenter are summarized in Table 1. The systems used by Danford and Dean depended on endogenous enzymes for metabolic activation; the other six investigators incorporated an S9 fraction from an induced rat-liver homogenate and, in most cases, conducted assays in both the presence and absence of S9.

Clastogenic activity of each chemical

The lowest concentrations to induce structural aberrations (LPC) and the highest inactive or negative concentrations (HNC) are given in Table 2 and the overall assessment of the chromosome data is shown in Table 3.

HMPA

Positive in three of seven assays.

Lymphocytes: Positive in both the presence and absence of S9. LPC = 100/500 μg/ml.

TABLE 1

IN VITRO CHROMOSOME ABERRATION ASSAYS

Protocol Details

	Richardson	Douglas	Natarajan	Palitti	Gulati	Ishidate	Danford	Dean
Cell type	Human lymphocytes	CHO	CHO	CHO	CHO	CHL (lung) fibroblast	CH-L (liver) fibroblast	RL_4 (rat liver) epithelial like
Medium	RPMI 1640	MEM	Ham's F10	Ham's F10	McCoy's 5A	MEM	Dulbecco's MEM	Dulbecco's MEM
Serum	Fetal calf (10%)	Fetal calf (7.5%)	Newborn calf (15%)	Newborn calf (15%)	Fetal calf (10%) (Heat inact.)	Newborn calf (10%) (Heat inact.)	Fetal calf (15%)	Fetal calf (10%)
Cell cycle	24 h (variable)	12–14 h	12–14 h	12–14 h	12–14 h	15 h	18–19 h	13 h
Metabolism (system)	Rat S9 (2%)	Rat S9 (4%)	Rat S9 (1.5%)	Rat S9 (1.5%)	Rat S9 (1.5%)	Rat S9 (5%)	Endogenous	Endogenous
Inducing agent	Aroclor 1254	Aroclor 1254	Aroclor 1254	Phenobarbital + β-naphthoflavone	Aroclor 1254	PCB (KC-400)	–	–
Toxicity measurement	Mitotic index	Growth inhibition	None	Mitotic index	None	Growth inhibition (50%)	Growth inhibition (50%)	Plating efficiency; Mitotic index
Period of exposure −S9	3 h after	1 h	1 h	3 h	8–10 h	24 and 48 h	36 h	24 h
+ S9	3 h 44 h	1 h (no serum)	1 h	3 h	2 h (no serum)	6 h	–	–
Period, exposure to harvest (h)	28 h	25 h	(a) 12, 15 and 18 h	3, 12 and 18 h	10–12 h	24 and 48 h (−S9) 24 h (+S9)	36 h	24 h
Repl. cult./ dose level	2/3	2	3	2	1	2	2/3	2/3
Repl. expts.	2	1	1	1	2	1 or 2	1	1
Number of cells scored per culture	100	500 (Micronuclei)	100	100	100	100	100	100
Positive −S9	MMC	MMS	MMS	CP	MMC	MNNG, MMC	2-AAF, BP, EMS,	DMBA
controls +S9	CP	DMN	CP	CP	CP	BP, DMN	CP, DMN	
Statistical technique	Fisher's exact test (1-sided) and analysis for trend	None	None	Fisher's exact test	None	None	Fisher's Exact Test, Regression	Dunnett's modified t-test

TABLE 2

LOWEST CONCENTRATIONS INDUCING STRUCTURAL ABERRATION (LPC) AND HIGHEST NEGATIVE CONCENTRATION (HNC)-μg/ml

		Douglas		Natarajan		Palitti		Gulati		Richardson				Ishidate		Danford	Dean
										Donor 1		Donor 2					
		−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	−S9
										−S9	+S9	−S9	+S9				
HMPA	LPC	–	–	NT	NT	–	–	–	−500	500	500	100	6000	NT	2	–	
	HNC	1792	7168	NT	NT	10000	10000	5000	5000	100	100	100	0	4000	NT	0	2000
TOL	LPC	–	–	–	–	–	–	500	250	NT	NT	NT	NT	–	1250	12	700
	HNC	1072	4286	2142	2142	900	900	250	100	NT	NT	NT	NT	1500	1000	0	350
BEN	LPC	–	–	–	–	–	200	–	–	9	9	88	44	–	1100	–	–
	HNC	7811	7811	937	937	1800	0	3000	5000	0	0	9	9	4400	550	250	1000
SAF	LPC	–	–	NT	NT	250	83	–	–	NT	NT	NT	NT	–	175	–	–
	HNC	162	162	NT	NT	83	28	100	100	NT	NT	NT	NT	150	150	60	100
CAP	LPC	–	–	–	–	–	–	–	–	1370	270	270	270	–	12000	–	–
	HNC	1132	1132	16980	16980	2500	2500	5000	5000	270	0	0	0	8000	10000	2000	1000
ACN	LPC	1327	1327	53	53	NT	NT	–	100	NT	NT	NT	NT	12.5	NT	12.5	–
	HNC	651	651	Q	0	NT	NT	50	75	NT	NT	NT	NT	3.1	NT	6.25	10
DEHP	LPC	–	–	NT	NT	NT	NT	–	–	NT	NT	NT	NT	–	–	–	–
	HNC	3905	3905	NT	NT	NT	NT	5000	5000	NT	NT	NT	NT	4130	4130	50	1000
ZOIN	LPC	–	–	NT	NT	NT	NT	–	–	–	2	–	–	–	20	–	–
	HNC	21200	21200	NT	NT	NT	NT	500	2000	2	1	2	2.	40	10	200	400
DES	LPC	–	–	NT	NT	NT	NT	1	7.5	0.1	0.1	0.2	0.2	–	NT	3	–
	HNC	2.8	2.8	NT	NT	NT	NT	0	5	0.02	0.02	0.1	0.1	10	NT	0	10
PB	LPC	–	–	3483	1741	NT	NT	1000	500	NT	NT	NT	NT	–	NT	100	–
	HNC	282	282	1741	871	NT	NT	500	250	NT	NT	NT	NT	2000	NT	0	1000

TABLE 3

STRUCTURAL ABERRATIONS: OVERALL ASSESSMENT

	Richardson	Douglas	Natarajan	Palitti	Gulati	Ishidate	Danford	Dean
Cells	Lymphocyte	CHO	CHO	CHO	CHO	CH lung	CH liver	RL_4
HMPA	+	–	NT	–	–	+	+	–
TOL	NT	–	–	–	+	+	+	+w
BEN	+	–	–	+	–	+	–	–
SAF	NT	–	NT	+	–	+	–	–
CAP	+	–	–	–	–	+	–	–
ACN	NT	+	+	NT	–	+	+	–
DEHP	NT	–	NT	NT	–	–	–	–
ZOIN	–	–	NT	NT	–	+	–	–
DES	+	–	NT	NT	+	–	+	–
PB	NT	–	+	NT	+w	–	+w	–

CH lung: Positive in absence of S9 only. LPC = 6000 μg/ml.
CH liver: Positive in absence of S9 only. LPC = 2 μg/ml.

All studies with CHO cells were negative.

Conclusion. HMPA is a clastogen in cultured cells. Rat S9 is not an appropriate metabolizing system for HMPA. Positive results appear to be dependent on the nature of the endogenous metabolism.

TOL

Positive in four of seven assays.

CH lung: Positive only in presence of S9. LPC = 1250 μg/ml.
CH liver: Positive in absence of S9. LPC = 12 μg/ml.
CHO (Gulati): Positive +S9 and −S9. LPC = 250/500 μg/ml.
RL_4: Weak positive in absence of S9. LPC = 700 μg/ml.

Conclusion. TOL is clastogenic in cultured cells; it was negative in three of four CHO assays. There was no lymphocyte data. The Gulati protocol used 8–10 h exposure of cells to the chemical in the absence of S9, compared to 1–3 h in the other CHO assays. In addition, Gulati incubated compound plus S9 for 2 h in medium without serum; two of the other three investigators conducted this step with complete medium. The results suggest that a relatively long (8–10 h) incubation of cells with the compound is necessary for the endogenous metabolizing system to be effective and that the presence of calf serum in the S9 incubation mixture eliminates the activity of any clastogenic metabolites. TOL induced significant mitotic delay at high doses and extended harvest times were necessary to detect clastogenic activity.

BEN

Positive in three of eight assays.

Lymphocytes: Positive +S9 and −S9. LPC = 9–88 μg/ml.
CH lung: Positive in presence of S9 only. LPC = 1100 μg/ml.
CHO (Palitti): Positive in presence of S9 only. LPC = 100 μg/ml.

Conclusion. Benzene clastogenicity can be detected in cultured cells but was clearly negative in five assays. It was evident that positive results were only obtained using protocols that took account of mitotic delay. In the lymphocyte system, cells were treated for a 3-h period, 44 h after culture initiation, and harvested 72 h after initiation. It was necessary to agitate the cultures during incubation with the test chemical, since preliminary experiments showed benzene to float on top of the medium. Without agitation benzene was not in contact with the cells and very different levels of cytotoxicity were observed. Thus, lymphocytes were harvested 24 h after treatment and CH lung cells were incubated for 18 h after removal of the compound. The importance of this factor was clearly demonstrated in CHO cells in

which Palitti detected aberrations only after 18 h incubation. Gulati only incubated CHO cells for 12 h before harvesting; Palitti obtained negative results after 12 h.

SAF

Positive in two of six assays.

CH lung:	Positive in presence of S9 only. LPC = 175 μg/ml.
CHO (Palitti):	Positive + S9 and − S9.

Conclusion. SAF induced chromosome aberrations in two Chinese hamster cell lines. Surprisingly, aberrations were detected in one CHO assay in the absence of S9, after only a 3-h exposure to 250 μg/ml SAF. This conflicts with data from five other assays, in which contact of SAF with the cells ranged up to 36 h. It appears probable that SAF will only be reproducibly detected as a clastogen when its in vitro metabolic requirements are more fully characterised.

CAP

Positive in two of eight assays.

Lymphocytes:	Positive both in presence and absence of S9. LPC = 270 μg/ml.
CH lung:	Positive in presence of S9 only. LPC = 12000 μg/ml.

Conclusion. CAP was convincingly clastogenic in lymphocytes with no requirements for S9. The CH lung fibroblast data must be interpreted with some caution as an increase in chromosome breakage was only observed at an extremely high dose level (12 mg/ml) in the presence of S9. Without S9 this concentration was completely cytotoxic and the significance of these findings is difficult to assess. However, the lymphocyte data were clearly positive, and this may reflect a metabolic pathway missing from established cell lines.

ACN

Positive in four of six assays.

CH lung:	Positive without S9. LPC = 12.5 μg/ml.
CH liver:	Positive without S9. LPC = 12.5 μg/ml.
CHO (Douglas):	Positive + S9 and − S9. LPC = 1326 μg/ml.
CHO (Natarajan):	Positive + S9 and − S9. LPC = 53 μg/ml.

Conclusion. ACN was clearly a clastogen in cultured cells and was activated by endogenous enzyme systems. The negative results in RL_4 cells may also be explained by the fact that 10 μg/ml was the highest concentration tested suggesting that additional testing at high doses are required in this system.

DEHP

Negative in five of five assays.

Conclusion. DEHP was not considered to be a clastogen.

ZOIN

Positive in one of six assays.

CH lung:	Positive in presence of S9 only. LPC = 40 μg/ml.

Conclusion. ZOIN generally appeared non-clastogenic in lymphocytes, CH liver cells, RL_4 cells and two CHO assays. However, the chromatid aberrations in CH lung fibroblasts increased with dose up to 28% cells with aberrations at 40 μg/ml and this finding cannot be ignored.

DES

Positive in three of six assays.

Lymphocytes:	Positive + S9 and − S9. LPC = 0.1–0.2 μg/ml.
CH liver:	Positive without S9. LPC = 3 μg/ml.
CHO (Gulati):	Positive + S9 and − S9. LPC = 1 μg/ml.

Conclusion. DES was clastogenic at relatively low concentrations with no requirement for an exogenous metabolizing system. Some data suggested that S9 may slightly reduce the clastogenic activity of DES. This compound is another example of the need for extended incubation times to accommodate compound-induced mitotic delay.

PB

Positive in three of six assays.

CH liver: Weak response, no S9.
LPC = 100–1000 μg/ml.
CHO (Gulati): Weak response in presence of S9, equivocal result in absence of S9.
LPC = 500 μg/ml.
CHO (Natarajan): Positive +S9 and −S9.
LPC = 1741–3483 μg/ml.

Conclusion. This chemical showed relatively weak clastogenic activity. Further investigations are suggested to confirm these findings.

Induction of polyploidy and aneuploidy

Three assays were extended to include recording of polyploidy; the frequency of aneuploidy was analyzed in the CH liver cell system. The concentrations of compounds inducing numerical chromosome changes are given in Table 4 and Table 5 shows the summarized findings with the three assays.

TOL induced polyploidy in CH lung fibroblasts in the absence of S9 while polyploid cells were observed after exposure of these cells to CAP both in the presence and absence of S9. DES induced polyploidy in CH-lung fibroblasts, CH-liver cells and rat-liver (RL_4) cells.

The CH-liver cell system was also used in a comprehensive study of aneuploidy induction by the ten chemicals. Five of the carcinogenic chemicals — HMPA, TOL, BEN, DEHP, and DES — induced significant increases in the number of aneuploid cells. The two noncarcinogens — CAP and ZOIN — and the remaining three carcinogens — SAF, ACN, and PB — were negative in the aneuploidy assay.

TABLE 4

LOWEST CONCENTRATION INDUCING NUMERICAL CHROMOSOME CHANGES (LPC) AND HIGHEST NEGATIVE CONCENTRATION (HNC) - μg/ml

		Ishidate Polyploidy		Danford		Dean Polyploidy
				Polyploidy	Aneuploidy	
		−S9	+S9	−S9	−S9	−S9
HMPA	LPC	–	NT	–	10	–
	HNC	8000	NT	20	5	2000
TOL	LPC	1000	–	–	60	–
	HNC	<100	1500	120	30	700
BEN	LPC	–	–	–	62.5	–
	HNC	4400	4400	250	25	1000
SAF	LPC	–	–	–	–	–
	HNC	150	200	60	60	100
CAP	LPC	6000	12000	–	–	–
	HNC	<6000	10000	2000	2000	1000
ACN	LPC	–	NT	–	–	–
	HNC	12.5	NT	25	25	10
DEHP	LPC	–	–	–	50	–
	HNC	4130	4130	50	25	1000
ZOIN	LPC	–	–	–	–	–
	HNC	40	40	200	200	400
DES	LPC	7.5	NT	15	3	5
	HNC	5.0	NT	7.5	0	2.5
PB	LPC	–	NT	–	100 [a]	–
	HNC	2000	NT	1000	1000	1000

[a] Significantly increased at lowest concentration only.

TABLE 5

POLYPLOIDY AND ANEUPLOIDY

	Ishidate CH Lung Polyploidy	Danford CH Liver		Dean RL_4 Polyploidy
		Polyploidy	Aneuploidy	
HMPA	-	-	+	-
TOL	+	-	+	-
BEN	-	-	+	-
SAF	-	-	-	-
CAP	+	-	-	-
ACN	-	-	-	-
DEHP	-	-	+	-
ZOIN	-	-	-	-
DES	+	+	+	+
PB	-	-	-	-

Performance of the cytogenetic assays

For the purpose of this assessment, the assays are grouped by cell type. Four investigators used CHO cells, and the other investigators presented data from either lymphocytes, CH-lung cells, CH-liver cells, or RL_4 cells.

Human lymphocytes. Data were presented on five chemicals from studies using lymphocytes from two donors, i.e. each compound was tested in cells from one male and one female subject. Although there were quantitative differences in response between cultures from the two donors, they gave similar qualitative results. The three carcinogens tested were clearly positive in this assay, i.e. HMPA, BEN and DES, although the latter response was relative weak. ZOIN did not induce aberrations, but CAP proved to be a strong clastogen in lymphocytes with no requirement for exogenous metabolic activation.

Since the meeting to review these results, additional data from human lymphocytes have been presented by G. Obe. These studies are described elsewhere in this volume but the complete data were not available for consideration by the working group and thus are not assessed in this report.

CHO cells. When the enumeration of micronuclei was used as a measure of chromosome breakage (one assay), only ACN gave a positive response of the ten chemicals tested. In the other three assays with CHO cells, conventional metaphase analysis was used to assess chromosome damage. The overall assessment shows that agreement among the three data sets was far from complete (Table 3). One investigator (Gulati) detected TOL, DES and PB (weak) as clastogenic in the ten chemicals tested. Palitti and Natarajan had negative results with TOL but Palitti identified BEN and SAF as clastogens. CAP was negative in the three assays; ACN, DES and PB induced aberrations in CHO cells, although not all investigators produced data on these three chemicals.

CH-lung fibroblasts. This assay gave positive results with eight of the ten compounds tested. Both CAP and ZOIN, the noncarcinogens, induced chromosome aberrations. DES, which produced chromosome aberrations in three other systems, failed to induce structural damage in CH-lung cells, but induced a significant frequency of polyploidy. Only two carcinogens, DEHP and PB, were not detected in the combined aberration/polyploidy assay (Table 9).

CHL liver cells. This was the only assay that included aneuploidy among its observations. Five chemicals induced structural aberrations. DES also induced polyploidy and HMPA, TOL; BEN, DEHP and DES induced aneuploidy. SAF was the only carcinogen to remain undetected, and both ZOIN and CAP failed to induce either aberrations, polyploidy or aneuploidy (Table 9).

Rat-liver (RL_4) cells. Only TOL induced a positive (weak) clastogenic response. DES caused a significant increase in polyploidy. Thus, six of

TABLE 6

IN VITRO SISTER-CHROMATID EXCHANGE ASSAYS

Protocol details

	Phillips	Douglas	Natarajan	Gulati	Van Went	Dean
Cell type	CHO-K1-BH4	CHO	CHO	CHO	V79 (lung)	RL4 (Rat liver)
Medium	Hams's F12	MEM	Ham's F10	McCoy's 5a	Ham's F10	Dulbecco's MEM
Serum	Fetal calf (5%) (heat inact.)	Fetal calf	Newborn calf (15%)	Fetal calf (10%) (heat inact.)	Newborn calf (15%)	Fetal calf (10%)
Cell cycle	12–14 h	12–14 h	12–14 h	12–14 h	11–12 h	13 h
Metabolism (system)	Rat S9 (10%)	Rat S9 (4%)	Rat S9	Rat S9	Rat S9 (10%)	Endogenous
Inducing agent	Aroclor 1254	Aroclor 1254	Aroclor 1254	Aroclor 1254	Aroclor 1254	–
Toxicity measurement	Growth inhibition	Growth inhibition	None	None	Growth inhibition	Plating efficiency:
Period of – S9	5 h	1 h	1 h	26 h	2 h (no serum)	22 or 32 h
Exposure – S9	5 h	1 h	1 h	2 h	2 h (no serum)	
Period, expos. to harvest (h)	29 h	27 h	25, 30 and 36 h	26 h +	26 h	22 or 32 h
Repl. cult./ dose level	1	2	1	1	1	2/3
Repl. expts.	2	1	2	2	2	1
Number of cells scored per culture	30	30	25	50	30	30
Positive – S9	EMS, 4-NQO, NMMG	MMS	MMS	MMC		DMBA
Controls + S9	CP, BP, 2-AAF	DMN	CP	CP	BP, DEN	
Statistical technique	*t*-test	None	None	Fisher's Exact Test	*t*-test	Dunnett's modified *t*-test
BrdUrd Concentration	10 μM	10 μM	5 μM	10 μM	10 μM	15 μM
Period in BrdUrd (h)	29 h	26 h	25–36 h	26 h +	24 h	24 h

the carcinogens failed to induce either structural or numerical changes in this assay.

Sister-chromatid exchanges (SCE)

Six investigators presented SCE data based on three different cell types, including CHO cells (Phillips, five chemicals; Douglas, six chemicals; Natarajan, five chemicals; Gulati, ten chemicals), V79 cells (van Went, five chemicals) and RL_4 cells (Dean, ten chemicals).

Protocols

Technical details of the assays are summarized in Table 6. The RL_4 system depended on endogenous enzymes for metabolic activation; the remaining five assays incorporated on S9 fraction from Aroclor-induced rat-liver homogenates.

Activity of each chemical in SCE assays

The lowest concentrations to induce SCE (LPC) and the highest inactive or negative concentrations (HNC) are summarized in Table 7 and the results of the SCE assays are shown in Table 8.

HMPA

Positive in three of five assays.

CHO (Gulati):	Positive in absence of S9 only.
	LPC = 1000 μg/ml.
V79:	Positive in presence of S9 only.
	LPC = 896 μg/ml.
RL_4:	Positive (weak), no. S9.
	LPC = 2000 μg/ml.

Conclusion. HMPA was positive in CHO and RL_4 cells with no requirement for S9, but positive in V79 cells only in the presence of S9. With the exception of the V79 assay, the results confirm the importance of endogenous metabolism in the genotoxicity of HMPA in cultured cells. Negative results from two investigators using CHO cells may be explained by the short treatment times in the absence of S9. Gulati's positive results were obtained after a treatment period of 26 h, while Phillips used 5 h and Douglas exposed the cells for only 1 h.

TOL

Positive in four of six assays.

CHO (Phillips):	Positive in absence of S9 only.
	LPC = 125 μg/ml.
CHO (Gulati):	Positive + S9 (weak) and − S9.
	LPC = 750 μg/ml. (+ S9)
	LPC = 250 μg/ml. (− S9)
V79:	Positive + S9 and − S9.
	LPC = 268 μg/ml.
RL_4:	Positive, no S9.
	LPC = 21.8 μg/ml.

Conclusion. TOL is capable of inducing SCE in most of the systems investigated. No requirement for S9 which reduced or eliminated activity of TOL in CHO cells. Endogenous metabolic activation benefits from extended exposure time (i.e. Gulati obtained SCE-induction only at a harvest time of 43 h).

BEN

Negative in six of six assays.

Conclusion. Benzene did not induce SCE under these experimental conditions. Other studies (Morimoto, 1983) suggest that benzene can induce SCE but under very critical metabolic activation conditions.

SAF

Positive in two of five assays.

CHO (Gulati):	Weak positive, + S9 and − S9.
	LPC = 30 μg/ml.
V79:	Positive in presence of S9 only.
	LPC = 81 μg/ml.

Conclusion. SAF appears capable of inducing SCE in cultured cells. The response of CHO cells was variable: the only positive result was very weak with no requirement for S9, suggesting that endogenous enzymes were responsible for the biotransformation. However, SCE were induced in V79 only in the presence of S9, i.e. a similar finding to that of HMPA.

CAP

Negative in six of six assays.

Conclusion. CAP is not an SCE-inducing compound under these experimental conditions.

ACN

Positive in two of three assays.

CHO (Gulati):	Positive + S9 and − S9.
	LPC = 10/25 μg/ml.

TABLE 7

LOWEST CONCENTRATIONS INDUCING SCE (LPC) AND HIGHEST NEGATIVE CONCENTRATIONS HNC (HNC), μg/ml

		Phillips		Douglas		Natarajan		Gulati		Van Went		Dean
		−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9
HMPA	LPC	–	–	–	–	NT	NT	1000	–	–	896	2000
	HNC	5200	5200	1792	7168	NT	NT	500	5000	8960	–	1000
TOL	LPC	125	–	–	–	–	–	16	750	268	268	21.8
	HNC	250	1000	107	1071	2142	2142	5	500	–	–	0
BEN	LPC	–	–	–	–	–	–	–	–	–	–	–
	HNC	1000	1000	781	781	937	937	1000	5000	3905	3905	1000
SAF	LPC	–	–	–	–	NT	NT	30	75	–	81	–
	HNC	100	100	81	130	NT	NT	25	30	81	16.2	1000
CAP	LPC	–	–	–	–	–	–	–	–	–	–	–
	HNC	5000	5000	1132	1132	16950	16950	3000	5000	5658	5658	1000
ACN	LPC	NT	NT	NT	NT	106	106	10	25	NT	NT	–
	HNC	NT	NT	NT	NT	53	53	5	16	NT	NT	10
DEHP	LPC	NT	NT	–	–	NT	NT	–	–	NT	NT	–
	HNC	NT	NT	391	3905	NT	NT	5000	5000	NT	NT	1000
ZOIN	LPC	NT	NT	NT	NT	NT	NT	–	–	NT	NT	–
	HNC	NT	NT	NT	NT	NT	NT	500	2000	NT	NT	400
DES	LPC	NT	NT	NT	NT	NT	NT	–	–	NT	NT	–
	HNC	NT	NT	NT	NT	NT	NT	5	5	NT	NT	5
PB	LPC	NT	NT	NT	NT	1741	870	–	–	NT	NT	–
	HNC	NT	NT	NT	NT	870	0	500	4000	NT	NT	1000

TABLE 8

SCEs, OVERALL ASSESSMENT

Cells	Phillips CHO	Douglas CHO	Natarajan CHO	Gulati CHO	Van Went V79	Dean RL_4
HMPA	–	–	NT	+	+	+w
TOL	+	–	–	+	+	+
BEN	–	–	–	–	–	–
SAF	–	–	NT	+w	+	–
CAP	–	–	–	–	–	–
ACN	NT	NT	+	+	NT	–
DEHP	NT	–	NT	–	NT	–
ZOIN	NT	NT	NT	–	NT	–
DES	NT	NT	NT	–	NT	–
PB	NT	NT	+	–	NT	–

CHO (Natarajan): Positive + S9 and – S9.
LPC = 106 μg/ml.

Conclusion. ACN can be detected by conventional SCE procedures.

DEHP

Negative in three of three assays.

Conclusion. DEHP did not induce SCE in CHO or RL_4 cells.

ZOIN

Negative in two of two assays.

Conclusion. No evidence of SCE induction.

DES

Negative in two of two assays.

Conclusion. No evidence of SCE induction.

PB

Positive in one of three assays.

CHO (Natarajan): Positive + S9 and – S9.
LPC = 1741 μg/ml (– S9).
LPC = 870 μg/ml (+ S9).

Conclusion. There was a clear indication of SCE induction in one CHO study but it requires confirmation.

Performance of the SCE assays

The performance of the assays is considered by cell type, i.e. CHO, V79 or RL_4.

CHO cells. The variation in results among the four investigators probably reflects the stringent metabolic requirements of these chemicals (Table 8). HMPA only induced SCE in one assay, with two negative results. Similarly, TOL was positive in two of four CHO assays, while BEN did not produce SCE in four different laboratories. SAF gave a weak positive result in one assay and ACN was clearly positive in the two laboratories in which it was tested. PB was also positive in one assay of two. Thus, only five of the carcinogens appeared capable of inducing SCE in CHO cells, and only two of these results were reproducible between two laboratories. However, the two non carcinogens, CAP and ZOIN did not produce any positive findings.

V79 cells. Of the five compounds tested in V79 cells, three (HMPA, TOL, and SAF) induced a significant frequency of SCE. BEN was negative as was the noncarcinogen CAP.

RL_4 cells. Two of the carcinogens, HMPA and TOL, induced SCE in RL_4 cells.

Combined data: chromosome aberrations and SCE

Four investigators presented data on both chromosome aberrations and SCE from parallel experiments (Table 10). In Douglas's hands, the combined data from micronuclei and SCE in CHO cells detected only ACN. Natarajan had identical results for SCE and structural aberrations in CHO cells in the five compounds reported. Combined data from Gulati's laboratory showed that the CHO assay detected six of the eight carcinogens;

only BEN and DEHP failed to induce either aberrations or SCE. Both ZOIN and CAP were negative. The RL_4 assay gave positive results for either SCE or structural or numerical aberrations with three of the carcinogens.

The results from Gulati's laboratory, in particular, suggest that the procedures, i.e. structural aberrations and SCE, complement each other in the detection of activity in these compounds.

Mitotic spindle disturbances

Although this was not strictly a cytogenetic technique, data were presented by Elizabeth Parry on the results of an investigation of mitotic spindle disturbances induced in CH-liver cells by the ten compounds. The studies were conducted in parallel with the aneuploidy experiments presented by Danford and provide a useful insight into the mechanism of action of some of the carcinogens. The technique, described by Parry elsewhere in this volume, allows observation of the mitotic spindle at different mitotic stages. Four compounds were judged to cause unequivocal spindle disturbances. These were DES, which appeared to induce a direct effect on the spindle structure; and DEHP, PB and BEN, which produced increases in the numbers of abnormal divisions, including increases in chromosome cluster groups. Of these four compounds, DES, BEN and DEHP increased the number of aneuploid cells in this cell line, (Danford, Table 9). HMPA and TOL also induced aneuploidy. A small number of abnormal division stages were detected in cells exposed to HMPA, but TOL showed no evidence of mitotic disturbances in this assay.

Discussion

At first glance, the most striking conclusion from both the chromosome aberration and SCE studies is the lack of agreement in the results from different laboratories, even when, ostensibly, the same procedure and cell line had been used. However, close scrutiny of the protocols suggests a number of factors that may have contributed toward the variability. Four data sets were presented from both the CHO/aberration assay and the CHO/SCE assay, but not from the same four laboratories. It was apparent from studies with HMPA, TOL, BEN, SAF and DES that two factors were of critical importance in the detection of clastogenic activity in CHO cells.

(a) In the case of HMPA and TOL in particular, positive results depended on endogenous metabolism of the compounds rather than S9 activation, and clastogenicity was only detected reproducibly where the cells were incubated with

TABLE 9

COMBINED RESULTS: STRUCTURAL AND NUMERICAL CHANGES, AND MITOTIC SPINDLE DISTURBANCES

	Ishidate CH lung		Danford CH liver			Dean Rat liver		Parry CH liver
	Aberrations	Polyploidy	Aberrations	Polyploidy	Aneuploidy	Aberrations	Polyploidy	Spindle disturbance
HMPA	+	–	+	–	+	–	–	–
TOL	+	+	+	–	+	+w	–	–
BEN	+	–	–	–	+	–	–	+
SAF	+	–	–	–	–	–	–	–
CAP	+	+	–	–	–	–	–	–
ACN	+	–	+	–	–	–	–	–
DEHP	–	–	–	–	+	–	–	+
ZOIN	+	–	–	–	–	–	–	–
DES	–	+	+	+	+	–	+	+
PB	–	–	+w	–	–	–	–	+
Concordant [a] results	6/10		9/10			4/10		6/10

[a] Agreement with carcinogenic/noncarcinogenic activity.

the compound for periods in excess of 8–10 h. Incubation times of 3 h or less failed to give positive results. (b) Aberrations were often only detected at concentrations of the test material that induced considerable cell-cycle delay. This was particularly noticeable with TOL, BEN and DES where extended harvest times of 22–48 h after beginning exposure were necessary to detect activity. Two investigators (Gulati and Douglas) conducted the exposure of cells to compound in the presence of S9 in serum-free medium. This appeared to increase the effectiveness of the S9 supplement in some of Gulati's experiments.

In general, Gulati's protocol for both aberrations and SCE performed well, detecting six of the eight carcinogens. BEN, which was not detected by Gulati, induced aberrations in CHO cells in Palitti's laboratory. DEHP was not detected by any investigator using CHO cells.

It is difficult to assess the performance of the human lymphocyte system based on the results of five compounds using one comprehensive protocol, and data from ten compounds using another protocol (Obe). The positive result with CAP causes some concern as it is clearly not carcinogenic in laboratory rodents. However, the compound is activated by human lymphocytes to a clastogenic form and this raises the question of a metabolic route for CAP in human cells that is inactive in rodent cells. This finding may merit investigations in other human cell types.

The CH-lung fibroblast assay performed well with the carcinogens in this group of compounds, detecting aberrations or polyploidy with six of them. However, it also gave positive results with both CAP and ZOIN. This assay is supported by an extensive data base in the literature, though even here a number of compounds that are either proven or assumed non carcinogens produced significant chromosome damage (Ishidate, 1983).

As data from only five compounds were presented on the V79 system, any assessment of the assay on the basis of this study would be premature. The V79 line provided some interesting contrasts to the CHO line, indicating that they are not interchangeable. In tests with HMPA in particular, V79 cells required S9 for the demonstration of SCE while CHO gave positive results for both aberrations and SCE in the absence of S9. This may simply reflect the short incubation time (2 h) of V79 in the absence of S9 rather than significant differences in endogenous metabolic capabilities; but the results with HMPA, TOL and SAF in the presence of S9 suggest that V79 cells are more receptive of S9-mediated metabolites than CHO cells.

In spite of a competent performance in the previous collaborative study and a fairly extensive in-house data-base, the RL_4 assay detected clastogenic activity in only one carcinogen, polyploidy

TABLE 10

COMBINED RESULTS: STRUCTURAL AND NUMERICAL CHROMOSOME CHANGES AND SCE

	Douglas CHO		Natarajan CHO		Gulati CHO		Dean Rat liver	
	Chromosome	SCE	Chromosome	SCE	Chromosome	SCE	Chromosome	SCE
HMPA	–	–	NT	NT	–	+	–	+w
TOL	–	–	–	–	+	+	+w	+
BEN	–	–	–	–	–	–	–	–
SAF	–	–	NT	NT	–	+w	–	–
CAP	–	–	–	–	–	–	–	–
ACN	+	NT	+	+	–	+	–	–
DEHP	–	–	NT	NT	–	–	–	–
ZOIN	–	NT	NT	NT	–	–	–	–
DES	–	NT	NT	NT	+	–	+	–
PB	–	NT	+	+	+w	–	–	–
Concordant results [a]	3/10		3/5		8/10		5/10	

[a] Agreement with carcinogenic/noncarcinogenic activity.

in another, and SCE induction in two compounds. The failure to detect five carcinogens may be due to protocol shortcomings similar to those identified in the CHO assay. Two factors that should be examined are extended harvesting times and the use of higher test concentrations, to accomodate compounds that induce damage at concentrations associated with considerable cell-cycle delay and thus, partial growth inhibition.

Probably the most promising data were derived from the CH-liver cell assay, which combined a high endogenous metabolic activity with a technique that allowed accurate counting of chromosomes providing data on aneuploidy and structural aberrations in the same assay. The high endogenous enzyme activity was demonstrated by the relative low concentrations of HMPA (2 μg/ml) TOL (12 μg/ml) and DES (3 μg/ml) required to produce a positive effect. Seven of the eight carcinogens were detected, five inducing aberrations and five causing aneuploidy. BEN and DEHP, in particular, induced numerical chromosome changes in the absence of structural aberrations.

The data presented by Parry on the effects of the compounds on mitotic spindle structure and function provide useful supplementary information on the mechanism of action of some of these chemicals. DES clearly damages the mitotic spindle in a dose-related manner and this is probably the direct cause of aneuploidy and polyploidy with this chemical. In addition, BEN and DEHP, which induced aneuploidy in Danford's study, were shown by Parry to interfere with spindle function as demonstrated by an increase in abnormal division stages.

Conclusions and recommendations

The data considered in this study have not provided unequivocal evidence for the clear categorization of the different cytogenetic and SCE assays. However, certain conclusions have been reached and a number of recommendations for limited additional investigations are given below.

Human lymphocytes

The utility of the human lymphocyte system is demonstrated by its widespread use and proven performance over many years. However, data were only presented from one laboratory on five of the compounds. Using the categorization criteria (Ashby et al., 1983) the assay cannot therefore be placed in categories A, B or C (see Introduction, pp. 3–9) until additional data are generated on the ten test compounds, and until the significance of the positive findings with CAP is established.

CHO cells

With the exception of DEHP, all of the carcinogens induced SCE or aberrations, or both, in at least one CHO assay. This suggests that a modified protocol to take into consideration the needs of endogenous activation (i.e. an exposure time of at least 10 h in the absence of S9) and cell cycle delay (i.e. multiple harvest times to be used, extending up to 48 h after the beginning of treatment), will provide an assay that falls clearly into Category A.

V79 cells

Further studies are required before this assay can be considered for inclusion in Category A and it should therefore be regarded as a Category B procedure.

CH-lung fibroblasts

Although this assay responded very positively to the carcinogens in the ten-chemical group, the positive results with the noncarcinogens suggest that the protocol should be re-examined. In particular, unusually high concentrations of compounds (e.g. 12000 μg/ml CAP) are tested (see also Ishidate, 1983) and the indirect physical and chemical effects of such high concentrations on the integrity of the test system should be investigated. In view of its sensitivity to six of the carcinogens and its extensive data base, the CH-lung fibroblast assay is provisionally placed in Category A.

Rat-liver (RL_4) assay

Although this method has functioned satisfactorily as a Category A assay for a number of years, its performance with this group of carcinogens suggests that protocol modifications are necessary to meet the requirements of clastogens that induce cell-cycle delay at relatively high concentrations. Further experiments should be conducted with the

compounds to characterize the cell cycle-related effects. The assay is provisionally placed in Category B.

CH-liver assay

The initial promise of this combined aberrations/aneuploidy assay should be explored further. It is recommended that this proceeds in two phases: the procedure should first be established in at least two other laboratories and validated using the same ten test compounds; and the data base should then be extended by testing a series of carefully selected carcinogen/noncarcinogen paired compounds in at least three laboratories. The contribution of aneuploidy towards the carcinogenic process has long been suspected and with the introduction of an appropriate mammalian cell assay, the opportunity should be taken to exploit it to the full.

Cytogenetic assays in cultured cells are recommended by many authorities for use in parallel with bacterial mutation tests in an initial screening package. The results of this study have not significantly affected this view, and with a more stringent experimental design, assays for chromosome aberrations, including the induction of polyploidy, should be confirmed as the leading candidate for Category A. Although the SCE data were fewer in number than those from cytogenetic assays, it is apparent that the carcinogens did not consistently induce SCE in cultured cells. The results suggest that SCE tests are more suitable for use as supplementary assays rather than Category A, though a combined aberration/SCE procedure has distinct advantages over the investigation of aberrations alone.

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7

Summary Report on the performance of gene mutation assays in mammalian cells in culture

Work Group Chairman
R. Colin Garner

Work Group Participants
D. Amacher, W. Caspary, C. Crespi, G. DeLow, A. Knaap, Y. Kuroda (Absent, but data considered), T. Kuroki (Absent, but data considered), C. Lee, B. Myrh, T. Oberly, J. Styles and M. Zdzienicka

This work group considered reports from twelve different laboratories. Two laboratories reported data on two different cell lines thus making a total of fourteen assay systems evaluated. No attempt was made to standardise protocols in the various assay systems reported, nor was any attempt made to discriminate between results from the various cell lines used, i.e. results of mutation studies in human cells were not regarded as being more relevant to human cancer than those from rodent-derived cell lines. Various genetic end points were measured in this series of experiments and these are outlined in Table 1.

All the genetic end-points were to drug resistance and scoring was by means of counting numbers of drug-resistant mutants in the treated population compared with controls.

The study provided an opportunity unique to this date, of assessing the activity of ten standard test chemicals in a great variety of in vitro mammalian cell mutation test systems. The study design enabled comparisons to be made between similar test systems, i.e. interlaboratory variation between results obtained using the same genetic end-point, namely trifluorothymidine resistance in mouse L5178Y cells, as well as a comparison with results from cell lines such as Chinese hamster V79 cells and human lymphoblastoid cells such as TK6. Besides the qualitative comparisons that could be made between differing assay systems, quantitative comparisons could also be made, as well as studying the effects of different metabolic activation systems.

The published data base on the use of mammalian cell mutation assays is not large. Most workers, to this date, have not used these systems for routine screening purposes but more to elucidate the mechanism of mutation and to study the effects of DNA repair-deficiencies. Those data which have been published relate mainly to the $TK^{+/-}$ system in mouse lymphoma L5178Y cells developed by Clive and his coworkers. Six of the laboratories in this study reported on the use of this system, albeit with some substantial differences in protocol (cloning in soft agar versus, for example, plating out in microtitre wells). The remainder of the other laboratories reported data on a variety of other cell lines, one studied mutations at the HGPRT locus in Chinese hamster V79 cells, two studied mutations to ouabain resistance in L5178Y $TK^{+/+}$ cells, and one laboratory reported on mutation in two different human lymphoblastoid cell lines, one of which had some metabolic activation capability. The group felt that the results of this study did not enable one to make any firm recommendation about the utility of one assay system compared with another. However, it was felt that assays in which resistance to ouabain was measured are less likely to detect mammalian cell mutagens than the other end-points examined, because of the nature of this mutation. Only base-substitution mutagens appear

TABLE 1

SUMMARY OF ASSAY SYSTEMS

Laboratory	1	2	3	4	5	6	7	8	9	10	11	12
Cell line	Mouse L5178Y $Tk^{+/-}$	(i) Human TK6 (ii) human AHH-1	Hamster V79	Mouse L5178Y ($Tk^{+/+}$)	Mouse L5178Y $Tk^{+/-}$	Hamster V79	Hamster V79	(i) Hamster V79 (ii) mouse L5178Y $Tk^{+/-}$	Mouse L5178Y $Tk^{+/-}$	Mouse L5178Y $Tk^{+/-}$	(i) mouse L5178Y $Tk^{+/-}$ (ii) mouse L5178Y $Tk^{+/+}$	Hamster CHO
Medium	RPMI 1640	RPMI 1640	Eagles MEM	Fisch	Hams F10 (−Hx)	Eagles MEM	Eagles MEM	Eagles MEM	Fisch	RPMI 1640	RPMI 1640	Hams F-10 (−Hx)
Serum	Horse	(i) horse (ii) horse	Fetal Bovine	Horse	Newborn Bovine	Fetal Bovine	Fetal Bovine	(i) Fetal Bovine (ii) Horse	Horse	Horse Heat Inact	Horse Heat Inact	Newborn Bovine
Selective Agent	TFT	(i) TFT (ii) 6TG	6TG	(i) oua (ii) 6TG	(i) TFT (ii) 6TG	(i) oua (ii) 6TG	Oua	(i) 8AzG (ii) TFT + BudR	TFT	TFT	(i) TFT (ii) oua	(i) oua (ii) 6TG
Expression time (h)	48	(i) 72 (ii) 144	120, 192, 288	(i) 48 (ii) 168	(i) 72 (ii) 168	(i) 48 (ii) 144	72–120	(i) 96 (ii) 72	48	48	(i) 48 (ii) 48	(i) 96 (ii) 192

* Laboratory code:

1 D.E. Amacher and G.N. Turner.
2 C.L. Crespi, C.G. Ryan, G.M. seixas, I.R. Turner and B.W. Perman.
3 M. Fox and G. Delow.
4 R.C. Garner and J. Campbell.
5 A.G.A. Knaap.
6 Y. Kuroda.
7 I. Kuroki
8 C.G. Lee.
9 B. Myrh and C. Caspary.
10 T. Oberly.
11 J.A. Styles, P. Clay and M.F. Cross.
12 M.Z. Zdzienicka and J.W.I.M. Simons.

to be detected; mutagens can also be readily detected at the HGPRT or Tk locus with greater ease by virtue of the larger DNA target.

Considerable differences in the ways of analysing data were presented and this led to protracted discussion. Later on in this report some recommendations have been made ensuing from these discussions. It should be strongly emphasised at this point that the compounds chosen to be tested were ones that are negative in the Salmonella/microsome assay but for which there is some evidence of animal carcinogenicity.

Test protocols

An attempt has been made to summarise in Tables 1–3 the various test systems which each investigator used in this study. Some points of interest in the various assays are pointed out to the reader. A more detailed account can be found in the investigators' individual reports.

Cell-lines

Six laboratories used mouse lymphoma L5178Y cells $Tk^{+/-}$ originally obtained from D. Clive. One laboratory had carried out a chromosome analysis using banding techniques to confirm that their line was identical to that described by Clive. All

TABLE 2

METABOLIC ACTIVATION SYSTEMS

Laboratory	Activation system	Induction method	Final conc. (%)	Co-factor system
1	Rat Sprague–Dawley	Non-induced	1.25	3 mM NADP 18 mM isocitrate
2	Rat Sprague–Dawley	Aroclor-1254	1.25	1.3 mM NADP 3 mM G-6-P
3	Rat Albino	PB and β-naphtho-flavone	2.0	5 mM NaDP 5 mM 6-G-P
4	Rat Sprague-Dawley	Aroclor-1254	0.5	3 mM NADP 6 mM NADP
5	(i) Rat (ii) Rat hepatocytes (iii) Syrian hamster embryo cells (iv) Chick embryo hepatocytes	Aroclor-1254	(i) 1.25	14 mM NADP 5 mM G-6-P
6	Rat Sprague–Dawley	PB and β-naphtho-flavone		NADH NADPH G-6-P
7	(i) Rat (ii) Irradiated Syrian hamster embryo cells	(i) PB and β-naphtho-flavone	2.0	(i) 0.8 mM NADP 0.8 mM NADPH 0.8 mM NADPH 5 mM G-6-P
8	Rat Porton	Aroclor-1254	0.25	2 mM NADP 2 mM G-6-P
9	Rat Fisher	(i) Uninduced (ii) Aroclor-1254	1.25	1 mM NADP 5 mM isocitrate
10	Rat Fischer	Aroclor-1254	0.25	3 mM NADP 18 mM isocitrate
11	Rat Wistar	Aroclor-1254	1.25	4 mM NADP 5 mM G-6-P
12	Irradiated Syrian hamster embryo cells (4000 rad)	NA	NA	NA

TABLE 3

SUMMARY OF TEST PROTOCOLS

	1	2	3	4	5	6
Determination of dose	% Total relative growth	Preliminary survival curve	Preliminary cytotoxicity test	Preliminary cytotoxicity test	Cloning efficiency post treatment	Preliminary cytotoxicity test
Treatment +S9	3	TK6: 3	1	2	2	4
time (h) −S9	3	TK6: 20 AHH-1: 28	1	2	2	4
Replicate cultures	Yes	Yes	No	Yes	No	
Repeat experiments	Only for inconclusives	Positive responses confirmed	Yes	No	Sometimes	
Number of cells exposed/ replicate	6×10^6	4×10^7	2×10^6	2.5×10^7	$5-10\times10^6$	
Number of cells plated/replicate	3×10^6	TK6: 9×10^6 AHH-1: 5×10^7	5×10^5	OUA: 8×10^6 6TG: 2×10^6	3×10^6	
Positive +S9	3MC	BP	Cyclophos	BP	BP, DEN	
controls −S9	EMS	NQO	EMS	NQO	EMS, MNNG, ENU	EMS, MNNG
Plate development period (days)	7–9	12	10	10–14	10	
Scoring method	Electronic colony counter	Counting wells with growth	Colony count by eye	Counting wells with growth	Colony count by eye	Colony count by eye

laboratories using $Tk^{+/-}$ grew their cells up in suspension culture using a variety of different growth media, and differing concentrations of horse-serum. Some investigators used heat-inactivated serum while others did not. Some workers using $Tk^{+/-}$ purged their population of cells prior to use in an experiment with selective media to reduce the number of spontaneous mutants. While it was generally agreed that it is essential to reduce the number of spontaneous mutants in the population to as low a number as possible, (see analysis of data) there was considerable discussion about purging as a routine procedure.

Most of the remainder of investigators used either hamster V79 or CHO cells for this study enabling some comparisons to be made between the performance of these cells in testing of the ten test chemicals. Some attempt has been made to summarise the spontaneous mutation frequencies normally found for the cell lines used. Frequencies for resistance to trifluorothymidine tended to be some ten-fold higher than a marker such as ouabain.

Only data from one laboratory was submitted using cultured human cell lines. Both these lines appeared to have a lower spontaneous mutation frequency for Tk and HGPRT when compared with the rodent lines. These lines also appeared to have slightly longer cycle times than those rodent lines reported on.

Treatment with the test chemical

Most investigators performed preliminary toxicity tests to determine the concentration range over which the test chemical was to be assayed. Compounds were usually dissolved in dimethyl sulphoxide for testing. Treatment periods varied from 1 to 4 h in the rodent cell assays and up to 28 h for the human cells. Some investigators treated their cells in vessels which were not enclosed. For volatile compounds this is a practice which cannot be encouraged.

However, in terms of the results from this study this fact did not appear to account for the inconsistencies seen. Some workers exposed their cells to the test chemical in suspension culture while others

7	8	9	10	11	12
Cloning efficiency post treatment	(i) Cloning efficiency post treatment (V79) (ii) % Total relative growth (L5178Y)	Prelim. toxicity test: % total relative growth	Preliminary test: % total relative growth	Preliminary toxicity test	% Total relative growth
3	2	4	4	2	4 days
3	2	4	4	2	4 days
	(i) No (ii) Duplicates	Yes	No		No
	Three (minimum of two)	Yes		When considered necessary	Sometimes
5×10^5	(i) 5×10^5 (ii) 1×10^6		6×10^6	10^7	1×10^6
8×10^5	(i) 2.5×10^6 (ii) 1×10^6	3×10^6	3×10^6	OUA: 8×10^6 TFT: 4×10^5	OUA: 2.5×10^6 6TG: 2×10^6
DMN	BP MNNG	3MC MMS, FMS	3MC EMS	BP EMS	BP EMS
12–14	(i) 8 (ii) 10		9–13	10	8–9
Colony count by eye	(i) Colony count by eye (ii) Image analyser	Electric colony counter	Electronic colony counter	Counting wells with growth	Colony count by eye

exposed cells on a monolayer. The former technique is likely to lead to a more uniform exposure than the latter.

Provision for metabolic activation

In Table 2 are set out the metabolic activation conditions which each investigator used. The most common procedure was to use a rat-liver 'S9' mix from either Aroclor or phenobarbitone/α-naphthoflavone induced animals. Most workers stored batches of liver 'S9' at $-90°$ prior to use. The final concentration of liver 'S9' varied considerably from laboratory to laboratory (0.25–2.0%). In one laboratory uninduced rat liver 'S9' was used while in another activation was performed not only with liver 'S9' but also with various primary cell cultures (rat hepatocyte, Syrian hamster embryo cells and chick embryo hepatocytes). As will be seen later these co-cultivation procedures enabled the Working Group to assign HMPA as a gene mutagen. Various NADPH-generating systems were used but since these are unlikely to be rate-limiting they are probably not important in assay variation. On the other hand it was the view of the Group that more work was needed to examine the role of metabolic activation in these assays. Many laboratories participating did not have a sufficiently large data base on known carcinogens and non-carcinogens to know whether or not the metabolic activation conditions used were optimum.

Replicate cultures and population size

It was the concensus view of the investigators that the above are of critical importance in the design of a good assay. Unfortunately, some investigators either failed to use replicate cultures or insufficient cells to demonstrate a mutagenic effect with anything but the most potent mutagens. As a general guide one should treat ten times the inverse of the spontaneous mutant frequency. Replication of cultures was thought to be desirable on the grounds that a greater indication of variability at the various treatment doses could be obtained.

Repeat experiments

While it was felt highly desirable that experiments should be repeated to confirm both positive and negative responses only a few investigators were able to do this. The chief reason for this was the cost of repeat testing; many of the investigators were participating in this study at their laboratory's expense. The disadvantage of this approach is spelt-out in the General Conclusions. For this group of weak mutagens the ideal case would be to perform at least one repeat assay.

Dose levels

Most investigators selected their dose levels on the basis of a preliminary toxicity test. One or two studies did not test up to a reasonable toxic level. For some of the compounds, such as caprolactam which were non-toxic, heroic doses of chemical were sometimes tested. All investigators tested at a minimum of five concentrations and some up to ten. Ideally the concentration range tested should go down to at least ten percent survival.

Expression times

Expression times varied depending on the drug-resistance marker under investigation. For trifluorothymidine resistance this ranged between 48 and 72 h while for 6-thioguanine the range was between 144 and 192 h. One laboratory tested at several different expression times but this procedure failed to convert negative to positive responses. The Working Group was not convinced that sampling should be performed at varying expression times. No data from this study served to reinforce this idea.

Scoring

Depending on the assay methodology various scoring procedures were used in this study. In the L5178Y $Tk^{+/-}$ assay no investigator split the mutant colonies analysed into 'large' and 'small' but counted the whole size spectrum as individual clones. When electronic colony counters were used the aperture was set wide open for colony counting.

TABLE 4

METHODS OF DATA ANALYSIS FOR INDIVIDUAL INVESTIGATORS

	1	2		3	4		5		6
		TK6	AHH-1						
Spontaneous mutation frequency ($\times 10^{-6}$)	TFT^R 17 ± 4	TFT^R 1.29 ± 0.61	$6TG^R$ 3.91 ± 1.49	$6TG^R$ 4.9	Oua^R 0.03 ± 0.07	$6TG^R$ 13.2 ± 5.1	TFT^R 30.0	$6TG^R$ 9.0	$6TG^R$ 4.0
Positive criteria	Acceptable data meeting 3 criteria (see text)	Acceptable assays and meeting two statistical criteria set out in text		Reproducible dose–response or trend in repeat experiments. Five-fold increase	Statistical increase above background at two or more dose levels		Dose–response replicate experiments		
Statistical method	*t*-test on pooled mutant yield data over active dose-range	(1) *t*-test $p < 0.05$ (2) greater than 99% upper confidence limit of historical control		None	Chi^2 test		None		None

Analysis of data

This aspect proved to be the most contentious, there being some heated arguments about how mammalian cell mutation data should be analysed and what constitutes a positive response. In Table 4 are listed the mean spontaneous mutant frequencies and statistical criteria used by each laboratory.

The Working Group took the approach that it would consider a compound positive if the individual investigator had classified the compound as positive on the basis of dose-dependent increase in mutation, an absolute increase in mutant numbers and some increase (statistical or two-fold) over spontaneous mutant values. The experiment should have also been performed more than once. The Working Group did not have time to examine each individual compound in more than a rudimentary manner. For one compound, safrole, results which some investigators had declared positive were shown not to be statistically-significant. If this process had been continued for all ten chemicals, something that would have taken several weeks to perform, it is not known if the overall conclusions for the individual compounds would have been modified. As mentioned earlier on but worth reiterating at this point, an ideal test would be one in which: (1) sufficient cells would be treated, passaged and plated to allow enough mutant cells in the cell population to be maintained at all steps of the assay; (2) standard procedures should be adopted to reduce the background level of mutant cells in the cell population before chemical exposure; (3) independent repeat experiments should be performed, and (4) replicate cultures at each test concentration should be used. A discussion of some of these points can be found in the UKEMS Guidelines on mutagenicity testing.

Concensus summary on the activity of the ten test chemicals in mammalian cell gene mutation assays

As the evaluation criteria for a significant response varied considerably between investigators and as all the different assay systems could not be considered equivalent in sensitivity or adequacy of design, the approach of the Working Group was to consider the evaluations of each investigator. These individual evaluations are recorded in Table 5. The data for each compound was then evaluated

7	8		9	10	11		12	
Oua^r	TFT^R	$8Azg^R$	TFT^R	TFT^R	TFT^R	Oua^R	Oua^R	6TG
1.0	10	30	37 ± 15	2.8 ± 1	10	10 4.8	$5.1 \pm$ 0.9	$13.7 \pm$
	Dose–response Absolute increase in mutants before correcting for survival (at several doses)		Individual dose analyses and trend analysis meeting 95% probability Uses replicate doses and replicate experiments (see text)	Two-fold increase above background at two or more dose-levels > 10% survival	Dose-response Some evidence of toxicity but not excessive i.e. > 20% survival			
None	None		Statistical analysis based on pair-wise comparison of doses against solvent control. A trend of dose–response	None	None		None	

in total for evidence of convincing responses indicating mutagenesis. It was not required that a compound be active in more than one assay type or laboratory to be considered mutagenic. A well-defined response obtained, with one or more systems for example, could outweigh the negative responses seen in a majority of laboratories if the negative experiments were not definitive. Conversely, a series of weak or indefinite responses could lead, as it did in the case of benzene, to an inconclusive evaluation and the recognition of the need for additional experiments. The concensus view, therefore, did not generally reflect a simple sum of the individual positive and negative evaluations. Instead the concensus view, which is briefly described below, represents the Group's best effort to determine the activity of each test chemical.

List of summary statements

Hexamethylphosphoramide. The concensus of the working group was that HMPA was a mutagen. Three positive results were obtained only with some form of exogenous metabolic activation. In one laboratory, the results for HMPA were inconclusive when tested with S9 yet positive when L5178Y cells were cocultivated with any of 3 different metabolically competent cell types. Positive results for two laboratories in the absence of S9 were obtained at the TK and ouabain-resistance locus of L5178Y cells, and for one laboratory in V79 cells (active concentration range 250-10210 micrograms/ml).

o-Toluidine. The majority of the investigators failed to detect this compound as a mutagen, however, positive results for *o*-toluidine were reported by five laboratories leading to the consensus view that this chemical was a mutagen. In V79 and L5178Y cells, the positive results were obtained in the absence of S9 but at low cell survival in one case. With human lymphoblasts, positive results were obtained in one line with S9 and in another metabolically competent line without S9 at high cell survival (active concentration range 10–1000 μg/ml).

Benzene. Five laboratories scored benzene as a positive mutagen. two investigators obtained their positive results with S9 activation and one positive was obtained in a metabolically competent cell line in the absence of S9. Positive results were obtained at the TK, HGPRT, and ouabain-resistance markers in 3 different cell lines. The consensus of the working group was that there was no convincing evidence for the mutagenicity of benzene (tested up to 3000 μg/ml).

Safrole. Safrole was a mammalian cell muta-

TABLE 5

SUMMARY OF MAMMALIAN CELL GENE MUTATION DATA

	1		2			3		4		5		6		7	
			TK6		AHH										
	a	b	a	b	b	a	b	a	b	a	b	a	b	a	b
HMPA	+	–	–			–	–	–	–	+	–		+	–	–
TOL	–	–	+	–	+	–	–	+	–	–	–	–	+	–	–
BEN	–	–	–		+	–	–	–	+	–	–	–	+	–	–
SAF	+	–	+	–	–	–	–	–	+	I	–		+	–	–
CAP	–	–	–	–	–	–	–	–	–	–	–			–	–
ACN	+	+	+	–	+			+	+						
DEHP	–	I	–	–	–			–	–						
ZOIN	–	–	–	–	–			–	–			+	–		
DES	–	–	–	–	+			+	+						
PB	–	–	–	–	+			–	–			–	+		

I, inconclusive.

a, +'S9'.

b, –'S9'.

* Most investigators found this compound to be the most active of those tested.

gen that the majority of the laboratories failed to detect. Only two laboratories obtained acceptable positive results in the presence of S9, but the magnitude of the response was small. Other laboratories obtained negative or equivocal results for this chemical. Evidence for mutagenicity at the ouabain-resistance locus, HGPRT locus, or the TK locus without S9 was reported, but was not convincing (active concentration range 1–625 μg/ml).

Caprolactam. Caprolactam was declared a normutagen in all assay systems when tested under varied metabolic activation conditions even at toxic concentrations (tested up to 11 320 μg/ml).

Acrylonitrile. Acrylonitrile was a mammalian cell mutagen in both human lymphoblast or mouse lymphoma cells at the TK and HGPRT loci. The concensus view was that this compound was the most potent of the ten test chemicals. Mutagenic activity was observed both in the presence and/or absence of S9 (active concentration range 6–225 μg/ml).

Diethylhexylphthalate. There was little evidence of mutagenicity for DEHP in these assay systems. Most laboratories reported negative or inconclusive data. Positive mutagenicity was reported in a single assay. By consensus DEHP was considered as normutagenic due to the lack of sufficient supporting evidence of its activity (tested up to 10 000 μg/ml).

Benzoin. Benzoin was declared a positive mutagen in four laboratories in studies using two cell types and three gene loci. Mutagenicity was obtained only in the presence of S9 prepared from Aroclor or phenobarbital-benzoflavone treated rats. Good dose-related increases in mutant frequencies were reported by all four laboratories. The consensus of the group was that benzoin was a mammalian cell mutagen (active concentration range 3–3000 μg/ml).

Diethylstilboestrol. It was the consensus of the working group that DES was a mammalian cell mutagen. Convincing evidence for the mutagenicity of this chemical was reported by five laboratories in a variety of assays and under different metabolic activation conditions (active concentration range 6–50 μg/ml).

Phenobarbitone. It was concluded that phenobarbitone could act as a mutagenic agent in mammalian cells. Some investigators obtained positive results in the presence of S9 while in other laboratories positive results were reported in the absence of S9 (active concentration range 250–2000 μg/ml).

8				9		10		11				12		Consensus
L5178Y		V79						$TK^{+/-}$		$TK^{+/+}$				
a	b	a	b	a	b	a	b	a	b	a	b	a	b	
–	–	–	–	+	–		+	–	+		+	–	–	+
–	–	–	–		+	–	–	–	+	–	–	I	–	+
–	–	–	–	–	–	+	–	+		+		–	–	I
–	–	–	–	I	–	–	–	–	+	+	+	–	–	+
–	–	–	–	–	–	–	–						–	– –
+	+	–	–		+	+	–			–				+ *
–	–	–	–	–		+	–	–	–	–	–			–
–	–	+	–	+	–	+	–	–	–		I			+
–	–	–	–		+	–	–	+	–	+	–			+
–	–	–	–	–	+	+	–	+	–	I	+			+

b, –'S9'.

* Most investigators found this compound to be the most active of those tested.

General conclusions on the performance of mammalian cell gene-mutation assays

This study provides a unique opportunity to compare mammalian cell gene-mutation assay responses in a variety of labs. Six laboratories used the same genetic end-point, namely resistance to trifluorothymidine in the same cell-line, mouse lymphoma L5178Y. Three laboratories used hamster V79 cells. it should be stated at the outset of this discussion that the results of this study were disappointing. There was very little concordance in assay response even when assays were very similar. Nevertheless, the compound that appeared to be the most potent genotoxin, ACN, was active in six of the eight laboratories that tested this compound. On the other hand CAP, a non-carcinogen, was uniformly negative in all laboratories. The remainder of the compounds tested fell in-between this range of activity presumably reflecting potency differences. For some compounds, such as HMPA, the general lack of response could be explained, while for others no obvious explanation was apparent. Members of the Working Group discussed at some length the criteria for an adequate mutation assay. These can be summarised as follows:

(1) Sufficient numbers of cells should be present after treatment with the test agent to be able to detect mutations in the surviving population.

(2) Replicate cultures should be used.

(3) Cells should be treated at sufficient concentration that cytotoxicity is observed (10–30% survival).

(4) An adequate dose-range should be studied.

(5) Provision for metabolic activations should be optimised for a selected group of positive control compounds.

(6) The experiment should be repeated.

(7) Positive controls with and without liver 'S9' should be assayed alongside the test chemical.

(8) The spontaneous mutation frequency for the experiment under consideration should be within the historical range.

(9) Analysis of the data should use a suitable statistical test. A positive response is one that is dose-dependent and reproducible.

No one laboratory in the present study satisfied all the above. It did not prove possible, due to insufficient time, to examine the raw data from each laboratory in any detail. When the data for one compound, SAF, which was called positive by several laboratories, was rigorously examined using suitable statistical tests then the result called by some of these labs was found to be incorrect. Were this process to be done for all ten chemicals then some of the inconsistencies between labs would probably be removed. It is hoped that it will be possible in the future to take the raw data from all the labs submitting L5178Y results and analyse it using a uniform procedure. It would be foolish not to use this opportunity in a more than superficial manner.

The participants in our Group did not feel as down-hearted as the Steering Group about the results that were obtained in this trial. It is not surprising that mammalian cell gene-mutation assays fail to detect carcinogens such as DEHP. These assay systems are quite specific in what they can detect, i.e. chemicals that interact with the cell's genome. On the other hand, we cannot say that compounds such as DEHP will do this. Cancer is a multifactorial process that occurs through largely unknown mechanisms. On the other hand gene-mutation appears to be a one-hit phenomenon, at least in the assay systems which were examined in this trial.

Far from being discouraged, therefore, the Working Group wished to make the following recommendations for further research:

(a) A study be set up in which compounds are tested blind.

(b) An attempt be made to standardise protocols at least as far as the number of cells treated, replicate cultures, etc.

(c) The test compounds selected should be of carcinogen/non-carcinogen pairs.

(d) Analysis of the results to be performed using standardised statistical techniques, preferably at some central location.

(e) The concentration of liver 'S9', cofactors, etc. be optimised.

(f) Varying expression times be examined.

(g) Some attempt be made to find a robust system which could be recommended for general screening.

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8

Summary Report on the performance of the inhibition of metabolic cooperation assay

Work Group
Eugene Elmore and Andrew J. Nelmes

Summary

All investigators in the IPCS program used the Chinese hamster V79 inhibition of metabolic cooperation assay. One investigator evaluated five of the chemicals and the other two investigators evaluated all ten chemicals. The chemicals evaluated were: *o*-toluidine (TOL), acrylonitrile (ACN), diethylhexylphthalate (DEHP), safrole (SAF), phenobarbital (PB), benzoin (ZOIN), diethylstilbestrol (DES), hexamethylphosphoramide (HMPA), benzene (BEN) and caprolactam (CAP). Test criteria were devised to assess the performance of each chemical in the assay. Two chemicals, TOL and ACN, were evaluated as positive; four chemicals, DEHP, SAF, PB and ZOIN were evaluated as inconclusive because they gave weak and inconsistent responses; the remaining four chemicals, DES, HMPA, BEN, and CAP, were evaluated as negative.

The inhibition of metabolic cooperation assay is relatively new and insufficient data are available to qualify it as a complementary assay for routine use in conjunction with the Salmonella assay. Nevertheless, based on the existing information on V79 and other cell types, there is an apparent association between the inhibition of metabolic cooperation in vitro and in vivo tumor promotion, cocarcinogenesis, and perhaps teratogenesis. In the collaborative studies, chemicals that produced positive responses were strong inhibitors of metabolic cooperation and those that produced inconclusive responses were weak inhibitors of metabolic cooperation. The chemicals that were evaluated as negative in this assay may require metabolic competency beyond that which is present in V79 cells. The assay was evaluated as category 'C' based on the criteria established by the Assay Coordinating Committee; however, upon completion of the validation studies currently in progress, there is a strong possibility that the assay can be reclassified as category 'A'. Based on the current data from the evaluation of a variety of chemicals in the inhibition of metabolic cooperation assay with V79 cells, the assay appears to offer many of the properties that are necessary to complement the bacterial tests for genotoxicity.

The IPCS assays have provided important insight into the design of future validation studies. A series of guidelines were offered for the benefit of those who wish to develop improved methods to increase the sensitivity and reproducibility of the inhibition of metabolic cooperation assay.

Introduction

Initially it was necessary to define the assay system and the endpoints relating to the phenomenon of metabolic cooperation. The assay used in this system was originally developed by Yotti et al. (1979) and utilized wild-type (hypoxanthine guanine phosphoribosyl transferase proficient, HG-PRT$^+$) and mutant (HG-PRT$^-$) Chinese hamster lung (V79) cells. The HG-PRT$^+$ cells can phosphorylate the purine base analogues including 8-azaguanine and 6-thioguanine (Chu, 1971;

Caskey and Kruh, 1979) to permit their incorporation into DNA and/or RNA (Nelson et al., 1975) thereby resulting in cell death. HG-PRT$^-$ cells cannot phosphorylate the purine analogues and are therefore resistant to the toxic effects of these analogues. However, when mammalian cells are cultured at a cellular density that permits direct contact between cells, gap junctions can form at the point of contact. Cells interconnected with each other via gap junctions can transfer nutrients, ions and other molecules to one another resulting in a form of metabolic cooperation (Cox et al., 1976; Pitts and Simms, 1977; Finbow and Pitts, 1981). In the current model in vitro assay, low numbers of HG-PRT$^-$ cells are cocultivated with high numbers of HG-PRT$^+$ cells in purine analogue selective media. The high HG-PRT$^+$ cell densities ensure that most of the mutant cells are in frequent contact with wild-type cells. Under these conditions, the mutant cells that are cross-fed with the toxic phosphorylated purine analogues will be killed while the noncontacting mutant cells will survive and proliferate to form colonies.

The data from experiments that measure the inhibition of metabolic cooperation between cells in vitro can be easily quantitated. The experiments of Yotti et al. (1979) clearly demonstrated that the addition of the known tumor promoter, 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA), to the selective medium can inhibit the toxic effects resulting from phosphorylated purine analogue transfer through metabolic cooperation, thereby permitting the enhanced recovery of mutant cells. Utilizing a system that permitted direct measurements of cell-to-cell transfer, Fitzgerald and Murray (Murray and Fitzgerald, 1979; Fitzgerald and Murray, 1980) demonstrated that TPA inhibited the direct communication between cells. These and other workers have suggested that tumor promoter inhibition of metabolic cooperation may be an integral step in multistage chemical carcinogenesis. The V79 inhibition of metabolic cooperation assay has also been suggested to have broad applicability for evaluating chemicals that are known to cause a variety of human health effects. This assay responds to many chemicals that produce epigenetic as well as genetic effects (Elmore et al., this volume). This report will evaluate the data presented by the collaborators to determine if the assay should be recommended to complement the bacterial bioassays for environmental toxicants.

Materials and methods

The compounds evaluated in these studies were: *o*-toluidine (TOL), acrylonitrile (ACN), diethylhexylphthalate (DEHP), safrole (SAF), phenobarbital (PB), benzoin (ZOIN), diethylstilbestrol (DES), hexylmethylphosphoramide (HMPA), benzene (BEN) and caprolactam (CAP).

The protocols from the individual investigators in this study were compared (Table 1). There were marked differences in the serum concentrations, selective agent, cloning efficiencies, cell numbers and treatment times.

The data from individual investigors were converted to a common form to permit an assessment of the individual variability. In an effort to standardize the evaluation of the data from each investigator, the following valid-test criteria were established to evaluate the raw data. These criteria were not intended to be applied generally, but only to permit an assessment of the data generated in this study.

(1) The percentage of mutant colony recovery with the solvent controls in the metabolic cooperation assay should not exceed 33% of the colonies observed in the solvent control mutant-cloning efficiency assay.

(2) The mutant colony recovery with the positive control should represent a two-fold, or greater, increase in mutant recovery efficiency relative to the solvent control.

(3) The cytotoxicity should not exceed a 30% reduction in mutant cloning efficiency.

The following response definitions were used to describe the activity of the test chemicals:

(1) For a response to be positive at any concentration, a statistically significant enhancement of mutant cell recovery relative to the mutant cell recovery of the solvent control must be demonstrated.

(2) An experiment was considered positive when two or more consecutive concentrations produced positive responses.

(3) A test chemical was termed positive on the basis of confirmatory information in two or more experiments within the same laboratory.

TABLE 1

TEST PROTOCOLS AND ENDPOINTS OF INHIBITION OF METABOLIC COOPERATION ASSAY

	Investigators		
	Elmore et al.	Scott et al.	Umeda et al.
Cell type	V79 (Trosko)	V79 (Newbold (4-K1)	V79
Mutant cells	6-TG^r (X-ray induced)	8-A_zG^r (V79-M13)	Subline T2-14
Selective agent	6-TG (10 μg/ml)	8-A_zG (30 μg/ml)	6-TG (10 μg/ml)
Medium	MEM with 50% increase in NEAA and 100% increase in pyruvate	DMEM	MEM
Serum	3% FBS	10% FBS	10% FBS
Trypsin	Worthington (TRL3) 0.01%	Gibco 0.25%	Mochida crystalline 50 u/ml
Culture dishes	6 cm	9 cm	24-multiwell plates
Metabolic system	None	None	None
Toxicity measurement	Preliminary screen CE for MC	Consecutive assay CE for MC	Preliminary screen
Number of cells	10^2 6-TG^r for CE 10^2 6-TG^r + 4×10^5 6-TG^s for MC	3×10^2 8-A_zG^r for CE 3×10^2 8-A_zG^r + 8×10^5 8-A_zG^s for MC	50 6-TG^r for CE 50 6-TG^r + 10^4 6-TG^s for MC
Repeat experiments	2–3	3	3–6
Positive control	TPA (4 ng/ml)	TPA (200 ng/ml)	TPA (100 ng/ml)
Negative control	DMSO (0.5%, v/v)	DMSO (0.5%, v/v)	DMSO (0.5%, v/v)
Cell usage time	2 months	4 months	1 month
Cell cycle time	11–13 hr	11–13 hr	11–13 hr
Treatment time	3 days	Continuous	4 days
Growth time	CE 7 days MC 7–8 days	CE 14 days MC 14 days	5 days
Dose levels	5	6	5–6
Replicate cultures	5 (CE) 4–8 (MC)	3 (CE) 6 (MC)	4–6 wells
de novo mutation control	6-TG^s cells only	8-A_zG^s cells only	6-TG^s cells only

CE, cloning efficiency. MC, metabolic cooperation assay. 6-TG, 6-thioguanine. 6-TG^r, 6-TG-resistant. 6-TG^s, 6-TG-sensitive. 8-A_zG, 8-azaguanine. 8-A_zG^r, 8-A_zG-resistant. 8-A_zG^s, 8-A_zG-sensitive. NEAA, non essential amino acids. TPA, 12-O-tetradecanoyl-phorbol 13-acetate. FBS, fetal bovine serum.

(4) A test chemical was considered to produce a weak or inconclusive response when only one of two or more experiments within the same laboratory was considered positive.

(5) A test chemical was considered negative when no positive responses were observed in two or more experiments within the same laboratory.

The overall evaluation of an individual chemical was based on the workgroup-evaluated responses from all laboratories. Each chemical's potential to inhibit metabolic cooperation was classified positive, inconclusive, or negative utilizing the following guidelines.

(1) For a chemical to be considered positive, the data from two or more laboratories must be evaluated as positive.

(2) Chemicals that were evaluated positive for only one laboratory were considered inconclusive regardless of the relative strength of the response in any one or more experiments. This criterion is relatively conservative and may require that the data be reevaluated when the sensitivity of the assay is better understood.

(3) Chemicals that were evaluated as weak (inconclusive) or negative for all laboratories were considered as negative.

Results

The results reported by individual laboratories demonstrated marked differences. Elmore et al. reported six of ten chemicals as active or weakly

active, Scott et al. reported one of five chemicals as active; and Umeda et al. reported two of ten chemicals as active or weakly active. The workgroup evaluated the data from each laboratory using the established criteria and compared the findings to that reported by the original investigators (Table 2).

On the basis of the criteria, Umeda's conclusion for ZOIN was revised and reevaluated as positive. The investigators, Umeda et al., averaged the result of four experiments, two of which were positive. For some test chemicals, the absence of a positive control made interpretation of the results tenuous. In other cases, the assays were regarded as equivocal if the percentage of recovery of the solvent controls in the metabolic cooperation assay was high or the mutant colony recovery of the positive control was below the established two-fold increase over the solvent control.

The workgroup's evaluations of the test chemicals' responses in the inhibition of metabolic cooperation assay with V79 cells are as follows.

Positive chemicals

TOL

The results with TOL in the inhibition of metabolic cooperation assay were positive. There were considerable differences in the protocols (Table 1) that resulted in the variability in the responses observed between laboratories. The cytotoxicity appeared to be greater at lower serum concentrations (Fig. 1). Also, the assay appeared to be more sensitive in the detection of enhanced mutant recovery when a lower serum concentration was used in the selective medium (Fig. 2). No threshold level for inhibition enhancement could be established from these studies. Within each laboratory, the positive responses of individual assays were comparable.

ACN

The results for ACN were positive. When the inter laboratory findings of Elmore et al. and Umeda et al. were compared, the cytotoxicity of ACN was low. In both studies, the relative enhancement of mutant colony recovery was comparable over the independently determined test chemical concentration range. These findings were more consistent than those for TOL and reflect similar assay protocols (Table 1).

Inconclusive chemicals

DEHP

DEHP was considered inconclusive utilizing the established criteria; however, the strong positive responses reported by Elmore et al., the positive response in one experiment by Umeda et al., and the response reported by Malcolm and Mills (1984)

TABLE 2

SUMMARY OF WORKGROUP EVALUATION OF THE INHIBITION OF METABOLIC COOPERATION ASSAY

Chemical	Investigator reported activity			Workgroup evaluation			
	Elmore	Scott	Umeda	Elmore	Scott	Umeda	Consensus
TOL	+	+	−	+	+	+/−	+
ACN	+	NT	+	+		+	+
DEHP	+	NT	−	+		+/−	+/−
SAF	+/−	−	+/−	+/−	−	+/−	+/−
PB	+/−	NT	−	+/−		−	+/−
ZOIN	+/−	NT	−	+/−		+	+/−
DES	−	NT	−	−		−	−
HMPA	−	−	−	−	−	−	−
BEN	−	−	−	−	−	−	-
CAP	−	−	−	−	−	−	−

NT, not tested. +, positive. −, negative. +/−, weak or suspect.

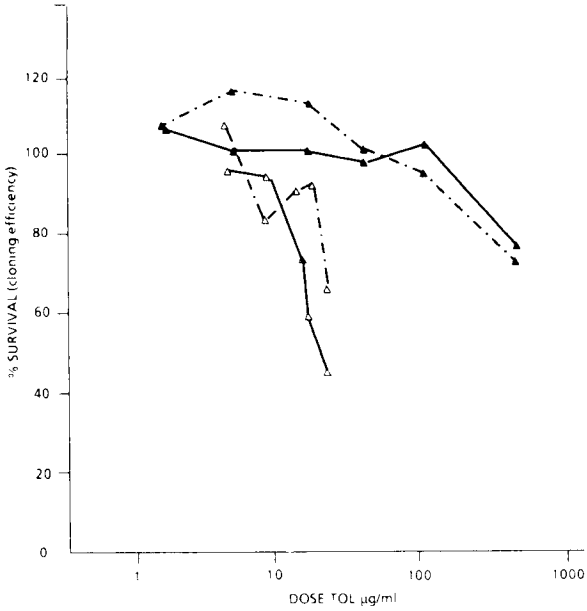

Fig. 1. Inter- and intra-laboratory evaluation of *o*-toluidine. The effect of TOL on the percent survival of mutant colonies (CE). The percent survival was determined relative to the solvent controls in each experiment. Individual assays: △———△, △–·△, Elmore et al.; and ▲———▲, ▲–·▲, Scott et al.

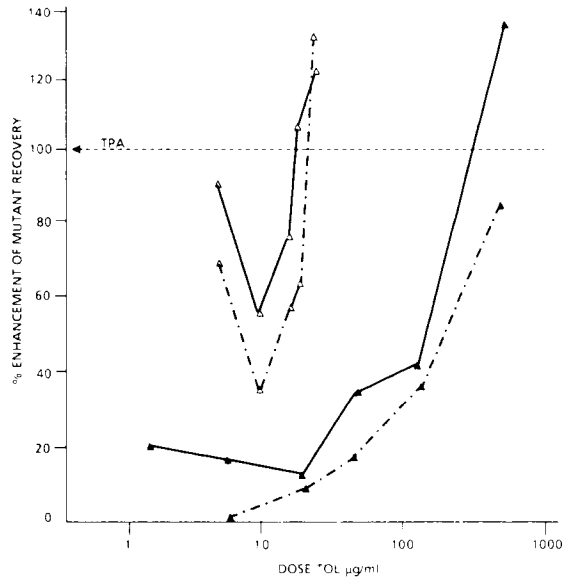

Fig. 2. Inter- and intra-laboratory evaluation of *o*-toluidine. The effect of TOL on the percent enhancement of mutant colony recovery. The enhanced mutant colony recovery was calculated by the methods described by Elmore et al. (this volume). Briefly, the percent enhancement was determined by subtracting the solvent control mutant colony recovery value from the test chemical mutant colony recovery value and determining the enhanced recovery relative to that observed in the positive (TPA) control (100%). Individual assays: △———△, △–·△, Elmore et al.; and ▲———▲, ▲–·▲, Scott et al.

suggest that this chemical should be considered positive. The concentrations that provided positive responses were similar.

SAF

SAF produced positive responses in one experiment by Elmore et al. and in one by Umeda et al. The concentrations that produced positive responses were similar. SAF was weakly positive based on the degree of response observed in each laboratory.

PB

PB produced weakly positive responses in only one experiment by Elmore et al. and was therefore considered inconclusive. Information from the literature is conflicting (Trosko et al., 1981; Umeda et al., 1980). Determination of the response of this weakly active chemical in the inhibition of metabolic cooperation assay may require additional studies utilizing cells that are known to be capable of metabolically activating this chemical.

ZOIN

ZOIN produced positive resonses in two experiments by Umeda et al. and in one experiment by Elmore et al. The concentrations that produced positive responses were similar. Based on the degree of response, ZOIN was weakly positive in inhibiting metabolic cooperation.

Negative chemicals

DES

DES was evaluated as negative. The highest concentration evaluated exceeded 1 µg/ml. This evaluation was in agreement with the published work of Barrett et al. (1983).

HMPA

HMPA was evaluated as negative based on results from all laboratories participating in this

study. The highest concentration evaluated was greater than 3 mg/ml.

BEN

BEN was evaluated to be negative. This finding was probably due to the metabolic capacity of the V79 cells, since Malcolm and Mills (1984) have reported that the metabolites of benzene are active in this assay. The highest concentration evaluated in these studies was 500 μg/ml.

CAP

CAP was evaluated to be negative. The highest concentration evaluated exceeded 2 mg/ml.

Overview and discussion

The observations of Yotti and others (Yotti et al., 1979; Umeda et al., 1980; Newbold and Amos, 1981; Trosko et al., 1981; Trosko et al., 1982; Malcolm and Mills, 1984; Welsch and Stedman, 1984) suggest that tumor promoters, teratogens, carcinogens and other environmentally significant toxicants may inhibit metabolic cooperation resulting in the transient loss of normal cellular control mechanisms that are mediated by cell–cell communicaiton. Alterations of normal cellular control mechanisms are known to play an important role in the regulation of cellular differentiation, and are important in the expression of cancer, birth defects and neurological and reproductive diseases.

A diverse range of chemicals has been evaluated in this assay (Table 1, Elmore et al., this volume); however, additional data are needed to confirm the proposed association between in vivo effects, e.g., tumor promotion, cocarcinogenesis, and teratogenesis, and in vitro inhibition of metabolic cooperation. The currently available data suggest that the inhibition of metabolic cooperation assay may be an important complementary assay for evaluation of chemicals for potential carcinogenicity, cocarcinogenicity and teratogenicity. A National Institute of Occupational Safety and Health inter laboratory validation study, utilizing 25 test chemicals, is in progress and should provide additional information for this association.

The IPCS assays have provided important insight into the design of future studies to measure the inhibition of metabolic cooperation. The following comments are offered to those wishing to evaluate chemicals in this assay.

(1) The Chinese hamster lung fibroblast (V79) cell line is a suitable cell type for such a study, although this line is limited by its metabolic capacity.

(2) Frozen stocks of a master culture shuld be prepared and used within a period of two months of thawing.

(3) All cultures should be free of mycoplasma.

(4) 6-Thioguanine (6-TG) is recommended as the selective agent; however, other purine analogues may be used when proper controls for serum lot and concentration are included.

(5) It is essential to obtain a single cell suspension prior to counting and seeding.

(6) It is important to determine the seeding density of the purine analogue-sensitive cells that permits the optimal recovery of purine analogue-resistant cells. The method outlined by Dorman and Boreiko (1983) may be used in this determination.

(7) The density of the cell cultures at the time of addition of test chemical should be determined by fixing and staining representative dishes. This information would provide an estimate of the actual cell densities present at the time of treatment, which can affect the 6-TG^r colony recovery efficiency in the presence of 6-TG^s cells.

(8) The cell number present at each dose level should be determined at the end of the treatment period to provide a direct estimate of cytotoxicity.

(9) A clonal cytotoxicity assay should be done in parallel to the metabolic cooperation assay to provide an additional cytotoxicity control.

(10) The greater the difference between the mutant cell recovery efficiencies of the positive and negative controls, the greater the confidence in a negative result for a test chemical.

(11) The sensitivity of the assay may be influenced by the concentration of serum in the selective medium.

(12) Relevant information on the minimum duration of chemical treatment is not available, but at least a 20–24 h treatment is recommended (until such time as the optimal treatment duration is established).

(13) The pH of the media containing test chemicals should be maintained at 7.2–7.4.

(14) Water or serum-free medium are the solvents of choice; however, ethanol or other organic solvents may be used. Caution must be used with solvents such as DMSO and ethanol which may inhibit metabolic cooperation at relatively low concentration. When such solvents are used, the solvent volume should be held constant for all test chemical concentrations.

(15) If test chemicals are volatile, these should be tested in closed culture vessels. It may be desirable to consider the development of gaseous exposure methods.

(16) The test chemicals should be investigated up to the limit of their solubility or their maximum nontoxic dose.

(17) Many cell types and other in vitro assay systems may be used, e.g., Table 3; however, in each case, care must be taken to ensure that the monitored endpoint is related to the metabolic cooperation phenomenon.

Additional experiments are needed to evaluate the assay's utility for detecting specific classes of chemicals that act primarily through nongenetic mechanisms. Validation of the utility of this assay as a complementary assay for the bacterial and other tests will require carefully designed and controlled experiments to optimize the sensitivity and reproducibility of the assay for detecting various classes of environmental toxicants. The assay was evaluated as category 'C' based on the criteria established by the Assay Coordinating Committee (Ashby et al., 1983); however, upon completion of the validation assays that are currently in progress, there is a strong possibility that the assay can be reevaluated to category 'A'.

TABLE 3

EXAMPLES OF OTHER CELL TYPES USED TO INVESTIGATE METABOLIC COOPERATION

Murray and Fitzgerald (1979) Cocultured HEL/37 (mouse epidermal cells) and 3T3 (Swiss albino mouse cells)
Fitzgerald and Murray (1980) Cocultivated HEL/37 and PG-19 (mouse fibroblasts)
Williams et al. (1981) Rat hepatocyte primary culture
Dorman et al. (1983) C3H/10T1/2 cells
Mosser and Bols (1982) Human fibroblasts
Parkinson and Emmerson (1982) Human epidermal cells

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9

Summary Report on the performance of the cell transformation assays

Work Group Chairmen
D. McGregor and J. Ashby

Work Group Participants
C. Barrett, G. Hatch, D. McGregor, S. Nesnow, J. Rundell, T. Sanner, W. Suk and M. Zdzienicka

Introduction

Tests from six assay categories were submitted from eight participating laboratories.

The assays used to demonstrate a transformation in cell behavior were divided into the following categories, depending upon the characteristics of the cells, the assay endpoints and the involvement of transforming (DNA) or infective (RNA) viruses.

(A) Colony morphology

(1) Primary Syrian hamster embryo (SHE) colony assay (Barrett, Sanner). (2) C3H10T½ cell focus assay (McGregor, Nesnow). (3) BALB/c 3T3 cell focus assay (Rundell).

(B) Viral dependence

(1) Primary SHE cell cultures: enhancement of transformation by Simian adenovirus (SA7), following pretreatment of cells with chemical or physical agents (Hatch). (2) Fischer rat embryo cell cultures: enhancement of survival of Rauscher leukemia virus (RLV)-infected cells on agar substrate following pretreatment of cells with chemical or physical agents (Suk).

(C) Altered growth in agar

(1) Chinese hamster ovary (CHO) cell invasiveness assay (Zdzienicka).

SHE colony assay

Morphological transformation has been achieved in primary Syrian hamster embryo cells (Di Paolo et al., 1969). A significant advance was the demonstration that the cells can be cryopreserved, thereby eliminating the need to prepare cell batches of unkown transformability before each assay (Pienta et al., 1977).

Two kinds of cell cultures are used in this assay; these are designated feeder cells and target cells.

'Conditioning' of the medium by the use of X-irradiated feeder cells from either homologous or heterologous sources is used to increase the plating efficiency of SHE target cells from approximately 1–3% to 20% or more. However, the feeder cells may also contribute to the metabolism of some chemicals (Huberman, 1975; Kouri and Schechtman, 1978; Curren et al., 1981).

Conditions under which experiments are conducted must be carefully controlled and optimised, e.g., by pre-testing all batches of medium (especially serum) and plastic vessels.

Not all batches of SHE cells are susceptible to transformation (Pienta et al., 1977), but responsive batches can be identified by parallel testing of samples against a reference carcinogenic transforming agent. The assay is known to be sensitive to selected carcinogens from among the PAHs,

nitrosamines, aromatic amines, azo compounds, heterocyclic compounds, mycotoxins and metallic salts. The stringent technical requirements of the assay have contributed to its failure to gain widespread acceptance for testing chemicals of unknown carcinogenic potential.

In this assay, 150–500 reconstituted cryopreserved target cells in complete medium are seeded into 60-mm Petri dishes containing $6–8 \times 10^4$ X-irradiated (4500 r) feeder cells. After 24 h the cultures are dosed with the test chemical in complete medium for 6 days. The cultures are then washed, fixed and stained. Morphological transformation is defined as altered colony morphology, consisting of criss-crossing and piling up to cells not observed in control dishes.

At least 130 chemicals have been subjected to the assay, although there were only 2 proven non-carcinogens in this number (Pienta et al., 1983). The majority of the remainder were chemicals of undetermined carcinogenic potential. Of the 37 carcinogens, 24 induced transformation, 4 did not and 9 induced questionable responses.

C3H10T½ cell focus assay

This cell line was derived in Heidelberger's laboratory by Reznikoff et al. (1973) from a C3H mouse embryo culture. It is passaged every 7 days at 0.75×10^5 cells per 60-mm plate, or a similar density in a different type of flask. Its growth is strongly cell–cell contact inhibited, and these cells do not form tumours when injected into syngeneic mice. The endpoint in this assay is the appearance of foci of cells piled-up to various degrees and graded as type I, II or III. It is normal only for type II and III foci to be scored. Type II foci are cells which show extensive piling up and form tumours at a frequency of about 50% when inoculated into X-irradiated C3H mice (Reznikoff et al., 1973b). Type III foci show, in addition, a stellate cellular morphology, marked criss-crossing and swirling of cells at the edges of the growth foci and an even higher frequency of tumour formation (80–90%) when inoculated into mice. These focus classifications are made for convenience, there being intermediate types which make assignment of foci to II or III a subjective procedure. Blind scoring, therefore, is essential. However, it would be premature to ignore type I foci until it is shown conclusively that they do not progress to type II and III.

Most studies using this system have been mechanistic in nature, rather than validatory. The system has been used to study promotion (Frazelle et al., 1983), initiation (Mordan et al., 1982, 1983), cocarcinogenesis (Nesnow et al., 1981), DNA binding (Amstad et al., 1983) and metabolism (Nesnow et al., 1981).

In a typical assay, cultures seeded at 1000 or fewer per 60-mm flask are exposed to the chemicals for 24 h, after which time the cells are washed and plated in medium containing 10% foetal calf serum (FCS). This is changed weekly until confluence is reached, when the FCS concentration is reduced to 5%. After about 6 weeks, the cultures are fixed, stained and examined. Statistical analysis of the data is particularly difficult in this assay because the control cultures normally do not form type II or III foci.

Problems with the assay (possibly associated with either short chemical half-life or cell-cycle stage specificity) have been encountered with alkylating agents, but these can be largely overcome by the use of synchronous culturies, re-seeding treated cells, or by exposing growing cell colonies. The activation systems to be used with some major classes of chemical carcinogens to produce transforming agents require further investigation. The addition of S9 can lead to transformation induction by 2-acetylaminofluorene, while the transforming potential of benzo[*a*]pyrene is reduced (Lawrence and McGregor, this volume). Successful efforts have been made to enhance the endogenous activating potential of the cells by pre-treatment with 5,6-benzoflavone, benz[*as*]anthracene or phenobarbital. Aroclor 1254 does not improve the activating potential (Nesnow et al., 1981).

The system responds well to polycyclic aromatic hydrocarbons, some alkylating agents, aflatoxin B_1 and 4-nitroquiniline-*N*-oxide (Nesnow et al., this volume). There is a large data base associated with this assay, but approximately 70% of the chemicals tested are PAH-related (Heidelberger et al., 1983).

BALB/c 3T3 cells focus assay

Balb/c 3T3 A-31 clone 1–13 cells were originally derived by Kakunaga (1973) and have been

employed to assess chemically-induced transformation activity since 1976. There is a data base for the assay consisting of at least 50 chemicals including chemical carcinogens representative of many chemical groups (Heidelberger et al., 1983).

The 3T3 transformation assay has many qualitative features that resemble the C3H10T$\frac{1}{2}$ assay system, i.e., (1) both cell types are aneuploid and are of mouse origin; (2) both assays have quantitation of transformed foci as endpoints; (3) cells derived from transformed foci (in contrast to untreated parent cells) readily form tumours in immuno-suppressed animals; and (4) the serum concentration in the medium used is reduced following chemical treatment. The more notable biological differences between the two assays are (1) types I, II and III foci have not been characterised in 3T3 cells; and (2) 3T3 transformation assays frequently have a low, but readily detectable, control group transformation frequency, which facilitates the use of statistical tests.

In the assay, suitable subpopulations and passage number cells are distributed to dishes; 24 h later they are exposed to the test compound for three days, then they are washed and fed with medium containing 7.5% FCS. After 3–6 weeks, the cultures are fixed, stained and examined. Colonies which are scored are at least 1–2 mm diameter, invade the surrounding monolayer and are composed of a minimum of three cell layers.

The detection of chemically-induced 3T3 transformation activity is most frpequently seen at chemical doses that yield 10–100% survival, while the most prominent positive responses normally occur at 50–20% cell survival.

In this programme, none of the chemicals was scored as transforming agents, but time restrictions limited testing to experiments in the absence of exogenous metabolic activation systems. Data generated since the study using rat hepatocytes as an activating system are shown elsewhere in this volume (Matthews et al.).

SHE/SA7 assay

The assay is based on the observation that treatment of cells with DNA-damaging agents leads to an enhancement in the frequency of virally-mediated transformation (Simian adenovirus, SA7). The papers of Casto et al. (1973, 1974, 1976) and Hatch et al. (1983) linked to studies conducted in the present laboratory have led to a database of approximately 150 chemicals representing 31 different chemical classes described in approximately 25 papers. Compounds evaluated include particulates, complex mixtures and volatile and gaseous agents. Reproducibility of data between laboratories has been investigated by the NTP. Nine known positive or negative model chemicals and a further 24 coded compounds representing diverse classes are under joint evaluation at present. A high degree of intra- and inter-laboratory agreement has been reported on 20 of 21 chemicals. An advantage of the assay is its sensitivity to metal carcinogens (Ni, Cd, As), certain chlorinated hydrocarbons (CCl_4, chloroform), steroid hormones and hydrazines.

The test protocol involves plating primary SHE cells followed 72–120 h later by addition of SA7 virus and adsorption for 3 h. Virus-treated cells are trypsinised, washed and replated, after which they are exposed to the test chemical at between 72–120 h after plating. S9 mix or hepatocytes may be added with the chemical, although this is generally not required due to the high general metaboliccompetence of the cells. Several carcinogens that require auxiliary metabolism (DMN, DEN and urethane) are activated best by S9 mix or hepatocytes derived from liver of animals pre-treated with these agents. The test chemical is removed and the cells incubated for 3–4 weeks with 1–2 media changes per week. Parallel cell toxicity studies are conducted to ensure that the assay includes both toxic and non-toxic doses. The spontaneous frequency of transformation of the cells ($2–4 \times 10^4$) is increased approximately 20-fold by the virus.

Minor problems with the assay include the requirement to reduce the period of exposure to the test agent from approximately 20 h to 2 h for hydrolytically unstable (reactive) alkylating agents which may induce rapidly repairable lesions, and the fluctuations in the transformation frequency induced by the same virus pool in different lots of primary cells. Cells with higher sensitivity to the virus usually have higher sensitivity to transforming agents. Cryopreserved or passaged cells are insensitive to virus transformation. The assay has limited commercial availability at present.

FRE/RLV assay

The test is based on the observation of Freeman et al. (1970) showing that Fischer rat embryo (FRE) cells infected with Rauscher leukemia virus are sensitive to chemical transformation after subsequent passage. Virus alone, chemical treatment alone or chemical treatment followed by viral infection do not lead to transformation. Moreover, virus-specific antisera inhibit chemical transformation. In the early experiments, a focal endpoint was employed. Interestingly, streptonigrin, a gene-mutagen, the detection of which is unique to strain TA102 of *S. typhimurium*, blocks the expression of transformation in the assay. Traul et al. (1981) developed the assay using a new Fischer rat cell line such that the endpoint was simplified to monitor cell survival in complete, liquid medium. Enhanced viability is associated with a pre-transformation state. Although cells at this stage are not transformed, if they are collected and carried through the assay a further time, they do express features of the transformed phenotype (growth in agar, focal morphology, tumourigenicity).

The good correlation between the viability results generated by the assay and the carcinogenicity of the test chemical has led to the second cycle being omitted from the standard test protocol. Nonetheless, this compromise reduces the stature of this assay as it is not definitive of transformation.

5×10^5 cells infected with RLV are seeded on to 75-cm^2 flasks, then 24 h later they are exposed to the test chemical for three days. Treatment for this period may be a single application or by three daily applications of test chemical-containing medium. This is followed by a three-day recovery period, after which the cells are trypsinised and plated on to 1% agar at a density of 6×10^5 viable cells per dish. The cells do not spread out or attach to the agar substrate, and there is no evidence of DNA or RNA synthesis during the four days they are incubated, when many cells die. The aggregates of cells, which are not anchored to the agar, are sucked off the agar surface, resuspended, and their viability estimated using the trypan blue dye exclusion technique. The endpoint is enhanced viability, in relation to the solvent controls.

In the early studies of Freeman et al. (1973) a range of carcinogens was detected as transforming agents, including some nitrosamines, over 30 polycyclic hydrocarbons, Adriamycin, certain azo dyes and aromatic amines, etc. Compounds evaluated in the present assay include 3MCA, DMBA, B(a)P, MNNG, 4-NQO and DEN (extended exposure). Certain alkylating agents give a better response with limited exposure periods (4 h) and stronger responses are observed in the 3-day exposure assay if this is split into 3×1-day treatments. No experiments have been conducted with S9 mix, up to the present time.

CHO cell invasive growth in agar (IGA-transformation)

This is a new assay and is to be considered as being in an early phase of development. The only agents tested prior to this study were UV light, X-rays, ethylnitrosourea, ethyl methanesulphonate, 4-nitroquinoline-*N*-oxide, *N*-acetoxy-2-acetylaminofluorene and benzo[*a*]pyrene, each of which acted as transforming agents.

The CHO cells are a pseudodiploid line widely used for mutagenicity testing; an extensive data base in the mutagenicity of chemicals in this cell line is available. CHO cells are already transformed in that they are capable of growth in soft agar (0.32%), they have an indefinite lifespan, and they are tumourigenic. The endpoint of the assay is further progression to invasing growth in agar scored as amoeboid-shaped colonies in the background of solid clumps of cells forming globular colonies. The transformed phenotype correlates with an increased tumourigenicity, both in nude mice and newborn rats. Limited data also indicate that IGA-transformed cells have increased metastatic potential in newborn rats treated with antithymocyte globulin.

For 4 of the 5 chemicals previously tested, exposures were of suspensions for 1 h in Ham's F10 medium with 10% newborn calf serum, followed by plating in 0.4% foetal calf serum containing medium and soft agar for three weeks. For benzo[*a*]pyrene and the five chemicals tested in the current assays, a different exposure system including metabolic activation was used. 10^6 CHO cells were incubated in a roller bottle, then 4 h later 5×10^6 X-irradiated SHE cells were added,

along with the test chemical, and incubated for four days. At the end of this period, the cells were suspended and cloned in soft agar.

4000 cells in 40 ml medium are plated per 90-mm dish (5 dishes per treatment). The cloning efficiency is about 30%. The spontaneous frequency of $0-5 \times 10^4$ results in about 0–10 invasive colonies in the control group. If survival is reduced, then more plates are poured and included in the eventual assessment. The invasive phenotype is stable, but its expression is dependent upon serum batch and concentration and upon agar and bicarbonate (0.08%) concentrations. High quality agar is not a requirement.

Response criteria

In view of the very different types of assay employed, it was not possibly to apply uniform criteria for result acceptance in this group. Consequently, and, it is believed, correctly, the criteria used by the investigators were accepted *without* modification as a result of group discussions. This fact is sufficient reason for such assays not being presently suitable for widespread use as standard screening assays.

SHE colony assay. In Barrett's laboratory, there was a control transformation frequency of 0.014%. A positive response was recorded if a compound induced more than 4 morphological transformed colonies per 2000 surviving colonies, while a negative response was recorded if no transformed colonies per 1000 survivors occurred. Since group sizes in any particular experiment were seldom large enough to permit proper evaluation, significance attaches only to data pooled from a number of experiments. Sanner, on the other hand, had a measurable control frequency of about 0.25%. In this instance, a 1% frequency was required in 2 independent experiments before a transforming agent was defined.

C3H10T$\frac{1}{2}$ cell focus assay. In both laboratories, the significant statistical unit was the flask, not the foci it contained. Nesnow did not find any transformed (Type II or III) foci in any of the 84 dishes in this series of experiments, which represents a spontaneous frequency of $< 0.005\%$. No dose-related responses were observed, so, Fisher's Exact Test was used to establish the significance of transformation induction. At a single concentration, 3 focus-containing plates out of 20 was positive evidence for transformation induction. McGregor did not observe any Type III foci in control flasks, but 3 Type II foci occurred in 111 DMSO-treated cultures. Against this background, a single flask containing a Type II or III focus was labelled questionable, while 2 such flasks within a group was taken as positive evidence for cell transformation. It is probable that these different criteria would coincide, given a greater data base.

BALB/c 3T3 cell focus assay. An appreciable transformation frquency occurs in this cell line, the unit of measurement being the number of foci. Treated and control groups were compared using a modified Student's t test. Positive evidence for transformation induction requires a $6-8\times$ background increase in the absolute number of foci.

SHE/SA7 assay. Using number of foci as the unit of measurement, the statistical test applied was a Lorenz table of critical ratios (Lorenz, 1962), which is based upon the Poisson distribution. Increased transformation was considered significant at the 5% or 1% confidence level if the viral enhancement ratio exceeded the appropriate Lorenz table value. Positive evidence of transformation was a statistically significant dose-dependent enhancement of viral transformation at two or more treatment concentrations (where survival was $\geq 10\%$), with a significant absolute increase in viral foci at one or more treatment concentrations. Suspect evidence for enhancement relied entirely upon significant viral enhancement at one or more concentrations.

FRE/RLV assay. Any enhancement of cell survival was calculated as follows:

$$\frac{\text{Viable treated cells} - \text{viable control cells}}{\text{viable control cells}} \times 100$$

The control culture used was normally the solvent (DMSO) control. However, if this culture gave an absolute value outside of a predetermined range, then the medium control was used. The mean numbers of cells in two sets of controls should be within a standard deviation of each other. Clearly positive evidence for cell survival enhancement was where a treated group survival was at least 100% higher than the control culture

at 2 or more test compounds concentrations.

CHO cell IGA assay. The frequency of colonies exhibiting invasive growth in agar is about 0–5 per 10^4 survivors. In the usual size of experiment, this results in about 0–10 amoeboid-shaped colonies per control group. For positive evidence of transformation induction, there should be a 3 × increase over the control level at 2 dose levels. A questionable response was recorded if there was an increase at a single dose level. Lack of such evidence was recorded if there was no increase in the number of invasive colonies.

Conclusions drawn by investigators

The weight that can be placed upon a reported significant or insignificant response is heavily dependent upon the reproducibility of the result in an assay system and the magnitude and quality of a dose-related response. Dose responses, or the lack of them, as the case may be, are detailed in the investigators' reports. The negative or positive responses called by the investigators in experiment-by-experiment are summarised in Table 1 (when reviewing the data from Barrett, it is important to remember that he attaches no significance to individual experiments, but only to summated data).

Where negative responses are recorded, a critical factor in their evaluation is knowledge of the highest dose level tested in the experiment; this information allows a confidence estimate to be placed upon the result category. When positive responses are recorded, the least effective dose

TABLE 1

CONCLUSIONS DRAWN BY INVESTIGATORS FOR INDIVIDUAL EXPERIMENTS

Category		Morphological transformation					Viral dependent assays		Invasive growth in agar CHO Z	Carcinogenic (r = rat m = mouse h = human)
		SHE Colony		C3H Focus		3T3 Focus	enhancement of transformation SHE: (DNA; SA7) H	Increased cell survival (RNA; RLV) S		
		B	S	M	N					
HMPA	−A	+ +	+ + + −	−	?	−	??	+?	?	+ R
	+A			+					?	
Tol	−A	??+	+ + + −	−	+	−	+ −	?	−	+ R, M
	+A			+					−	
SaF	−A	− + +	− − − +	+	+	−	− −	− −	−	+ R, M
	+A			−						
Ben	−A	+ +	+ + −	?	?	−	− −		−	+ H
	+A			−					−	
Cap	−A	???	??	+	−	−	− −	− −		R, M
	+A			?					−	
Acn	−A	+ +	+ + +	−	−					+ R
	+A			+						
DEHP	−A	+ +	+ + +	+	−	+	+		+ R	
	+A			+						
Benzoin	−A	− −	− −	−	−		−		− R, M	
	+A			+						
Des	−A	+ +	− −	+	−	??	+ + +		+ R, M, H	
	+A									
PB	−A	− −	+ + −	?	−					+ R, M
	+A			?						

TABLE 2

EFFECTIVE CONCENTRATIONS (POSITIVE RESPONSES) AND HIGHEST TESTED CONCENTRATIONS (NEGATIVE RESPONSES) OF TEST COMPOUNDS IN CELL TRANSFORMATION TESTS

	SHE (TS)			SHE (CB)			C3H10T½ (SN)			C3H10T½ (DM) [a]		
	Test Res.	Dose (μg/ml)	Surv.	Test Res.	Dose (μg/ml)	Surv.	Test Res.	Dose (μg/ml)	Surv.	Test Res.	Dose (μg/ml)	Surv.
HMPA	+	500	83	+	0.1, 1, 10	100, 110, 110	?	1000	138	−, +	3260, 280	105, 73
TOL	+	100	96	+	1	100, 110, 110	?	500	86	−, +	600, 600	71, 34
SAF	+	100	62	+	10a	83	+	100	35	+, −	50, 200	97, 28
BEN	+	20	100	+	10, 100	91, 94	?	1000	101	?, −	50, 320	75, 97
CAP	+	300	100	+	10, 100	100, 99	−	1000	93	+, ?	4570, 350	94, 83
ACN	+	2	95	+	0.01, 1	100, 95				−, +	10, 16	13,32
DEHP	+	4	85	+	10, 100	91, 91				+, +	40, 1000	114, 28
ZOIN	−	40	77	−	100	54				−, +	50, 20	4, 23
DES	−	3	83	+	1	100				+ Null	10	65
PB	+	100	97	−	100	100				?, ?	2000, 2000	63, 21
	Balb 3T3			SHE/SA7 [b]			FRE/RLV			CHO [c]		
HMPA	−	5000	10	?, ?	20,000; 20,000	41; 31	?, +	100; 1000	105, 800	?, ?	10?000; 10,000	10
TOL	−	330	10	+, −	1,000; 2,000	93; 29	?	50	71	−, −	250; 500	26
SAF	−	180	10	−, −	200; 140	0; 14	−	500	12	−, −	1000; 1000	10
BEN	−	4000	10	−, −	1 ml/4.6 L; 1 ml/4.6 L	0; 1				−, −	1000; 1000	87
CAP	−	5938	10	−, −	1,000:20,000	101; 1	−	50	110	−, −	2000; 2000	89
ACN	−	20	10									
DEHP	−	21	10	−, +	2,000; 500	14; 100	+	2000	77			
ZOIN	−	165	10				−	50	121			
DES	−	18	10	?, ?	8; 16	76; 76	+, +, +	10, 10, 20	12, 14, 25			
PB	−	1750	10									

[a] Data represent experiments without activation (left column) and with added S9 (right column).

[b] Data represent replicate experiments without exogenous activation.

[c] Data represent experiments without activation (left column and with use of irradiated SHE feeder cells (right column).

level should be known, since this allows a rough estimate to be made of the potency of the chemical in that particular assay. These data are given in Table 2.

All of the results from the invasive growth in agar assay (Zdzienicka), BALB/c 3T3 focus assay (Rundell), and the C3H10T½ focus assay (McGregor) should be considered as single, unconfirmed experiments with those chemicals tested. An assay performed once with and once without auxillary metabolism are not, strictly speaking, replicated experiments. The investigators who submitted BALb/c 3T3 focus assay data were anxious that conclusions should not be based upon these data until they had an opportunity to incorporate auxillary metabolic potential. However, in other assays, many experiments were repeated and special attention was paid to those. Particularly impressive were the consistent positive results from replicate experiments with DES in the FRE/RLV assay and the high proportion of positive results in the replicated SHE colony assays with, for example, DEHP, acrylonitrile, benzene and *o*-toluidine. On the other hand, the original data leading to the judgement should not be ignored. Caprolactam, for example, induced positive responses according to the investigators' criteria, in both SHE colony assays and one C3H10T½ assay. In these cases, high dose levels were required for the significant response, which is an indication of very low activity of caprolactam in these assays. It is important to consider these issues when presented with an investigator's judgement of whether or not he is dealing with an agent with transforming potential (Table 3).

The CHO invasive growth in agar assay and the BALB/c 3T3 focus assay as performed did not allow the investigators to identify any of the chemicals as transforming agents. Besides the lack of auxillary metabolism in the latter assay, it was considered by the investigators that the method used for statistical analysis was weighted towards a non-significant judgement. This assay in particular, therefore, ensured that a negative response would be recorded for all of the chemicals somewhere in this series of experiments.

Positive responses also were recorded for all of the chemicals and questionable responses occurred with most of the chemicals. These questionable responses indicate that effects were seen, but their significance is low for any of a variety of reasons, and further experiments are required to resolve the issues. It was not the investigators' stated intention that the chemicals should remain in this indeterminate category.

Due to the wide variety of assays conducted, there was little opportunity for the interlaboratory comparison of assay performance. Such comparison was possible for the C3H10T½ focus assay and the SHE colony assay. A third comparison which might be made is between the primary cell culture assays and the C3H10T½ focus assay with S9 mix.

TABLE 3

CONCLUSION OF INVESTIGATORS REGARDING THEIR ASSAY RESPONSES

	SHE (TS)	SHE (CB)	10T½ (SN)	10T½ (DM)		BALB	SHE-SA7	FRE/RLV	CHO	
	−S9	−S9	−S9	(i−S9	+S9	−S9 +S9	−S9	−S9	−S9	+S9
HMPA	+	+	?	−	+	−	?	+	?	?
TOL	+	+	+	−	+	−	+	?	−	−
SAF	+	+	+	+	−	−	−	−	−	−
BEN	+	+	?	?	−	−	−		−	−
CAP	+	+	−	+	?	−	−	−	−	−
ACN	+	+		−	+	−				
DEHP	+	+		+	+	−	+	?		
ZOIN	−	−		−	+	−		−		
DES	−	+		+		−	?	+		
PB	+	−		?	?	−				

? Represents data indicative of an effect: further experiments required to resolve.

An advantage of primary cells is that they retain a broader spectrum of xenobiotic metabolic potential than do cell lines.

(1) C3H10T½ focus assay. Out of the five chemicals tested in both laboratories, there was absolute agreement in the results with two compounds: safrole (+, +) and benzene (?, ?). Although overall agreement was also reached with *o*-toluidine (+, +), in one assay the result was obtained only in the presence of S9 mix. The disagreement found with caprolactam might be explicable in terms of dose, since the laboratory recording (−) restricted dosing of all chemicals to 1 mg · ml^{-1}.

(2) SHE colony assays. Both laboratories tested 10 chemicals. Absolute agreement was recorded with 8/10: HMPA (+, +), *o*-toluidine (+, +) benzene (+, +), acrylonitrile (+, +), saffrole (+, +), DEHP (+, +) and benzoin (−, −). Disagreement was found with DES (+, −) and phenobarbital (−, +). Outside of this study, Sanner has tested toluene and xylene as well as benzene. He cosistently obtains positive responses with benzene and negative responses with the alkyl benzenes.

(3) Activation potentials within various assays. Once cells are removed from an animal and cultured, they begin to lose a proportion of their metabolic potential. This regression of enzymic activity in culture has been known for many years and is illustrated by the following data for rat hepatocytes cultured on collagen membranes, in the presence of insulin (I) and hydrocortisone (H).

Days in culture	[a] Cytochrome b_5		[a] Cytochrome P_{450}	
	I	I+H	I	I+H
1	456 ± 26	606 ± 54	157 ± 14	365 ± 61
3	362 ± 63	280 ± 58	65 ± 4	110 ± 22
5	247 ± 32	178 ± 57	65 ± 8	54 ± 9
7	183 ± 28	211 ± 60	39 ± 9	80 ± 1
10	155 ± 26	244 ± 43	33 ± 5	123 ± 7
Normal liver	679 ± 23		480 ± 24	

[a] picomoles cytochrome/mg microsomal protein. (reproduced from Michalopoulos et al., 1976).

Even 24 h in culture is sufficient time to ensure reduction in these enzymic activities, particularly if hydrocortisone is not present in the culture medium. Therefore, the various cell types used in transformation tests are metabolically deficient for two reasons. Firstly, they are cultured cells and, secondly, they are derived from embryonic tissues in which certain enzymic activities were never maximally expressed. In the series of assays currently under consideration, the primary Syrian hamster embryo cells used in the viral enhancement of transformation test are likely to be the most metabolically competent: they are never frozen and they are in culture for only 70–120 h before use. However, different batches of SHE cells are not equally sensitive to transformation and this variation may, in part, be due to different activating potentials in the isolated, heterogeneous cell populations. While pre-testing selection of suitable cell populations is possible in the SHE colony assay, it is not so in the SHE/SA7 assay. In this latter assay, no batches of cells have been tested which were responsive to either diethylnitrosamine (DEN) or urethane unless a S9 preparation is included in the medium during exposure of the cells to test chemical. It may be significant that DEN is primarily a liver carcinogen, while urethane also, although it induces tumours in lung, is metabolised to the proximate carcinogenic species in the liver.

Unfortunately, there are no records of the metabolic potential in the batches of target cells actually used in these assays and even the biochemical measurements made on the S9 used in the C3H10T½ cell assay (protein, cytochrome P450, aryl hydrocarbon hydroxylase, dimethylaminoazobenzene *N*-demethylase) are of little value for the purposes of assay response comparison.

This preamble is necessary to point out the problems involved in comparing assay response because it is all too easy to devise explanations for differences which are based upon an unknown, net metabolic potential. For example, safrole was positive in the C3H10T½ cell focus assay without S9 in both laboratories; it also induced positive or at least questionable responses in the SHE colony assay; but it did not induce transformation in the C3H10T½ cell focus assay in the presence of S9 or in the SHE/SA7 assay both of which probably have higher metabolic potential than the other assays. A postulate which could be made is that, in these in vitro, more competent assays, safrole is metabolised more effectively to a non-transform-

ing species. We are then left with finding an explanation for the lack of response in the BALB/c 3T3, CHO/IGA and FRE/RLV assays, in which endogenous metabolic potential is low and no S9 or hepatocytes was supplied.

It is, therefore, difficult to make detailed comparisons of assays based solely upon their apparent metabolic potential, without resort to tortuous, highly speculative explanations. Thus, there is no obvious pattern of responses which emerges from close examination of Table 4, when assays are grouped according to their supposed metabolic potential (which may have little to do with the relevance of that potential). The assays can be divided into three groups:

(A) Low metabolic potential: C3H10T$\frac{1}{2}$ without S9, BALB/c 3T3 and CHO/IGA without S9.

(B) Intermediate metabolic potential: SHE colony assays and FRE/RLV.

(C) High metabolic potential: C3H10T$\frac{1}{2}$ + S9, SHE/SA7 and CHO/IGA + S9.

The proportions of positive responses were: group A, 20%; group B, 67%; and group C, 33% From this result, points of interest which emerge are:

(1) the ranking of supposed metabolic potential might be incorrect;

(2) unidentified features other than metabolic potential might be more important for the determination of transforming potential in certain assays.

Further discussion of these points is not warranted on the basis of the present study alone.

General considerations regarding the role of transformation assays in in vitro test batteries

The following comments are based on the experience of members of the working group with most of the currently available transformation assays. These comments are based upon the findings of the present study, but are made stronger, through the past experience of the investigators, than this limited exercise could itself make possible.

This study was designed specifically to identify eukaryotic assays suitable for routine use in conjunction with the Salmonella assay. The general requirement that such assays were technically well developed at the start of the study and that earlier studies should have established their *general* utility for the detection of genotoxins is not always achieved for transformation assays.

All of the currently available transformation assays, including those represented herein, are still in a state of development. There are several distinct groupings of these assays, in terms of the cell

TABLE 4

GROUPING OF RESULTS ACCORDING TO THE SUPPOSED METABOLIC COMPETENCE OF THE ASSAYS

	A 'Low' metabolic potential			B 'Intermediate' metabolic potential		C 'High' metabolic potential		
	C3H w/o S9	3T3	CHO w/o S9	SHE colony	Free/RLV	C3H +S9	SHE/SA7	CHO +S9
HMPA	? −	−	?	+ +	+	+	?	?
Tol	+ −	−	−	+ +	?	+	+	−
Saf	+ +	−	−	+ +	−	−	−	−
Ben	? ?	−	−	+ +		−	−	−
Cap	− +	−	−	+ +	−	?	−	−
Acn	−	−		+ +		+		
DEHP	+	−		+ +	?	+	+	
Benzoin	−	−		− −	−	+		
DES	+	−		− +	+		?	
PB	?	−		+ −		?		
	+ 6 ? 5 − 19			+ 18 ?2 − 7		+ 7 ?5 − 9		
	Total assays: 30			Total assays: 27		Total assays: 21		

type employed or the phenotypic endpoint studied. This means that the technical development of specific assays usually proceeds in isolation rather than as a concerted effort in several laboratories. The facilities and time required to conduct these assays are usually not greater than those required for a mammalian cell gene mutation or cytogenetic assay. A unique aspect of many of these assays, especially those employing primary cell strains, is the ability of the marker cells to activate some carcinogens without external metabolic assistance. However, a need remains in some cell lines and for some chemical classes for auxiliary metabolism. In general, this problem is not easily solved by the addition of S9 mix, hepatocytes or embryonic cells. Thus, given the present state of development of transformation assays in general, and the present ones in particular, the group recommended that such assays should not be selected as the sole complementary test for use with Salmonella. However, the restraint for the use of only two assays was itself seen as indefensible from a scientific viewpoint (cf. EEC guidelines and Mutation Res., 118 (1983) 227). The possible future rôles for transformation assays are as follows:

(a) After preliminary evaluation of a new chemical in the Salmonella assay, it will usually be necessary to extend studies in one of two directions. Firstly, if a negative response was seen, additional tests may be required to confirm this inactivity. Usually this will be achieved by using a small range of eukaryotic assays, one of which may be a transformation assay (see b). Secondly, in the case of positive responses, further definition of the genotoxicity of the chemical may be required, in which case a chromosomal, gene-mutagenic or transformation assay may be conducted.

(b) Irrespective of the definition of the mutagenic/clastogenic properties of a chemical, its ability to transform cells may be of particular value vis-à-vis the possible carcinogenicity of the agent. In particular, carcinogens that do not interact directly with DNA may be detected by such tests.

(c) Finally, a specific future use for which transformation assays may be uniquely suited is in the study of promotional/co-carcinogenic/onco-gene activation effects. Such studies will be commissioned in specific rationalised situations, i.e., not as part of a routine screen.

Conclusion

The main appeal of cell transformation assays lies in the fact that they have a biological endpoint that is visually discernable as related to cancer. Thus, in all such assays, cells are produced which have altered morphological or behavioural characteristics, such as a loss of contact inhibition of growth, and carcinogenic potential when inoculated into the host strain of animal from which the cells were originally derived. It was therefore to be expected that such assays would be evaluated as possible screening tests for new chemical carcinogens. Progress in realising this goal has been slowed by 3 major factors, as listed below:

(1) A failure to gain general agreement on a common cell line, test protocol and auxiliary source of metabolising enzymes.

(2) Technical difficulties associated with such assays that often become apparent only when additional laboratories attempt to adopt a particular test for routine use. Experience gained with the BHK assay as described by Styles (1977) and the SHE assay as described by Pienta et al. (1977) illustrates this problem.

(3) The failure of investigators, often by design, to approach the question of whether their particular assay is appropriate or ready for routine use in other laboratories. Thus, many assays have been reserved for research use or for studying particular classes of chemical at the expense of studying their response to a wide range of carcinogens, their interlaboratory reproducibility and performance in collaborative studies.

The designers of the present study were therefore faced with the dilemma that transformation assays presented an attractive but essentially untried prospect for the definition of a complementary assay. Mindful that the omission of all such assays from the study might have damaged their overall development, efforts were made to include a range of transformation tests. The results obtained confirm the potential usefulness of such assays while underlining the urgent need to approach the problems listed above (1–3) if cell transformation is to assume a place in screening strategies for possible new mammalian carcinogens.

In summary, transformation assays can be used

to advantage by technically competent investigators to assess specific aspects of the biological activity of a chemical.

In the current study, there was limited opportunity for assessing the readiness of any of the assays for routine use. Near duplication of protocol in different laboratories and replication of experiments within laboratories were practices limited to the SHE colony assay. Results from the two laboratories using this assay were very encouraging. Nevertheless, in view of the history of disappointments experienced elsewhere, it would be premature to recommend even this test above all others in attempts to demonstrate cell transformation in vitro. Perhaps the most valuable outcome of the efforts made by participants in this section will be if it stimulates wider investigation of cell transformation tests, promoted with urgency and tempered by effective coordination.

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Overview and conclusions of the IPCS collaborative study on in vitro assay systems

John Ashby, Frederick J. de Serres, Morrell Draper, Motoi Ishidate Jr., Barry H. Margolin, Bernard Matter and Michael D. Shelby

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1. Design and layout of overview

Almost 90 individual sets of test data are included in this study. Each investigator evaluated 5 or more of the test chemicals and most of them tested all ten. In many cases, repeat tests were conducted and most assays were carried out both with and without auxiliary metabolism (S9); the primary database is therefore large. This fact alone made it difficult to derive simple conclusions, but the situation was further complicated by the particular test chemicals chosen. These were selected to present the assays with a range of distinct and very different challenges.

When attempting to analyse the database it became clear that it could also contribute to an understanding of a range of other issues of relevance to the science of environmental mutagenesis and carcinogenesis. For example, the problem of what constitutes a pure chemical, the definition of non-carcinogenicity, how to define positive response; the question of whether non-genotoxic (sometimes referred to as epigenetic) carcinogens form a discrete sub-group of carcinogens and the question of how current legislative initiatives might be affected by the findings of this study. In view of these issues, it was decided to divide this overview into a series of separate discussions. These have been constructed to enable access to issues of specific personel interest, and additionally, arranged such that the complete text remained coherent.

2. The distinction between confirmatory and complementary in vitro assays

It is now general practice to commence the evaluation of a new chemical for potential human mutagenic/carcinogenic properties by subjecting it to the Salmonella microsome reverse mutation assay. Despite the efficacy of this assay in the detection of many established mammalian carcinogens there has been a growing tendency over the past few years not to rely solely on this test. In particular, whether or not a new chemical is a bacterial mutagen it is now usual to evaluate it in one or more eukaryotic in vitro assays. The number of additional tests conducted, their nature and the underlying reason for conducting them are usually not totally clear. It is therefore important at the outset of this discussion to set the present study within its correct and rather complex context.

Perhaps the greatest stimulus to the use of multiple in vitro assays derives from the several testing guidelines or legislative requirements of national governments. In such legislation it is usual to require that a new chemical be subjected to a range of genotoxicity tests selected from different phyla and/or representing different genetic end-

points; the implicit reason being to ensure that possible new carcinogens are not missed due to the specific genetic makeup or metabolic deficiencies of any one particular assay. Although such legislation has been the major factor in creating a general requirement for multiple assays to be conducted, the reasons for their commission is based indirectly on the relevant scientific literature. There are two distinct needs that can be identified for requiring multiple tests.

The first need is to provide *confirmation* in eukaryotic cells of genotoxic activity observed in bacteria, the second is to *complement* negative findings in the Salmonella assay with additional data from other in vitro assay systems. The scientific implication of the first need is that some chemicals produce mutations in bacteria that are specific to the prokaryotic genome and are therefore of no genetic consequence to mammals. It is difficult to support this concern with data, i.e., to cite examples of bacteria-specific mutagens that are unambiguously devoid of mammalian mutagenic/carcinogenic properties. Perhaps the most quoted examples are the DNA-intercalating agents. These compounds interact noncovalently with DNA and are mutagenic to certain frameshift strains of Salmonella (TA1537, TA97 and TA98) and yeast mitochondria, but are generally regarded as weak or inactive carcinogens [1]. However, the cancer bioassay data for such compounds are usually inadequate. The intercalating agent daunomycin is an animal carcinogen and some others are mutagenic to mouse lymphoma cells [2].

Despite reservations regarding the reality of the concept of bacteria-specific mutagens, a major role has developed for confirmatory eukaryotic assays. Most of the classes of assays represented in the present study are already established as capable of detecting the majority of bacterial mutagens that are mammalian carcinogens, therefore, by definition, most are already established as useful confirmatory tests. This point is particularly important to appreciate given the inadequacy of some of them as complementary tests (see later). The major problem associated with the use of confirmatory eukaryotic assays is that no agreed procedure exists for interpreting discordant results that may be produced. For example the genetic toxicities of 5-chloro-*N*-methylisothiazolone [3] and dopamine [4] demonstrate our current inability to assess such divergent data. These compounds are mutagenic in some in vitro assays but not in others. In the case of the thiazolone derivative the authors concluded that a negligible human hazard would exist upon exposure to this agent, but this conclusion was based primarily on negative mutagenicity data derived from subsequent experiments conducted in vivo (Drosophila SLRL and mouse bone marrow cytogenetics) [3]. Likewise, the genetic toxicity of dopamine in vitro was essentially dismissed because of the proven ability of humans to tolerate the genotoxic activity of this endogenous agent due to their possession of adequate levels of endogenous catalase. Both of these risk assessments were made without consideration of the range of activities observed in the confirmatory eukaryotic assays conducted in vitro. Thus, if assessment of the possible in vivo mutagenic/carcinogenic hazard posed by exposure to a bacterial mutagen cannot be significantly and rationally modified by 'confirmatory' in vitro eukaryotic assay data, then the conduct of such tests will frequently lead only to the problems posed by discordant results.

In contrast, a need exists for assays capable of *complementing* the Salmonella assay in the detection of new animal carcinogens, when a test chemical is found to be inactive in the standard Salmonella mutation assay. For most practical purposes this will consist of evaluations made in strains TA1535, 1537, 1538, 98 and 100, in the presence and absence of Aroclor-induced S9 mix and using the original plate incorporation assay protocol [5]. The fact that a selection of established animal carcinogens are known to be non-mutagenic in the standard Salmonella assay [6] makes it unwise to rely solely upon bacterial mutation assay data. Complementary assays have been sought, with justification, from among the range of available confirmatory eukaryotic genotoxicity assays, but a general failure to distinguish between the confirmatory and complementary roles of the same eukaryotic assay probably lead to the lack of clarity of purpose evident in most legislative guidelines (see Appendix 3).

The above uncertainties were already evident when the first International Study [IPESTTC;6] was designed. In that study, eleven carcinogens

known to be difficult or impossible to detect using the standard Salmonella assay were selected for evaluation. The results of that study provided a strong indication that eukaryotic assays in general were capable of detecting such 'non-mutagenic' carcinogens, but the database available was insufficient to define the most useful and practical assays from among the many considered. Most of the eukaryotic assays also detected as positive the many carcinogenic bacterial mutagens studied, thus the usefulness of these assays as confirmatory tests was also underlined by that study.

The stimulus for the International Program on Chemical Safety (IPCS) to support the present study was provided by the prospect for the definition of a small number of internationally agreed and appropriately validated in vitro assays for use in conjunction with the Salmonella mutation assay. The need perceived was not to satisfy any particular legislative requirement, but to offer clear guidance on the minimal experimental data required for the identification in vitro of possible new carcinogens.

Short-term in vivo genotoxicity assays such as the mouse micronucleus test were avoided in this study as they are being considered separately in the second IPCS study. This decision is consistent with the suggestion that in vitro assays are optimal for the definition of the *potential* carcinogenicity of a chemical, while those conducted in vivo are most well suited to assessing if this potential is likely to be *expressed* in mammals exposed to the agent in vivo [7].

The selection of in vitro assays potentially suitable to complement the Salmonella assay was dominated primarily by those available when the study was designed. With the exception of the metabolic cooperation, aneuploidy and polyploidy assays, each was from a class of assay already validated as suitable for the detection of genotoxic agents. The tests included in this study were additionally suitable for evaluation of several current postulates regarding the non-mutagenicity to Salmonella of some mammalian carcinogens.

(a) Technical inadequacy of the Salmonella assay. Several possible technical inadequacies of the standard assay are now established; volatile chemicals may require evaluation in a dessicator in order to ensure that a sufficient concentration of the test chemical reaches the bacterial genome, the auxiliary metabolizing system (S9 mix) may be inappropriately constituted or the exposure conditions may be suboptimal. Thus, certain agents (e.g. ACN) are only found to be mutagenic when evaluated in a closed system while others are optimally or only detected when using phenobarbital-induced S9 mix or a pre-incubation test protocol. Such agents are clearly genotoxic bacterial mutagens that can easily be found inactive for technical reasons. Of the eight carcinogens included in the present study, TOL, SAF and ACN are probably representative of this class (see later). It should be emphasized that although such agents can be detected as bacterial mutagens under specific conditions of bioassay, it may be easier to conduct a complementary assay than to conduct all of the many modifications of the Salmonella test for their detection.

(b) Specificity of genetic activity. The prospect that some chemicals may exert a *specific* genotoxic effect, rather than a general one involving all genetic end-points (e.g. UDS, SCE, aneuploidy, etc.) probably provides the main basis for the multi-assay legislation alluded to above. Because of the several major technical differences between separate classes of assays it is difficult to assess the evidence supporting the concept of genetic specificity of action of test agents. For example, it would be easy to compare a set of negative Salmonella data recorded for acrylonitrile with its ability to induce SCEs in CHO cells [8] and to conclude that it was an SCE-inducing agent that is incapable of producing point-mutations. However, the SCE data were derived in an assay that employed a feeder layer of rat hepatocytes because the purpose of the study was to demonstrate that the metabolic activation of this agent is of more critical importance than the genetic end-point being monitored for change. Such uncertainties beset most of the possible examples of specificity of action. When this study commenced, benzene (BEN) was one of the best defined cases of an established clastogen that appeared to be incapable of inducing gene mutations in vitro, and diethylstilboestrol (DES) was similarly defined.

(c) Non-genotoxic mechanism of carcinogenic action. The prospect that some chemicals can induce cancer in animals without perceptibly mod-

ifying the genome of the constituent cells of the target tissue has been widely discussed of late [9–12]. The present study therefore presented a unique opportunity to derive a complete genotoxicity data base on two such alleged agents, phenobarbital (PB) and diethylhexylphthalate (DEHP). Diethylstilboestrol (DES) is also included in this catagory by some authors [9,10].

3. Criteria for selection of the test chemicals

Eleven carcinogens were defined as either difficult or impossible to detect as bacterial mutagens in the previous study [6], and four of these were selected for the present study. These were hexamethylphosphoramide (HMPA), safrole (SAF), diethylstilboestrol (DES) and *o*-toluidine (TOL). The rodent carcinogenicity of these agents is well-established [reviewed in Appendix 2] and only a low proportion of the Salmonella assays conducted in the previous study [6] detected them as mutagenic [1/15, 4/17, 1/17 and 3/16, respectively]. Several of these responses were unreproducible, and each was weak. This led to these compounds being regarded as essentially non-mutagenic to Salmonella in that study.

Hexamethylphosphoramide (HMPA) is among the most potent of animal carcinogens producing metastasising nasal tumours in rats exposed by inhalation at 0.025 ppm. There is a wealth of data indicating it to be non-mutagenic to Salmonella yet the results of the last study [6] suggested that it was a general genotoxin in eukaryotic assays. One possible mode of action for this agent is *via* enzyme mediated formation of formaldehyde [13] which is also a rat nasal carcinogen, difficult to detect as mutagenic in the Salmonella assay but which is a gene-mutagen in mammalian cells [14,15].

o-Toluidine (TOL) is a relatively weak rodent hepatocarcinogen. Its activity in this respect is interesting because it weakens the earlier assumption that single ring aromatic amines, as opposed to multiple ring arylamines such as 2-naphthylamine and 4-aminobiphenyl, are noncarcinogenic. TOL was established as difficult or impossible to detect in the Salmonella assay in the first study [6], yet it was already known to be mutagenic to these bacteria if evaluated in the presence of norharman [6]. These collected findings suggested TOL to be a general genotoxin that required specific metabolic activation rather than being an agent showing specificity of genetic action.

Safrole (SAF) is a weak rodent liver carcinogen and has been studied extensively in the Salmonella assay, and although certain investigators have reported it to be mutagenic, it is generally found inactive in this assay. Both the α-acetoxy and the sidechain epoxide derivatives are mutagenic and these have been suggested as the metabolites responsible for the carcinogenic action observed. Safrole may, therefore, be a further example of a general genotoxin that requires specific metabolic activation. Set against this is the fact that it appears devoid of genetic activity in vivo, thus it gives a negative response in both the mouse bone marrow micronucleus assay [6] and the in vivo rat liver unscheduled DNA synthesis (UDS) assay [16]. Consequently, the possibility cannot be excluded that the tumours produced by this agent may be mediated *via* some disturbance of normal homeostasis in the test animals (i.e. by a non genotoxic mechanism) despite its ability to induce genetic changes in some in vitro test systems.

Diethylstilboestrol (DES) is carcinogenic to both humans and experimental animals. It could have been selected for this study based simply on a recent paper by Barrett that showed it to be capable of transforming cells and inducing chromosomal damage in the apparent absence of gene-mutations [17]. Underpinning this finding was the fact that in the previous study [6] DES was regarded by the investigators as a clastogen that was non-mutagenic to Salmonella. Therefore, DES, together with benzene [BEN; see below], were included in the study as agents that could possibly demonstrate the reality of genetic specificity of action in some chemical carcinogens.

Benzene (BEN) is a unique carcinogen. Its possible leukaemogenic activity in man has been discussed for many years, yet this effect has been difficult to reproduce in animals. The compound is nonetheless generally regarded as carcinogenic and extensive data exist on its clastogenicity, particularly when evaluated in vivo. Dean [18] has reviewed the literature to 1978 on the genotoxicity of this agent in short-term tests, and this, coupled

with subsequent studies clearly defines it as non-mutagenic to bacteria. The possibility of its complete inability to induce gene-mutations in vitro is implied in some papers, but its gene-mutagenicity in vivo has not yet been assessed.

Acrylonitrile (ACN) is grossly similar in structure to the carcinogen vinyl chloride, and this led Venitt to evaluate it for bacterial mutagenicity. The debate that ensued in *Mutation Research* [19,20] regarding the mutagenic activity of this agent in Salmonella and *Escherichia coli* can be summarized by describing ACN as a chemical that could easily be found non-mutagenic in a routine screening programme that employed only bacteria as marker cells. The carcinogenicity of this agent has been subsequently defined and reviewed. The question of whether ACN interacts directly with DNA *via* a Michael reaction, or *via* the intermediate metabolic formation of an epoxide derivative heightens interest in this agent.

Diethylhexylphthalate (DEHP) is carcinogenic to the rodent liver yet the majority of experimental data indicate it to be non-mutagenic to bacteria. It has been proposed that the carcinogenicity of this agent is associated with its ability to proliferate peroxisome microbodies in the rodent liver [21]. This explanation would not require DEHP itself to interact with nuclear DNA. The carcinogenicity of DEHP has therefore been considered as possibly 'epigenetic' in origin which increases the need to determine accurately its genotoxic status in vitro. The extent to which DEHP is hydrolysed to the corresponding mono-acid derivative (MEHP) could influence the outcome of certain assays as the latter chemical, unlike the former, is reported to be a clastogen and SCE-inducing agent in vitro [12,22,23].

Phenobarbital (PB), although active as a rodent liver carcinogen, also has significant tumour-promoting properties in the rodent liver. In fact, the issue of whether PB is a pure promoting agent devoid of cancer-initiating activity is of great current interest. In contrast to DES, the rodent carcinogenicity of PB appears not to be reflected in man, despite the extensive and controlled exposure of epileptic patients [24]. Although this chemical is generally regarded as non-genotoxic, limited evidence exists for its ability to induce SCEs in vitro [12,25]. This property may be related to its ionic composition (cf. sodium saccharin, MEHP above, lacchaic acid, sodium benzoate, etc for similar activity profiles; reviewed in [12]). An additional point of interest in this chemical is that Williams has presented data to support the claim that PB is an example of an epigenetic carcinogen [26].

The non-carcinogens caprolactam (CAP) and benzoin (ZOIN). The selection of non-carcinogens suitable for use in the evaluation of short-term tests has presented a stumbling block to all validation exercises. In the early validation studies non-carcinogens were simply selected from those compounds commonly regarded as being non-carcinogenic. In some cases, no data existed regarding their carcinogenicity, and this was taken as indicative of inactivity. In the recent collaborative study [6] the non-carcinogens selected were graded according to the extent and quality of the negative data, and although an advance, this made the interpretation of unexpected positive assay responses difficult. This issue is made particularly important by the widespread reference to 'false-positive' responses occuring in short-term tests — the credibility that can be accorded to a false positive response is directly proportional to the certainty associated with the compound's classification as a non-carcinogen. The fact that some assumed non-carcinogens may eventually be classified as either weak or organ/strain/sex/species-specific carcinogens might lead to the re-evaluation of many previous examples of 'false-positive' assay responses.

In order to circumvent this problem in the current study particular attention was paid to the selection of the two chemicals required to act as negative controls. The agents selected were benzoin (ZOIN) and caprolactam (CAP). The major criterion for their selection was inactivity in recent cancer bioassays conducted as part of the U.S. National Toxicology Programme [Appendix 2]. In the reports of these studies it was concluded that neither compound was carcinogenic in male or female Fischer 344 rats or B6C3F1 mice dosed at levels up to the maximum tolerated dose over their lifetimes. These two studies were taken as definitive as they represent the most detailed of cancer bioassay protocols currently in use. A more detailed appraisal of the negative carcinogenicity

data on these two agents is made later in this chapter and in Appendix 2. In addition, these agents were devoid of overtly DNA-reactive substituents and were known to be non-mutagenic to bacteria [27].

The ten chemicals selected cover a wide range of structural types and could therefore be considered representative of agents encountered in the environment and chemical industries. In addition, several of the carcinogens selected have been associated by other investigators with possible alternative mechanisms of cancer induction to the DNA-reaction/somatic mutation theory. Finally, the two non-carcinogens are sufficiently well supported by negative carcinogenicity data to ensure that clear decisions can be made regarding the significance of genotoxic activity observed for them in vitro.

4. Purity of the test chemicals

What constitutes a pure chemical is, and will remain, an issue of continuing study. As analytical techniques improve it is possible to find trace impurities in materials formerly considered pure. Set against this is the practical need to obtain large supplies of pure chemicals for a study such as the present without inordinate costs and delays. This dilemma is heightened by the history of the conduct of cancer bioassays where the test chemical is often, if not usually, assumed to be pure in the absence of appropriate analytical data. Many chemicals bioassayed for carcinogenicity have been of technical quality and therefore probably of no greater than 95% purity. Normally, this would not matter, but when the cancer bioassay data are to be the ultimate reference point, as in the present study, then the relative purity of the in vitro test chemical becomes of importance. At one extreme it could be argued that material of similar purity (or impurity) to that employed in the cancer bioassay should be assayed, but this may lead to a further confounding of the total database. At the other extreme it could be suggested that only ultrapure materials should be employed in vitro, whatever the cost and inconvenience incurred in their preparation. This approach carries the penalty that the carcinogenic response observed in mammals may have been produced by impurities, in which case, activities observed in vitro may fail to correlate with carcinogenic activity.

This consideration is particularly pertinent to the present study in the case of benzene. The most convincing carcinogenicity data for benzene were derived in humans exposed to it along with other chemicals whose number and type varied from situation to situation. The fact that the carcinogenicity of this chemical is difficult to define in rodents has led to the suspicion that it may not be benzene, but the chemicals used in association with it, that are carcinogenic. Pure benzene was used in this study; a risk was taken by doing so.

The issue of 'impurities' usually only becomes of interest when activity is observed in vitro for a reported non-carcinogen. This emphasis cannot be logically supported, therefore, equal attention was paid to the purity of all of the present 10 test chemicals.

The purity criteria adopted for the present study were essentially a compromise among the above considerations. This entailed the following assays of chemical purity:

(i) The highest grade commercial samples available, usually 99% or greater, were obtained.

(ii) The proton nuclear magnetic resonance spectrum, mass spectrum and infrared spectrum were determined and checked for consistency with the proposed structure and for the possible presence of impurities.

(iii) The elemental analysis (C, H and N) was determined for both liquids and solids, each was within $\pm 0.4\%$ of the theoretical value.

(iv) The melting point was determined and compared with previously reported values for all solids. Due to differences in thermometer calibrations, variations of less than $\pm 4°C$ are hard to interpret.

(v) In two cases (SAF and TOL) high pressure liquid chromatography (HPLC) was employed to evaluate trace impurities seen by earlier assay methods.

(vi) Thin-layer chromatography (tlc) was undertaken on each material as appropriate. A variety of eluants and detection systems were employed.

Based on the above determinations the present chemicals were deemed to be pure to a level of $> 99\%$. These techniques cannot eliminate the chance that some activities observed for some of

the agents (both carcinogens and non-carcinogens) were due to impurities. This admission is necessary but is not exceptional given the paucity of analytical data usual in such studies, including the referelce cancer bioassays. Nonetheless, trace impurities may have contributed to some activities, the weak gene mutagenicity of phenobarbital in Salmonella being an example of where ultrapurification and reassaying in vitro may yield useful additional data. Genotoxic impurities should not, however, be too easily invoked to explain unexpected genotoxic responses. Firstly, similar concerns should apply to positive responses observed in vitro for mammalian carcinogens, and second, such uncertainties reflect equally on previous studies, whose findings constitute the vast majority of the established database of this science. The experimental details supporting the stated purity of the 10 test chemicals are presented in Appendix 1.

5. Published genotoxicity data on the test chemicals

The Environmental Mutagen Information Center (EMIC) has provided a survey of the published mutagenicity and carcinogenicity data on the 10 test chemicals. This was undertaken by Elizabeth Von Halle and is described in Appendix 2. The data available are extensive in the case of some of the test chemicals, yet for others, such as benzoin and caprolactam, the present database constitutes the majority of the available data.

This literature survey will prove generally useful for those wishing to pursue the published genotoxic activity of individual chemicals. Several of the carcinogens studied have previously been defined as mutagenic to Salmonella, in contrast to the Salmonella data presented in this volume. This emphasizes that some genotoxic activities may be very difficult to reproduce in independent laboratories. This may be due to inadequacies in the original database or test protocol adopted, or it may represent the failure of the present bacterial investigators to adopt critical protocol features. For example, in the case of ACN, the previous literature indicates that the genotoxic activity of this chemical can be missed by a failure to contain the test agent in a closed exposure chamber.

6. Confirmation of the non-mutagenicity of the test chemicals to Salmonella

The most fundamental assumption made at the outset of this study was that the 8 carcinogens selected were either difficult or impossible to detect as positive using the standard Salmonella mutation assay, and that the two non-carcinogens would be equally inactive. This assumption was based in part on the results of the first International Study [6] and partly on a general perception of the published literature available in 1981–1982.

As the study progressed it was decided to re-evaluate the test chemicals in the Salmonella assay as an integral part of the study. This was triggered by a variety of factors. First, the detailed literature review undertaken by EMIC revealed reports on the mutagenicity of some of the chemicals to Salmonella. Second, use of the pre-incubation test was becoming increasingly common, and not all of the agents had been tested using this protocol. Third, two new strains of Salmonella were announced by Professor Bruce Ames at that time (TA97 and TA102) [5] and the possible activity of these chemicals therein became of interest.

Finally, these 10 agents had not been tested in parallel before, nor had any common criteria been applied for the assessment of their relative mutagenicity or chemical purity.

The bacterial study included both the plate-incorporation and preincubation assay protocols, a range of S9 mixes and the 7 major strains of Salmonella, including TA97 and TA102. The Salmonella forward mutation system of Skopek et al. [6] was included for purposes of comparison (strain TM 677; assay 1.1.2), and Zeiger (assay 1.1.4) employed uninduced hamster as well as induced rat S9 in his experiments because this is his standard practice (see Table 1).

Negative conclusions were recorded in 347 of 360 tests, the exceptions being as listed below:

TOL weak activity in 1 of 5 laboratories in TA1535 and TA100 and only when using hamster S9.

ACN weak activity in TA1535 and TA100 (+S9) in one laboratory, weak activity in TM677 in another laboratory (−S9) and questionable activity in TA102 in a third laboratory (−S9).

ZOIN weak activity in TA1535 (−S9) and

KEY TO SUMMARY OF QUALITATIVE RESULTS (TABLES 1–3)

Assay		Investigator	Assay description
1. Bacteria			
1.1.1	Salmonella	Baker	Histidine revertants, pour plate, TA97, 98, 100, 102, w/wo activation
1.1.2	Salmonella	Liber	Azaguanine resistance, treat/plate, TM677, w/wo activation
1.1.3	Salmonella	Matsushima	Histidine revertants, preincubation, TA97, 98, 100, 102, w/wo activation
1.1.4	Salmonella	Zeiger	Histidine revertants, preincubation, TA97, 98, 100, 1535, w/wo activation, and Syrian hamster liver S9
1.1.5	Salmonella	Rexroat	Histidine revertants, pour plate, TA1535, 1537, 1538, 98, 100, w/wo activation
2. Fungi			
2.1. Mutation			
2.1.1	D7	Arni	Yeast D7 isoleucine revertans, w/wo exogenous activation
2.1.2.	Asper. 35	Carere	Aspergillus 35 methionine mutants, no activation
2.1.3	D7	Parry	Yeast D7 isoleucine mutants, w/wo activation
2.1.4	XV185	Mehta	Yeast XV185-14C amino acid auxotroph revertants, w/wo activation
2.1.6	PI	Loprieno	Yeast P_1 adenine mutants, w/wo activation
2.1.7	D6	Parry	Yeast D6 adenine and isoleucine revertants, no activation
2.1.8	D61-M	Parry	Yeast D61-M adenine and isoleucine revertants, w/wo activation
2.1.9	Mito. D5	Ferguson	Yeast D5 petite mutants, no activation
2.2. Gene conversion			
2.2.1	D7	Parry	Yeast D7 tryptophan prototrophs, w/wo activation
2.2.2	D7	Parry	Yeast D7 tryptophan prototrophs, w/wo activation
2.2.3	D7-144	Mehta	Yeast D7-144 tryptophan prototrophs, w/wo activation
2.2.4	PV-3	Inge-Vetchtomov	Yeast PV-3 lysine prototrophs, w/wo activation
2.2.5	PV-2	Inge-Vetchtomov	Yeast PV-2 lysine prototrophs, w/wo activation
2.2.6	JD-1	Brooks	Yeast JD1 tryptophan and histidine prototrophs, w/wo activation
2.2.7	D7	Arni	Yeast D7 tryptophan prototrophs, w/wo activation
2.3. Crossing-over			
2.3.1	D7	Arni	Yeast D7 red or pink colonies, w/wo activation
2.3.2	Asper 35	Carere	Aspergillus P_1 yellow colonies and nutritional markers, no activation
2.3.3	D6	Parry	Yeast D6 cycloheximide resistance, no activation
2.3.4	D61-M	Parry	Yeast D61-M cycloheximide resistance, w/wo activation
2.3.5	D61-M	Zimmermann	Yeast D61-M cycloheximide resistance, no activation
2.3.6	D7	Parry	Yeast D7, red or pink colonies, w/wo activation
2.4. Aneuploidy			
2.4.1	D6	Parry	Yeast D6, w/wo activation, white, cycloheximide resistant colonies
2.4.2	D61-M	Zimmermann	Yeast D61-M, no activation, white, cycloheximide resistant colonies
2.4.3	D61-M	Parry	Yeast D61-M, w/wo activation, white, cycloheximide resistant colonies
2.4.4	Asper. 35	Carere	Aspergillus 35, no activation, yellow sectors
3. Drosophila somatic cells			
3.1.1	Wing Spots	Würgler	Somatic recombination and mutation, wing spots
3.1.2	Eye Spots	Fujikawa	Somatic mutation, eye spots
3.1.3	Eye Spots	Vogel	Somatic recombination and mutation, eye spots

KEY TO SUMMARY (continued)

Assay		Investigator	Assay description
4. Cultured mammalian cells (endpoints other than mutation)			
4.1. Metabolic cooperation			
4.1.1	V79	Elmore	V79 Chinese hamster cells, inhibition of metabolic cooperation, no activation
4.1.2	V79	Nelmes	V79 Chinese hamster cells, inhibition of metabolic cooperation, no activation
4.1.3	V79	Umeda	V79 Chinese hamster cells, inhibition of metabolic cooperation, no activation
4.2. Transformation			
4.2.1	BALB/C	Rundell	BALB/C3T3 mouse cells, focus assay, w/wo activation, w/wo TPA enhancement
4.2.2	C3H	McGregor	C3H10T$\frac{1}{2}$ mouse cells, focus assay, w/wo activation
4.2.3	C3H	Nesnow	C3H10T$\frac{1}{2}$ mouse cells, focus assay, no activation
4.2.4	SHE	Sanner	Primary Syrian hamster embryo cells, clonal assay, no activation
4.2.5	SHE	Barrett	Primary Syrian hamster embryo cells, clonal assay, no activation
4.2.6	SHE/SA7	Hatch	Syrian hamster embryo cells, chemical enhancement of viral (SA7) transformation, focus assay, no activation
4.2.7	RLV/FRE	Suk	Viral (RLV) infected rat embryo cells, viability assay, w/wo activation
4.2.8	CHO	Zdzienicka	CHO cells, invasive colonies in soft agar, w/wo activation
4.3. Single-strand breaks			
4.3.1	Rat Hepat.	Bradley	Rat hepatocytes, single strand DNA breaks, alkaline elution, no activation
4.3.2	CHO	Lakhanisky	CHO cells, single strand DNA breaks, alkaline sucrose sedimentation, w/wo activation
4.3.3	CHO	Douglas	CHO cells, single strand DNA breaks, alkaline sucrose sedimentation, w/wo activation
4.4. Unscheduled DNA synthesis (UDS)			
4.4.1	Rat Hepat. (auto)	Williams	Rat hepatocytes, unscheduled DNA synthesis, autoradiography, no activation
4.4.2	Rat Hepat. (auto)	Probst	Rat hepatocytes, unscheduled DNA synthesis, autoradiography, no activation
4.4.3	Rat Hepat. (scin.)	Glauert	Rat hepatocytes, UDS, scintillation counts, no activation
4.4.4	HeLa (scin.)	Martin	Human HeLa cells, UDS, scintillation counts, w/wo activation
4.4.5	HeLa (scin.)	Barrett	Human HeLa cells, UDS, scintillation counts, w/wo activation
4.5. Chromosomal aberrations			
4.5.1	CHO	Gulati	CHO cells, w/wo activation
4.5.2	CHO	Palitti	CHO cells, w/wo activation
4.5.3	CHO	Natarajan	CHO cells, w/wo activation
4.5.4	LYM	Richardson	Human lymphocytes, w/wo activation
4.5.5	CH1-L	Danford	Chinese hamster-liver fibroblasts, no activation
4.5.6	CHL	Ishidate	Chinese hamster-lung fibroblasts, w/wo activation
4.5.7	RL4	Dean	Rat-liver cells (RL4), no activation

KEY TO SUMMARY (continued)

Assay		Investigator	Assay description
4.6. Sister-chromatid exchange			
4.6.1	CHO	Gulati	CHO cells, w/wo activation
4.6.2	CHO	Phillips	CHO cells, w/wo activation
4.6.3	CHO	Natarajan	CHO cells, w/wo activation
4.6.4	CHO	Douglas	CHO cells, w/wo activation
4.6.5	V79	VanWent	V79 cells, w/wo activation
4.6.6	RL4	Dean	RL4 rat-liver cells, no activation
4.7. Micronucleus			
4.7.1	CHO-MN	Douglas	CHO cells, w/wo activation
4.8. Polyploidy			
4.8.1	CHL	Ishidate	Chinese hamster-lung cells, w/wo activation
4.8.2	CH1-L	Danford	Chinese hamster-liver fibroblasts, no activation
4.8.3	RL4	Dean	RL4, rat-liver cells, no activation
4.9. Aneuploidy			
4.9.1	CH1-L (aneupl.)	Danford	Chinese hamster-liver fibroblasts, no activation, aneuploidy
4.9.2	CH1-L (spindle)	Parry	Chinese hamster-liver fibroblasts, no activation, spindle effects
5. Mammalian cell mutations			
5.1. L5178 Y			
5.1.1	L51-TK	Garner	L5178Y mouse lymphoma cells, thymidine kinase muants, w/wo activation
5.1.2	L51-TK	Oberley	L5178Y mouse lymphoma cells, thymidine kinase mutants, w/wo activation
5.1.3	L51-TK	Lee	L5178Y mouse lymphoma cells, thymidine kinase mutants, w/wo activation
5.1.4	L51-TK	Amacher	L5178Y mouse lymphoma cells, thymidine kinase mutants, w/wo activation
5.1.5	L51-TK	Myhr	L5178Y mouse lymphoma cells, thymidine kinase mutants, w/wo activation
5.1.6	L51-TK	Styles	L5178Y mouse lymphoma cells, thymidine kinase mutants, w/wo activation
5.1.7	L51-TK	Knaap	L5178Y mouse lymphoma cells, thymidine kinase mutants, w/wo activation
5.1.8	L51-OUA	Styles	L5178Y mouse lymphoma cells, Ouabain resistant mutants, w/wo activation
5.2. V79			
5.2.1	V79-OUA	Kuroki	V79 Chinese hamster cells, Ouabain resistant mutants, w/wo activation
5.2.2	V79-TG	Kuroda	V79 Chinese hamster cells, HGPRT mutants, w/wo activation
5.2.3	V79-TG	Lee	V79 Chinese hamster cells, HGPRT mutants, w/wo activation
5.2.4	V79-TG	Delow	V79 Chinese hamster cells, HGPRT mutants, w/wo activation
5.3. CHO			
5.3.1	CHO-TG	Zdzienicka	CHO Chinese hamster ovary cells, HGPRT mutants
5.3.2	CHO-OUA	Zdzienicka	CHO Chinese hamster ovary cells, Ouabain resistant mutants
5.4. Human lymphoblasts			
5.4.1	Human Lym. TK	Crespi	TK6 human lymphoblasts, Thymidine kinase mutants, w/wo activation
5.4.2	Human Lym. TG	Crespi	AHH human lymphoblasts, HGPRT mutants, no activation

TABLE 1

RESULTS OF THE STUDY EXPRESSED BY ASSAY GROUP AND TEST AGENT

The order of assays is as described in the Introduction and as displayed in the associated key. The order of the chemicals is according to decreasing genotoxicity, as shown in Fig. 1. The results shown are those of individual investigators and may therefore differ from those shown in the assay workgroup reports.

IPCS CSSTT *IN VITRO* STUDY - SUMMARY OF QUALITATIVE RESULTS

ASSAY	ACN	TOL	HMPA	SAF	DES	BEN	PB	DEHP	ZOM	CAP
1. BACTERIA										
1.1.1 Salmonella	?	○	○	○	○	○	●	○	○	○
1.1.2 Salmonella	●	○	○	○	○	○	○	○	○	○
1.1.3 Salmonella	○	○	○	○	○	○	○	○	○	○
1.1.4 Salmonella	●	●	○	○	○	○	●	○	●	○
1.1.5 Salmonella	○	○	○	○	○	○	●	○	○	○
2. FUNGI										
2.1 Mutation										
2.1.1 D7	○	○	●	○	○	○	○	○	○	○
2.1.2 Asper. 35		○	○	○		○				
2.1.3 D7	○	○	○	○	○	●	○	○	○	○
2.1.4 XV185	●	●	●	●		●	●	●	●	●
2.1.5 XV185		○	●	●					●	●
2.1.6 P1	○	○	○	●	○	○	○	○	○	○
2.1.7 D6	●	○	●	○	○	○	○	○	○	○
2.1.8 D61-M	●	●	●	●	○	○	○	○	○	○
2.1.9 Mito. D5	●	●	○	●	○	●	○	○	○	○
2.2 Gene conversion										
2.2.1 D7	●	○	●	○	○	○	○	●	○	○
2.2.2 D7	●	○	○	●	○	○	○	○	○	○
2.2.3 D7-144	●	●	●	●		●	●	●	●	●
2.2.4 PV-3	○	○	○	○	○	○	○	○	○	○
2.2.5 PV-2	○	○	○	○	○	○	○	○	○	○
2.2.6 JD-1	●	○	○	○	○	○	○	○	○	○
2.2.7 D7	●	○	●	○	○	○	○	●	○	○
2.3 Crossing-over										
2.3.1 D7	○	○	○	○	○	○	○	○	○	○
2.3.2 Asper. 35	○	○	○	○	○	○	○	○	○	○
2.3.3 D6	●	○	○	○	○	●	○	○		○
2.3.4 D61-M	●	○	○	○	○	○	○	○	○	○
2.3.5 D61-M	●	○	○	○	○	○	○	○	○	○
2.3.6 D7	○	○	○	●	○	○	○	●	●	○
2.4 Aneuploidy										
2.4.1 D6	●	●	●	●	●	●	●	●	○	○
2.4.2 D61-M	○	○	○	○	●	●	○	○	○	?
2.4.3 D61-M	●	●	●	●	●	●	●	●	○	○
2.4.4 Asper. 35	●	○	○	●	○	○	○	○	○	○
3. DROSOPHILA SOMATIC CELLS										
3.1.1 Wing spots	●	●	●	●	○	●	○	○	○	●
3.1.2 Eye spots	●	○	●	○	○	○	○	?	○	●
3.1.3 Eye spots	●	●	●	●	?	?	○	?	○	●
4. CULTURED MAMMALIAN CELLS (endpoints other than gene mutation)										
4.1 Metabolic cooperation										
4.1.1 V79	●	●	○	?	○	○	?	●	?	○
4.1.2 V79		●	○	○		○				○
4.1.3 V79	●	○	○	?	○	○	○	○	○	○
4.2 Transformation										
4.2.1 BALB/C	○	○	○	○	○	○	○	○	○	○
4.2.2 C3H	●	●	●	●	●	?	?	●	●	●
4.2.3 C3H		●	?	●		?				○
4.2.4 SHE	●	●	●	?	○	●	●	●	○	?
4.2.5 SHE	●	●	●	●	●	●	○	●	○	●
4.2.6 SHE/SA7		●	?	○	?	○		?		○
4.2.7 RI-FRE		?	●	○	●			●	○	○
4.2.8 CHO		○	?	○		○				○

ASSAY	ACN	TOL	HMPA	SAF	DES	BEN	PB	DEHP	ZOM	CAP
4.3 Single strand breaks										
4.3.1 Rat Hepat	●	●	○	●	●	○	○	○	●	○
4.3.2 CHO	○	●	○	●	●	●		○	○	○
4.3.3 CHO	●	●	○	○	●	○	○	○	○	○
4.4 Unscheduled DNA synthesis (UDS)										
4.4.1 Rat Hepat. (autorad.)	○	○	●	○	○	○	○	○	○	○
4.4.2 Rat Hepat. (autorad.)	○	○	○	○	○	○	○	○	○	○
4.4.3 Rat Hepat. (scint.)	●	●	●	●	○	●	○	●	●	○
4.4.4 HeLa (scint.)	○	●	●	●	○	○	○	○	○	○
4.4.5 HeLa (scint.)		●	?	●		○				○
4.5 Chromosomal aberrations										
4.5.1 CHO	●	●	○	○	●	○	●	○	○	○
4.5.2 CHO		○	○	●		●				○
4.5.3 CHO	●	○				○	●			○
4.5.4 LYM			●		●	●			○	●
4.5.5 CH1-L	●	●	●	○	●	○	●	○	○	○
4.5.6 CHL	●	●	●	●	○	●	?	○	●	●
4.5.7 RL4	○	●	○	○	○	○	○	○	○	○
4.6 Sister chromatid exchange										
4.6.1 CHO	●	●	●	●	○	○	○	○	○	○
4.6.2 CHO		●	○	○		○				○
4.6.3 CHO	●	○				○	●			○
4.6.4 CHO		○	○	○		○		○		○
4.6.5 V79		●	●	●		○				○
4.6.6 RL4	○	●	●	○	○	○	○	○	○	○
4.7 Micronucleus										
4.7.1 CHO-MN	●	○	○	○	○	○	○	○	○	○
4.8 Polyploidy										
4.8.1 CHL	○	●	○	○	●	○	○	○	○	●
4.8.2 CH1-L	○	○	○	○	●	○	○	○	○	○
4.8.3 RL4	○	○	○	○	●	○	○	○	○	○
4.9 Aneuploidy										
4.9.1 CH1-L aneupl.	○	●	●	○	●	●	○	●	○	○
4.9.2 CH1-L spindle	○	○	○	○	●	●	●	●	○	○
5. MAMMALIAN CELL MUTATIONS										
5.1 L5178Y										
5.1.1 L51-TK	●	●	○	●	●	●	○	○	○	○
5.1.2 L51-TK	●	○	●	○	○	●	●	●	●	○
5.1.3 L51-TK	●	○	○	○	○	○	○	○	○	○
5.1.4 L51-TK	●	○	●	●	○	○	○	○	○	○
5.1.5 L51-TK	●	●	●	?	●	○	●	○	●	○
5.1.6 L51-TK		?	●	●	●	●	?	○	○	
5.1.7 L51-TK		○	●	?		○				○
5.1.8 L51-OUA	○	○	●	●	●	●	?	○	?	
5.2 V79										
5.2.1 V79-OUA		○	○	○					○	○
5.2.2 V79-TG		●	●	?		●	●		●	?
5.2.3 V79-TG	○	○	○	○	○	○	○	○	●	○
5.2.4 V79-TG		○	○	○		○				○
5.3 CHO										
5.3.1 CHO-TG		○	○	○		○				○
5.3.2 CHO-OUA		?	○	○		○				○
5.4 Human lymphoblasts										
5.4.1 Human lym. TK	●	●	○	●	○	○	○	○	○	○
5.4.2 Human lym. TG	●	●	○	○	●	●	●	○	○	○

● Positive ○ Negative

TABLE 2

RESULTS FROM SELECTED ASSAYS SHOWN IN TABLE 1

This reduction in the database is justified in the text and forms the basis for the selection of a generally applicable complementary in vitro assay.

IPCS CSSTT *IN VITRO* STUDY - SUMMARY OF REDUCED DATABASE

	ASSAY	ACN	TOL	HMPA	SAF	DES	BEN	PB	DEHP	ZOAN	CAP
2	FUNGI										
2.1	Mutation										
2.1.4	XV185	●	●	●	●		●	●	●	●	●
2.1.5	XV185		○	●	●					●	●
2.1.7	D6	●	○	●	○	○	○	○	○	○	○
2.1.8	D61-M	●	●	●	●	○	○	○	○	○	○
2.1.9	Mito. D5	●	●	○	●	○	●	○	○	○	○
2.4	Aneuploidy										
2.4.1	D6	●	●	●	●	●	●	●	●	○	○
2.4.2	D61-M	○	○	○	○	●	●	○	○	○	?
2.4.3	D61-M	●	●	●	●	●	●	●	●	○	○
2.4.4	Asper. 35	●	○	○	●	○	○	○	○	○	○
3.	DROSOPHILA SOMATIC CELLS										
3.1.1	Wing spots	●	●	●	●	○	●	○	○	○	●
3.1.2	Eye spots	●	○	●	○	○	○	○	?	○	●
3.1.3	Eye spots	●	●	●	●	?	?	○	?	○	●
4.	CULTURED MAMMALIAN CELLS (endpoints other than gene mutation)										
4.3	Single strand breaks										
4.3.1	Rat Hepat	●	●	○	●	●	○	○	○	●	○
4.3.2	CHO	○	●	○	●	●	●		○	○	○
4.3.3	CHO	●	●	○	○	●	○	○	○	○	○
4.4	Unscheduled DNA synthesis (UDS)										
4.4.3	Rat Hepat. (scint.)	●	●	●	●	○	●	○	●	●	○
4.4.4	HeLa (scint.)	○	●	●	●	○	○	○	○	○	○
4.4.5	HeLa (scint.)		●	?	●		○				○
4.5	Chromosomal aberrations										
4.5.1	CHO	●	●	○	○	●	○	●	○	○	○
4.5.2	CHO		○	○	●		●				○
4.5.3	CHO	●	○				○	●			○
4.5.4	LYM			●		●	●			○	●
4.5.5	CH1-L	●	●	●	○	●	○	●	○	○	○
4.5.6	CHL	●	●	●	●	○	●	?	○	●	●
4.6	Sister chromatid exchange										
4.6.1	CHO	●	●	●	●	○	○	○	○	○	○
4.6.2	CHO		●	○	○		○				○
4.6.3	CHO	●	○				○	●			○
4.6.5	V79		●	●	●		○				○
4.6.6	RL4	○	●	●	○	○	○	○	○	○	○
4.9	Aneuploidy										
4.9.1	CH1-L aneupl.	○	●	●	○	●	●	○	●	○	○
5.	MAMMALIAN CELL MUTATIONS										
5.1	L5178Y										
5.1.1	L51-TK	●	●	○	●	●	●	○	○	○	○
5.1.2	L51-TK	●	○	●	○	●	●	●	●	○	○
5.1.4	L51-TK	●	○	●	●	○	○	○	○	○	○
5.1.5	L51-TK	●	●	●	?	●	○	●	○	●	○
5.1.6	L51-TK		?	●	●	●	●	?	○	○	
5.1.7	L51-TK		○	●	?		○				○
5.1.8	L51-OUA	○	○	●	●	●	●	?	○	?	
5.2	V79										
5.2.2	V79-TG		●	●	?		●	●		●	?
5.4	Human lymphoblasts										
5.4.1	Human lym. TK	●	●	○	●	○	○	○	○	○	○
5.4.2	Human lym. TG	●	●	○	○	●	●	●	○	○	○

● Positive ○ Negative

TABLE 3

THE RESPONSES SHOWN IN THE REDUCED DATABASE (TABLE 2) ARE REPRESENTED AS NUMBER OF POSITIVE RESPONSES/NUMBER OF OBSERVATIONS MADE

Questionable responses have been eliminated from the numerator but included in the denominator. The carcinogen sensitivity of each class of assay has been calculated, but not their sensitivity (as only two carcinogens were employed) nor their accuracy (because of the unique handling of non-carcinogens in this study). At the foot of the Table the responses of the Salmonella assay and the transformation assays are shown.

CLASS OF ASSAY / CHEMICAL	SUMMARY OF QUALITATIVE RESULTS EXPRESSED AS POSITIVE TESTS / TOTAL TESTS												OVERALL PERFORMANCE FOR THE EIGHT CARCINOGENS
	ACN	TOL	HMPA	SAF	OVERALL	DES	BEN	PB	DEHP	OVERALL	*ZOIN*	*CAP*	OVERALL	
2. FUNGI														
2.1 Mutation	4/4	3/5	4/5	4/5	15/19 (79%)	0/3	2/4	1/4	1/4	4/15 (27%)	2/5	2/5	4/10 (40%)	50%
2.4/4.9.1 Aneuploidy	3/5	3/5	3/5	3/5	12/20 (60%)	4/5	4/5	2/5	3/5	13/20 (65%)	0/5	0/5	0/10 (0%)	62%
3. DROSOPHILA SOMATIC CELLS	3/3	2/3	3/3	2/3	10/12 (83%)	0/3	1/3	0/3	0/3	1/12 (8%)	0/3	3/3	3/6 (50%)	46%
4. CULTURED MAMMALIAN CELLS														
4.3 Single strand breaks	2/3	3/3	0/3	2/3	7/12 (58%)	3/3	1/3	0/2	0/3	4/11 (36%)	1/3	0/3	1/6 (17%)	48%
4.4 UDS (scintillation)	1/2	3/3	2/3	3/3	9/11 (82%)	0/2	1/3	0/2	1/2	2/9 (22%)	1/2	0/3	1/5 (20%)	55%
4.5 Chromosomal aberrations	4/4	3/5	3/5	2/4	12/18 (67%)	3/4	3/6	3/4	0/3	9/17 (53%)	1/4	2/6	3/10 (30%)	60%
4.6 SCE	2/3	4/5	3/4	2/4	11/16 (69%)	0/2	0/5	1/3	0/2	1/12 (8%)	0/2	0/5	0/7 (0%)	43%
5. MAMMALIAN CELL MUTATIONS (L51, V79, human cells)	6/7	5/10	7/10	5/10	23/37 (62%)	5/8	6/10	4/9	1/8	16/35 (46%)	3/9	0/8	3/17 (18%)	55%
OVERALL ACTIVITY	25/31 (81%)	26/39 (67%)	25/38 (66%)	23/37 (62%)		15/30 (50%)	18/39 (46%)	11/32 (34%)	6/30 (20%)		8/33 (24%)	7/38 (18%)		

94/145 (68%)

33/69 (48%)

17/62 (27%)

15/72 (21%)

132/214 (62%)

32/133 (24%)

149/276 (54%)

1. Salmonella	2/5	1/5	0/5	0/5	3/20 (15%)	0/5	0/5	3/5	0/5	3/20 (15%)	1/5	0/5	1/10 (10%)	15%
4.2 Transformation	3/3	5/6	4/6	3/6	15/21 (71%)	3/5	2/5	1/3	4/5	10/18 (55%)	1/4	2/6	3/10 (30%)	64%

questionable activity in TA100 (−S9) in 1 laboratory of 5.

PB S9 independent weak activity in 2 laboratories in TA1535 and in 1 laboratory using TA100. Questionable activity was also seen in TA100 (±S9) in one of the laboratories recording activity in TA1535.

HMPA, BEN, SAF, CAP, DEHP and *DES* gave no evidence of mutagenic activity.

The performance of the new strains TA97 and TA102 is commented on later under the section dealing with definition of positive responses. A detailed description of all Salmonella data is given in the workgroup report and in the relevant investigators reports.

The assay workgroup concluded that these data confirm that the present 10 test chemicals are either difficult or impossible to detect as bacterial mutagens using the routinely employed test protocols of the Salmonella assay; thus, their selection for the present study is endorsed.

7. Current legislative guidelines for genotoxicity evaluation

The results of this study may have an impact on current legislative initiatives in this area of preventive human toxicology. These guidelines are summarized in Appendix 3 to enable the findings of this study to be related to the practical world of routine screening with a minimum of effort. This task was undertaken by D. Berry and M. Litchfield.

8. Significance and use of workshop consensus conclusions

The extent to which assays can be grouped together under a common heading is unresolved but is of considerable interest. When, for example, reference is made to legislative guidelines it is usual to have a category of assay defined with few details of the endpoints, cell line or test protocol given. Thus, it may be considered advisable to produce data regarding the mammalian cell gene mutagenicity of a chemical, but whether or not these data are derived in V79 or CHO cells, or using a plate or microwell technique, etc., is not specified — the inference being that any suitably established mamalian cell gene-mutation assay will prove acceptable. A clear scientific basis for the assumed equivalence of formally related assays is lacking, nonetheless, the groupings adopted by the present investigators were considered justified. Of course, the broader the definition of an assay group, the larger the number of investigators it will encompass; this in turn allows the problem of interlaboratory variations to be approached mainly in these broad groups. This becomes of importance when reviewing the total database because selective (usually therefore small) assay groups may appear to give more consistent data than the broader groups; this may be an illusion.

In cases where unanimously positive or negative data were observed within an agreed class of assay it was easy to conclude that a given chemical either was or was not active. Problems were inevitably encountered in the majority of cases where discordant activities were encountered. Thus, benzoin, for example, was active in 2 of the UDS assays but negative in the remainder. The groups attempted to decide if such divergent findings were due to differences in the cell line, variations in protocol, thoroughness of testing, dose-levels employed, etc. Such considerations usually led to a consensus statement on the overall activity of a chemical within a class of assay.

While useful for defining specific protocol inadequacies of certain assays these consensus conclusions are necessarily ephemeral. For example, although most investigators found TOL capable of inducing gene-mutation in mammalian cells, some did not, and it would be expected that in some cases the latter investigators could repeat these assays and confirm their original negative observations at a future date. Thus, to an individual investigator, the consensus conclusion may have little or no relevance to his perception of that chemical in his assay.

The most challenging situation in the drawing of consensus conclusions was encountered in cases where isolated individuals presented positive findings in contrast to the majority of their peers. Clearly, simple arithmetic considerations would have dominated the selection of a negative consensus finding, but on several occasions this was unacceptable due to the apparent quality of the isolated positive data. Perhaps the main conclu-

sion of relevance to the defined aims of this study is that unless a class of assay can record a consensus positive response for a carcinogen that represents a majority view of the investigators, then that assay must be regarded as currently unsuitable for the detection of the chemical in question. However, such divergent data may, on occasion, give an insight into a modification of the assay that would allow the general and reliable detection of the chemical in question.

9. Assessment of assays with respect to their routine use as complements to the Salmonella mutation assay

The aims of this study were clearly stated at the outset [28], together with an outline of the criteria by which generally applicable complementary assays were to be defined. These criteria are reproduced in the Introduction to this volume.

Reference to the master table of results (Table 1) and the above-criteria indicates that no category A assays were defined. Primary attention therefore focuses on possible category B assays, i.e., those which may prove appropriate for routine complementary use given adequate attention to the definition of minimal criteria for their conduct. In order to achieve this, a sequence of selective criteria has been imposed on the total database leading to a reduced table of data (Table 2). The remaining assays were then assessed leading to the general conclusions listed at the end of this Chapter. Use of the specific categorization A–D has been avoided without, it is hoped, a loss of clarity in the conclusions.

It must be strongly emphasized at this point that the principal aim of this study was to identify one or more non-bacterial in vitro assays which have performed well in the detection of the present 8 carcinogens, and for which evidence of interlaboratory reproducibility is also available. The failure of some assays to meet these criteria leads to their not being recommended below for routine use in conjunction with the Salmonella assay. However, this suggestion does not necessarily devalue the assay or the end-point being studied when employed for research.

General principles adopted for assay assessment. Within the context of such a large database, certain basic assumptions had to be made to enable an overall assessment of assay performances to proceed. First, the qualitative responses displayed in the Master Table (1) were assumed to be correct. Some of these results had not been confirmed and may therefore represent false positive or negative observations (see Section 14 of this Chapter). A second assumption was that an assay should be capable of detecting at least two of the present test agents as positive before it could be assessed for possible use as a complementary test. The extent to which inadequacies of individual test protocols, as opposed to the insensitivity of the particular assay or its genetic endpoint, were responsible for negative responses can only be determined in cases where the same assay was conducted in 2 or more laboratories.

Statistical comparisons of the overall performance of assays have generally been avoided because, with the present rather unusual set of test chemicals, this could yield meaningless if not misleading conclusions. Two further decisions were made which influence the conclusions. These were:

(i) It was accepted that in vitro assays are, by their constitution, only appropriate for the definition of potential carcinogens. It is implied by this assumption that certain agents will show activity in vitro but will be unable to express this potential in vivo due to their non-absorption, rapid excretion, preferential detoxification, inappropriate partitioning, etc in mammals. In vitro assays cannot and should not be expected to reveal these possibilities that are, by definition, unique to living animals. Specifically, activity seen in vitro for the present two non-carcinogens has not been used when determining the overall performance of the assays in question (see Table 3), but rather, to emphasize the true role and generic predictive weaknesses of in vitro assays. To compensate for this rather unusual use of reference non-carcinogens, a detailed discussion of the activities observed for CAP and ZOIN is presented as a separate section.

(ii) Four of the 8 carcinogens selected for this study (HMPA, TOL, SAF and ACN) were known, at the outset, to be more likely to be detected by most assays as each was already established as genotoxic, albeit they are usually inactive in the

Salmonella mutation assay. These four chemicals have therefore been considered separately on several occasions, in particular, in Table 3. The remaining carcinogens with the possible exception of benzene, were loosely regarded as non-genotoxic prior to this study. The present database generally supports the segregation of these 2 groups of carcinogens and enables selective assessment of each assay to be made. Some assays performed well with the first 4 carcinogens but poorly with the others, and some were insensitive to this division and performed either generally well or poorly. A possible further sub-division of the second 4 carcinogens is presented in Table 3.

Reduction of database

The following considerations led to a reduction of the Master Table of results (from Table 1 to Table 2). Each step of this has been separately displayed to enable the reader to derive different conclusions should any disagreement with these criteria exist.

(a) The Salmonella data, by definition of complementary assays, have been omitted.

(b) The group that reviewed the transformation assays recommended that although certain of them performed impressively and were worthy of continued research and development, none was yet optimal for routine adoption by an investigator seeking an assay with which to complement the Salmonella test. Consequently, this class of assay has been omitted in Table 2. However, the unique position of transformation assays vis-a-vis carcinogenicity led to a more detailed discussion in a subsequent part of this section and to a display of the data generated using them at the foot of Table 3.

(c) The group that reviewed the metabolic cooperation assays concluded that due to the relative novelty of these tests and the limited extent of the total database, they were currently unsuitable to act as complementary genotoxicity assays; they have therefore also been omitted. Their unrelated use for detecting tumour-promoting agents is discussed in the assay work group report.

(d) The fungal assay group recommended that based on the results of this study, yeast crossing-over assays, as currently constituted, were inappropriate for use as complementary assays; they, too, have therefore been omitted.

(e) The fungal workgroup supported the use of the yeast gene conversion assay as a supplementary test with yeast point mutation assays. However, the current database is not consistent with this class of assay being sufficiently sensitive or reproducible between laboratories, at least as currently constituted, to act as a routine complementary assay. Thus, two investigators recorded ten negative responses, while another found that only ACN was positive out of 10 determinations. Three of the remaining investigators (2.2.1, 2.2.2 and 2.2.7, respectively) agreed on the activity of ACN but failed to agree on the other 5 positive responses observed in 27 joint test determinations. These performances suggest that this class of assay is too insensitive to act as a complementary assay at the present time, and these data have therefore been omitted. The mostly positive data represented in assay 2.2.3 appear to contradict this decision, however, these results are in conflict with those shown in the yeast assay workgroup report. After extensive subsequent discussion with the investigator and the assay workgroup Chairman it was decided to display the investigators conclusions in the Master Table, but yet not allow these to influence the decision to exclude this class of assay from those currently suitable for general use as a complementary assay. We refer the reader to the investigator report and hope that independent confirmation of this particular assay protocol will be forthcoming.

(f) The three investigators who recorded the incidence of polyploidy did so in the course of scoring chromosomal aberrations. Assessment of polyploidy should not, therefore, be regarded as an independent assay. Consequently these data do not appear in Table 2. Later in this discussion, however, the possible value of scoring polyploidy while conducting a cytogenetic assay is discussed.

(g) Individual data sets have been eliminated in cases where an investigator failed to detect any of the test chemicals as active, or where only a single positive response was observed in cases where all 10 of the compounds had been assayed. These exclusions are justified in terms of the demonstrated insensitivity of the particular assay. In a few cases, some investigators who tested only 5–6 compounds found only a single agent active and

these data have been retained. In some cases exclusion of data by this criterion had the effect of eliminating one or two investigators from an assay group. In such cases, the conclusion is that certain protocol deficiencies or cell line insensitivities prevented those investigators from producing 'representative' assay data. In other cases, however, the effect was to remove a 'class' of assay from consideration, as occurred with the autoradiographic detection of UDS in hepatocytes and the scoring of micronuclei in CHO cells. It is appreciated that the larger the number of investigators who offered data for a particular assay group, the smaller was the chance that that group would be completely eliminated from the database when applying this criterion, but this was accepted as a necessary constraint of the analysis.

Justification for the reduction of the database

Almost half of the data shown in Table 1 has been eliminated from Table 2. As the major conclusions of this study are based on the latter database it is appropriate to consider the validity of the elimination principles employed.

The decision to remove certain classes of assay from consideration because they are not yet suitably developed for routine use, or have proven difficult to establish in independent laboratories, represents a decision of the Editors, but this is usually supported by the conclusions of the appropriate assay group reports. The transformation assays were thus eliminated based largely on the conclusions of the workgroup, but it is relevant that in laboratories where certain of these assays are performing reliably, they appear to provide an efficient complementary assay. The metabolic cooperation assays were similarly eliminated, but these do not appear to be optimal for use as a complementary test. The removal of certain classes of assay that were generally insensitive to the present carcinogens is automatically justified by the aims of this study. Elimination of the rat hepatocyte autoradiographic UDS assays and the CHO micronucleus test are representative of the application of this principle.

Elimination of individual assays that failed to detect any or only 1 of the test chemicals in cases where all 10 had been evaluated requires separate justification. In most of these cases, other investigators using nominally the same assay detected several of the carcinogens; the inference is, therefore, that the particular protocol used is more at fault than the particular class of assay. The individual assays eliminated by this criterion are listed below, and it is clear that different underlying reasons may have led to their poor performance. Six assays only detected ACN as positive. The fact that this carcinogen was the most generally genotoxic of the present 8 carcinogens suggests that the sensitivity of the assay protocols in question was too low, rather than the assay class as a whole being of no potential value as a complementary assay. However, 4 of these assays formed part of 2 classes eliminated for reasons of general insensitivity (yeast gene conversion and yeast crossing over). These six assays were:

2.2.3, 2.2.6 } yeast gene conversion

2.3.4, 2.3.5 } yeast crossing-over

4.7.1 CHO micronucleus
5.1.3 L5178Y TK gene mutation

The ability of two of the polyploidy assays (4.8.2 and 4.8.3) to detect only DES may reflect the exceptional potency of this agent as a spindle poison. DES may therefore be too potent an agent to act solely as the monitor for sensitivity of polyploidy and aneuploidy assays.

Five assays gave isolated positive responses that appear to have no explanation. These effects may therefore reflect technical false positive responses of these tests. Whatever, the weakness of these responses, coupled to the generally negative context in which they occurred, suggests that these activities should only be related with caution to the carcinogenicity of the test agents. These activities were for the following chemicals and assays:

ZOIN, assay 5.2.3 (V79 TG gene mutation)
TOL, assay 4.5.7 (RL4 chromosomal aberrations)
HMPA, assay 4.4.1 (hepatocyte autorad. UDS)
assay 2.2.1 (yeast D7 gene mutation)
BEN, assay 2.1.3 (yeast D7 gene mutation)

The decision to eliminate certain assays and data were designed to optimize the relevance of the conclusions from the present study. The steps

outlined removed from consideration those assays that were not optimally performed or which were representative of a class of assay generally deemed unsuitable for routine adoption as a complementary assay at this stage of their development. Similar exclusion principles have been adopted by the several GeneTox review groups, and in those cases the resultant database provides a more reliable reflection of the current stature of individual assays. The positive effect that the above decisions had on the database is demonstrated in Fig. 1. From this it is evident that the composite sensitivity of the assays to the 8 carcinogens has been increased without any corresponding loss of specificity (see boxed area of Fig. 1). This figure also demonstrates that the degree of genotoxicity of the present 10 chemicals, relative to each other, was not affected by the elimination procedures employed, and thus provides a compelling justification for proceeding with a detailed assessment of the data shown in Table 2.

Assessment of performances of the reduced list of assays

This assessment represents the opinion of the Editorial Committee. Close attention was paid to the conclusions drawn by the assay groups regarding the usefulness of individual assays. However, not all of these workgroups approached an assessment of their assay within the context outlined originally. On occasion, the recommendations and conclusions derived below are at variance with the group conclusions, but more generally they are consistent with them or represent the only ones available.

2.1. Gene mutation in yeast. These assays are, phylogenically, the nearest to the Salmonella assay and share a similar genetic endpoint. Previous studies have also established that genotoxic agents found active in Salmonella are generally also active in the corresponding yeast gene mutation assays. It was therefore expected that these 2 classes of assay would share similar sensitivities. Fourteen positive responses were observed within the group comprising HMPA, TOL, SAF and ACN, while only 3 positive responses were observed for BEN, DEHP, DES and PB, and there was general agreement on the gene-mutagenicity of HMPA (4/5 positive) (see Table 3 and Fig. 2). The enhanced

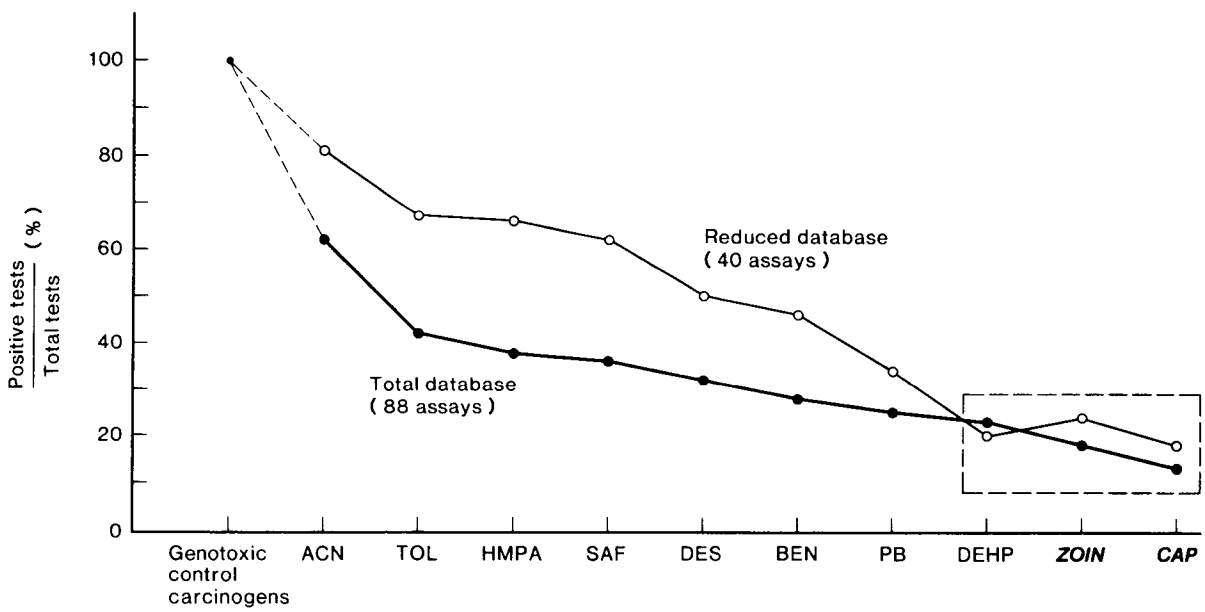

Fig. 1. Relative genotoxicity of the 10 test chemicals as calculated using the total database (filled circles) and the reduced database (open circles). The 2 non-carcinogens and DEHP gave a similar low level of genotoxicity (boxed area). Questionable responses were regarded as negative but counted as an observation.

sensitivity of the yeast gene-mutation assays, as opposed to the Salmonella mutation assay, to the four assumed genotoxins may be due to endogenous metabolism, especially evident when the cells are in active growth. It is therefore concluded that this class of assay may represent a possible alternative to the Salmonella assay, and may present advantages, on occasion, due to enhanced sensitivity to certain genotoxins. The mitochondrial mutation assay (2.1.9) has been included in this discussion, but should correctly be regarded as a distinct assay. Two potential problems are presented by these assays, and yeast assays in general. First, they are not yet conducted according to an agreed protocol. For example, the data shown in Table 1 represent the consensus gained after conducting assays within certain laboratories under a range of test conditions: low or high glucose, stationary, semi-stationary and log-phase cells etc. In addition, the largely positive database of assay 2.1.4 includes two classes of positive response (see assay group report and investigator report). Second, the Salmonella assay must remain the preferred primary assay for detecting gene mutagens in vitro owing to the extensive database available and the large number of laboratories involved (> 2000).

It is concluded that appropriate yeast assays present a useful method of detecting gene-mutations, and on occasion can be more sensitive than the Salmonella test. However, given the current state of the art, they would not be recommended as the primary gene-mutation assay and their usefulness as a single complement to the Salmonella test is reduced by the repetition of the genetic endpoint involved. The preferential detection by these assays of HMPA, TOL, SAF and ACN is commented on later herein.

3.1.3. Drosophila somatic cell mutation assays. New somatic cell mutation assays have recently been developed in Drosophila. Their novelty is evidenced by the fact that data supporting their general sensitivity to genotoxic agents (i.e. agents readily detected by the Salmonella assay) are only now being collected for publication. General sensitivity is, however, claimed (see workgroup report). Two different types of assay were represented.

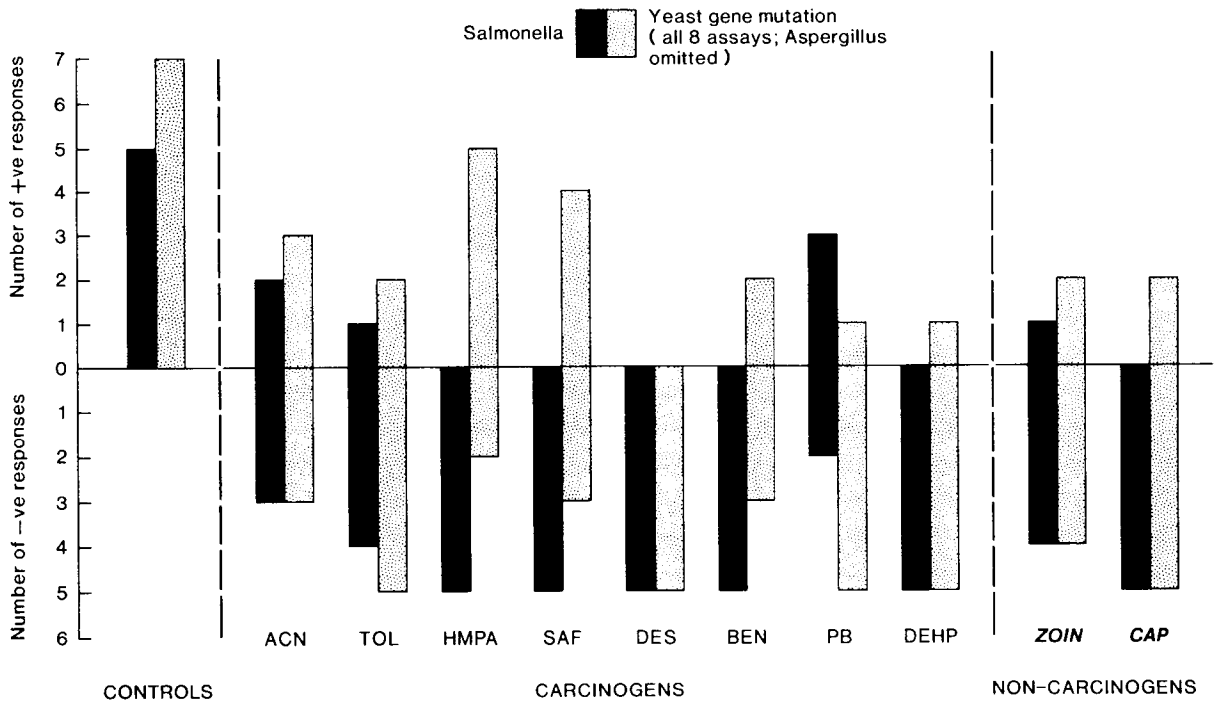

Fig. 2. Comparison of the performance of the Salmonella assay and the 8 yeast gene-mutation tests included in the study (i.e., from the total database, Table 1). Questionable responses have been ignored.

Assays 3.1.1 and 3.1.3, although based on different tissues, were capable of detecting a range of induced genetic events, i.e. chromosomal breakage, deletions and aneuploidy, gene mutations and mitotic recombinations. These two assays were, in fact, more sensitive to the present test chemicals than was assay 3.1.2 where an inappropriate route of administration was employed and the range of detected mutagenic events was limited to gene mutations and deletions. Nonetheless, the general concordance between the responses recorded in each of these 3 assays suggests that they are likely to yield reproducible data between laboratories.

Assays in Drosophila require minimal technical facilities but may prove relatively time-consuming. They present the appeal of conducting an assay on a multicellular organism with its inherent metabolic capability. Whether this capability is significantly related to that of a mammal cannot be automatically assumed, but the sensitivity of these assays to a range of genotoxic agents is consistent with their possession of a broad range of metabolic pathways.

The two most sensitive assays (3.1.1 and 3.1.3) detected 5 and perhaps 6 of the test agents, one of which was the non-carcinogen caprolactam (see subsequent discussion of CAP and ZOIN). Activity was mainly centred in the first group of carcinogens (Table 3 and Fig. 3). This performance suggests that somatic mutation in Drosophila, especially the assays capable of detecting a wide range of genetic events, presents a promising new assay. However, their preferential detection of HMPA, TOL, SAF and ACN reduces their value as a general complementary assay (cf. similar discussion of yeast gene-mutation assays, SCE assays and final conclusions). The present discussion only relates to the use of these assays for predicting the possible mammalian carcinogenicity of an agent; their use for predicting in vivo mammalian mutagenic events should be considered separately (see also discussions of mammalian cell gene mutation assays).

4.3/4.4. Assays for DNA damage [SSB (single strand breaks) and UDS (detected via autoradiography or scintillation counting). Although both of these classes of assay are designed to be sensitive to the consequences of the primary interaction of

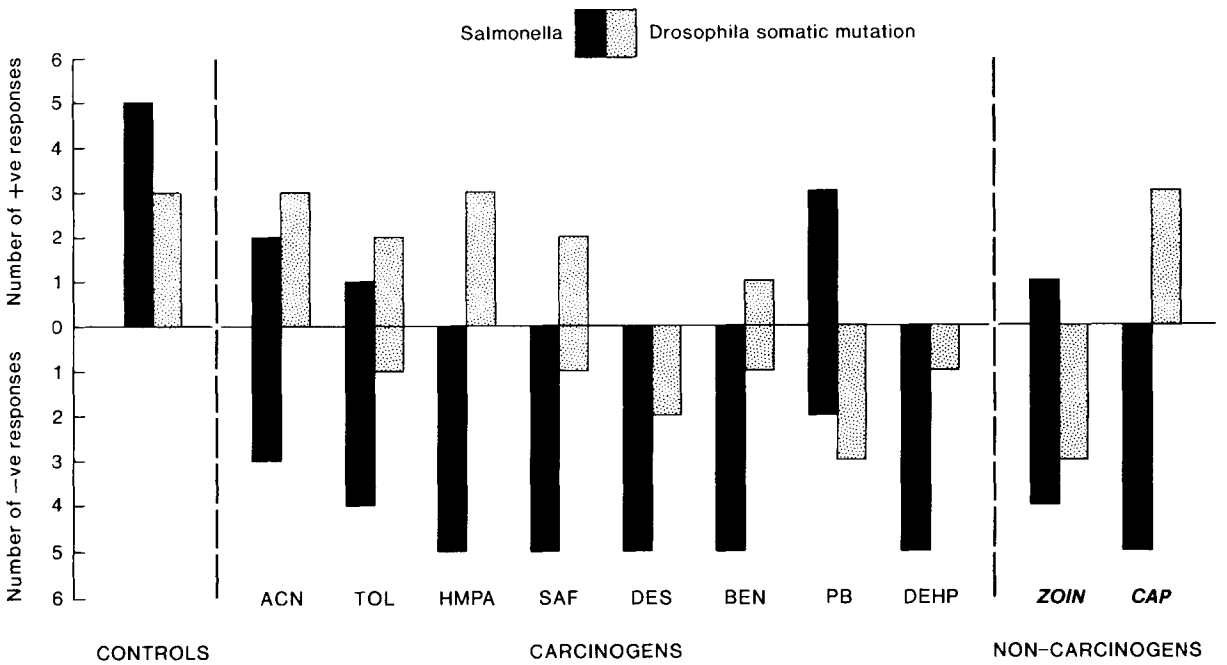

Fig. 3. Comparison of the performance of the Salmonella assay and the 3 assays for somatic mutation in Drosophila. Questionable responses have been ignored.

the test chemical (or a metabolite) with DNA, there are good reasons for not considering them as equivalent. These reasons are discussed in the assay workgroup report and are supported by aspects of the present database (Tables 1 and 3). For example, HMPA was inactive in the 3 SSB assays yet was active in the assays for UDS (2 + and 1?). In contrast, DES was uniformly active in the 3 SSB assays and inactive in the UDS tests. The fact that unanimity between these two assay groups can be achieved is evidenced by the responses seen for TOL (6/6 +), SAF (5/6 +) and PB (4/4 −). The relative sensitivity of these 3 classes of assay are shown in Fig. 4.

The decision to combine these assays in this discussion is partly supported by the conclusions of the workgroup report where the expediency of combining the responses observed in several of these assays is explored. However, the major reason for this decision was that several legislative authorities discuss the need for assay data indicating primary interaction of a test chemical with DNA (see Appendix 3). This requirement could be met with data generated from either of these groups of assay, i.e. the two are generally regarded as being different methods of assaying a similar phenomenon.

Perhaps one of the most surprising findings of this study is the insensitivity demonstrated by the two autoradiographic assays (4.4.1 and 4.4.2, deleted from Table 2). These were conducted according to similar protocols and are well represented in the previous literature. Their poor performance in this study suggests that they are unsuitable for use as a complementary assay, albeit their use for confirming the activity in mammalian cells of previously defined bacterial mutagens remains. Another intriguing aspect of their poor performance is that it provides suggestive evidence for the reduced sensitivity of the autoradiographic endpoint as compared with the others discussed herein (excepting the activity seen for HMPA by one assay, 4.4.1). This insensitivity is unlikely to be due to metabolic incompetence as the hepatocytes employed are primary isolates whose metabolic competence has been previously established. Further, the possibility that this divergence of data is due to these agents producing only

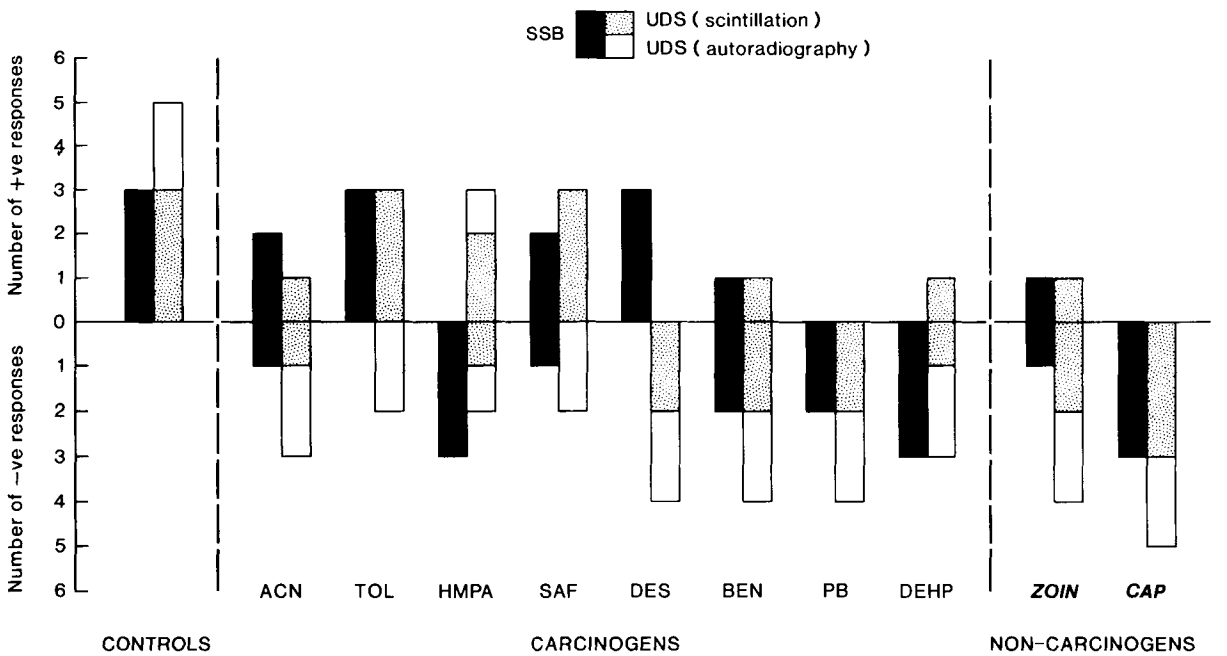

Fig. 4. Comparison of the relative performance of the 3 classes of DNA damage assay. The data are from the total database (Table 1). Questionable responses have been ignored.

short-patch repair is reduced, given the range of chemically different carcinogens employed. Whether some of the positive responses observed in the SSB assays are a direct result of cell necrosis is discussed below.

Assays for SSB and UDS would be expected to be primarily sensitive to the 4 confirmed genotoxins represented here, HMPA, TOL, SAF and ACN, and this was observed (cf. Table 3 and the discussion of the yeast and Drosophila gene-mutation assays in this section). The only confirmed success with the remaining 4 carcinogens was the ability of all of the SSB assays to detect DES. Non-reproducible activity was seen in both classes of assay for BEN (2/6), ZOIN (2/5) and DEHP (1/5). The latter isolated positive response was strongly endorsed by the investigator (4.4.3, see group report) and demands further study.

A topic for future research is the extent to which cell necrosis, with the consequent formation of broken DNA, contributes to the outcome of SSB assays. For example, the isolated positive response observed for benzene (assay 4.3.2) was only observed at higher dose levels than those employed by the other two investigators in this group. This response may therefore represent a technical false positive response induced solely by DNA-strand breakage as the result of toxicity. Equally, the extent to which 'mitotic' DNA synthesis has been successfully blocked during the conduct of a scintillation UDS assay relates directly to the credibility accorded to weak induced effects. The above two concerns may explain some of the divergencies of data evident within these 3 classes of assay for the present chemicals.

Given the simple requirement to provide evidence of the ability of a chemical to induce primary DNA damage in mammalian cells, the investigator is faced with the following decisions:

(a) If the agent is already defined as a bacterial mutagen, the use of any of the 3 classes of assay (SSB or UDS *via* autoradiography or scintillation counting) discussed herein could be supported.

(b) If the agent is inactive as a bacterial mutagen, use of either the SSB or scintillation counted UDS assays could present advantages depending on the chemicals involved. The evidence provided herein for HMPA and DES suggests that the employment of both assays should be considered if an assay for 'DNA damage' is required as a complement to bacterial mutagenicity.

It is therefore concluded that the simple legislative requirement for 'evidence of DNA damage' is too vague to be meaningful. If complementary data are being sought, the autoradiographic endpoint should be avoided and data derived from an SSB assay and/or a scintillation counted UDS assay. The failure of this study to indicate clearly a single class of assay within this group for routine adoption suggests that attention should be given to developing the sensitivity of DNA-damage assays to the point that only a single test is required. The relative performance of these 3 classes of DNA-repair assays is shown in Fig. 4.

2.4/4.9.1. Assays for the induction of aneuploidy. In the past, assays for the induction of aneuploidy have not been generally considered as predictive tests for carcinogenicity. In the first place, few, appropriate in vitro assays existed, and secondly, the induction of aneuploidy was assumed to have greater relative to the promotional, as opposed to the initiatory stages of carcinogenesis. Thus, most of the previous database has been focused on the evaluation of a few reference genotoxins such as MNU, but mainly cancer promoting agents such as the phorbol esters and saccharin, etc. Two investigators have provided the greater part of the present data base for this class of assay by presenting data derived from yeast assays (2.4.1–3). In addition, data derived using mammalian cells were presented (4.9.1) together with a set of data observed in Aspergillus (2.4.4).

The performance of the aneuploidy assays in the present study was impressive (see Tables 1 and 3). In the case of the two sets of yeast data provided from one laboratory (2.4.1 and 2.4.3) a 100% correlation between the reported carcinogenicity of the test agents and their activity in vitro was observed. In particular, the unanimous detection of both DEHP and PB by these aneuploidy assays is unique in this study.

The primary conclusion regarding this group of assays is therefore that they present high promise for development as a class of complementary assays. The developmental phase should be influenced by the following considerations, some of which are of sufficient importance to ensure that they are resolved before any particular aneuploidy

assay is recommended for general adoption.

(a) The findings of this study suggest that the induction of aneuploidy may be a critical step in the mechanism of action of complete carcinogens. These observations require independent confirmation in other laboratories for both the yeast and the mammalian cell systems.

(b) In the case of the D61-M yeast data from the two laboratories considered, major discrepancies were evident. These were all of the same nature and represented the failure of one laboratory to detect anything beyond BEN and DES. It is possible that this divergence was due to technical factors affecting the mitotic or metabolic activity of the yeast. Evidence is presented in the fungal workgroup report supporting the earlier observation that growing cells are more sensitive to genotoxins than are stationary cells, this being probably due to the reduced metabolic activity of stationary cells. Thus, this divergence may be due to a relatively minor technical consideration, but it must be defined, understood, corrected and defended in the literature before any form of general adoption of yeast aneuploidy assays commences. The question of whether the D6 strain can be regarded as repetitive of the D61-M strain requires resolution; if it is to be maintained, its performance in other laboratories requires study.

(c) The isolated set of Aspergillus data suggests that this assay is relatively insensitive. Clearly, if the induction of aneuploidy is to be pursued as a useful complementary endpoint, and if *Aspergillus nidulans* is to be selected as the marker organism, a separate database will have to be generated to justify that choice.

(d) Similar requirements to those in (c) above must apply to the isolated set of aneuploidy data generated in the Chinese hamster-liver fibroblast assay (4.9.1). This assay is relatively novel; thus, apart from an expansion of the database and the commissioning of independent studies, the following points require attention. The cells used in this study were between the 9–15th passage. The corollary to using such a spread of cell passages is that sufficient evidence must be presented on the chromosomal number and distribution in each cell line before test data can be interpreted with confidence. Also, the multiple scoring of hyper-, hypo- and poly-ploid cells presents a complex database whose statistical interpretation may not yet have been optimized. In addition, the possible karyotypic instability of higher passage cells suggests that the current practice of scoring only 200 cells each in test and control cultures may not be adequate.

The aneuploidy findings of this study therefore provide an exciting area of future research. However, the role of such assays as routinely employed complementary tests must await the acquisition of further data and the outcome of developmental studies (see discussion of polyploidy assays later herein).

5.1–4. Mammalian cell gene-mutation assays. Mammalian cell gene-mutation assays, together with chromosomal assays, are high on the list of optional secondary assays referred to by legislative authorities (Appendix 3). The current status of these assays is therefore of immediate interest and capable of being approached using the present data. In several ways this is an interesting assay group. Firstly, 16 sets of data were considered, six of which were based in nominally the same cell line (L5178Y) and employed the same selective agent (resistance to trifluorothymidine).

Qualitative inspection of the total database for the mammalian gene-mutation assays (Table 1), and of that produced following the primary reduction step (Table 2), is not, as pointed out in the workgroup report, initially encouraging. The overall sensitivity of the collected gene-mutation and chromosome aberration assays were similar (Table 3; 62 and 67% and 46 and 53% overall sensitivity, respectively, to the two groups of 4 carcinogens). The value of such numerical indices can be questioned, but they are supported by the individual comparisons drawn in Table 3 for the activity of each chemical in these two classes of assay. Thus, similar overall sensitivity was observed in both classes of assay for most of the chemicals (see Figs. 5 and 6 in subsequent section of this chapter for comparative sensitivity histograms).

The workgroup report on gene-mutation assays listed 9 major factors that could have led to the many disagreements in assay responses and which may lead to the development of improved assays if acted upon. Each of these points is concerned with protocol details, all of which are of relevance to all of these assays, irrespective of the cell line, selec-

tive agent, etc. employed. Some of these recommendations are of immediate relevance to the interpretation of weak assay responses (see subsequent discussion in this chapter of criteria for defining positive responses; Section 14). The common feature of the present test chemicals is that they tend to yield weak responses in vitro, therefore, it is impossible to discern at this stage which of the many weak positive responses recorded in this assay group are real and which are illusions created by inadequate experimentation (see points 1, 2, 6, 8 and 9 in assay group report). Equally, some of the negative responses observed may be attributable to experimental deficiencies rather than to an absolute inadequacy of the assay to detect the carcinogen.

The workgroup recommendations might be expected to lead to a general improvement in the sensitivity and reproducibility of all assays in this class. Further, such protocol modifications, especially those associated with the repetition of experiments and the use of appropriate cell numbers, should reduce the incidence of 'technical' false positive and negative responses. The database that would have been produced in this study had such technical modifications already been incorporated would doubtless have been much more self-consistent. But even that step forward, which can optimistically be anticipated, would leave two remaining questions.

The first is the extent to which this class of assay can realistically be reduced to a common cell line employing one or more common selective agents. This would obviously represent a major advance as it would enable meaningful inter-laboratory comparisons of data to be made and engender a feeling of confidence that once a test chemical had been evaluated for mammalian cell gene-mutagenicity, and a conclusion drawn, that this would not be reversed upon retesting in another, but similar assay. The need to attempt to devise such a system that could be recommended for general screening was, in fact, the final recommendation of the assay workgroup. Some of the divergences in test results evident in this study were probably due to the differential sensitivity and metabolic competence of the several cell lines employed, and this potential advantage may be reduced by adoption of a common cell line. However, the range of genetic loci monitored and the different selective agents employed in this study probably accounted for most instances of differential sensitivity. The need to employ at least two different genetic markers if a wide range of genotoxic agents are to be detected has been discussed elsewhere and instances of two loci being monitored concomitantly have been reported [17]. Appropriate experiments are required to discover the degree to which this class of assay can be standardized with a retention of overall sensitivity.

The second concern relates to whether these assays are suitable, by definition, to act as complements to the Salmonella assay. It was suggested earlier in this Chapter that the use of gene-mutation assays in yeast or Drosophila were not optimal for this purpose, owing to the similar nature of the genetic event being monitored. Similar concerns apply to mammalian cell gene-mutation assays. Thus, the concept that some agents may be specifically clastogenic would argue against the incorporation of only gene-mutation assays in a battery. Given that there is usually a requirement in legislative guidelines to assess a compound for clastogenic activity, the precise role of mammalian cell gene-mutation assays is worthy of assessment.

The above discussion has been concerned solely with the use of gene-mutation assays as routine screening tests. The problems they may face in this role do not necessarily detract from their use as confirmatory assays. Thus, if it is considered necessary to evaluate whether a bacterial gene-mutagen is similarly active in mammalian cells, their employment is obvious. The general protocol advice recommended in the assay group report would, of course, be equally applicable in the latter role.

The future use of mammalian gene mutation assays is therefore likely to be influenced by a variety of considerations. The first and most fundamental is the need for a precise exposition of their proposed role in the detection of potential carcinogens in vitro. If they are to be employed only to confirm activity observed in the Salmonella assay, then some justification for the implied existence of a group of established non-carcinogens that are mutagenic to Salmonella, yet inactive in a well conducted mammalian cell gene mutation assay should be provided (see Introduction to this Chapter). If they are to be employed as a safety

net to the Salmonella assay, i.e. act as a complementary assay, urgent attention should be given to protocol development along the lines outlined in the assay workgroup report. The selection of a single assay, or the development of a single multilocus assay, may form part of this development process. The wisdom of employing two gene-mutation assays for screening purposes should be considered carefully.

Set against these rather demanding requirements are the following two positive observations. First, it is significant that mammalian cell gene-mutation assays formed the largest group of closely related assays considered in this study. This is indicative of the current widespread use of these tests and implies that many laboratories have them performing to an acceptable standard for reference mutagenic carcinogens such as 2AAF, MNU, etc. The large number of laboratories performing these tests is of course, due mainly to the legislative stimulus, but had they proven generally insensitive or unreliable we would not have been able to collect so many together for this study. This is in contrast to most of the assays considered previously herein and provides a strong stimulus for further work to refine them to the point that they can be used with confidence for the detection of weak mutagenic responses. Second, although this study was concerned primarily with the detection in vitro of potential mammalian carcinogens, the possible in vivo somatic and germ cell mutagenic properties of chemicals are increasingly considered. If the specific requirement to assay for possible mammalian in vivo mutagens is a consideration, the role of in vitro gene mutation assays assumes a different complexion.

4.5. Chromosomal aberration assays. As discussed above, the performance of this class of assay, was similar to, but slightly better than that of the mammalian cell gene-mutation assays (see above and Table 3). As with the gene-mutation assays, chromosome-aberration tests are generally referred to in almost all legislative guidelines as suitable for use in conjunction with a bacterial mutation test when screening chemicals for new potential carcinogens. However, while it is currently normal practice to select the L5178Y cell line when conducting a gene mutation experiment, a range of possible cell lines could be chosen for cytogenetic studies. Thus, in this study, 4 distinct cell lines [CHO cells, human lymphocytes, Chinese hamster-liver fibroblasts (CHI-L) and Chinese hamster lung cells (CHL)] were selected, each of which had been used previously for such studies, and each of which could be considered suitable for use when submitting genotoxicity test data on a new chemical.

A distinct attribute of the cytogenetics assay data was that although a range of cytogenetic disturbances can be classified as chemically induced damage, the database was more ordered than might have been anticipated and this was due to the common reporting format employed by most investigators. This was achieved in advance by the assay group Chairman and was based substantially on the guidelines for assay data reporting suggested in the UKEMS criteria document [29]. Further sources of protocol variation can therefore be associated directly with technical decisions made by individual investigators, issues such as dose-levels, sampling times and statistical analysis methods employed, cell line adopted, etc.

As would be expected from the similar overall detection rates of the gene-mutation and chromosomal aberration assays (above, Table 3 and Figs. 5 and 6, later) simple inspection of the chromosomal aberration database (Tables 2 and 3) indicates a disappointing scatter of positive and negative observations. Certain consistencies were, however, evident. For example, general agreement was observed in the 3 cell lines studied for the clastogenicity of both ACN (4/4 +) and PB (3 + and 1? of 4). With the exception of a questionable response in one of the 3 CHO assays (4.5.3), DEHP gave negative results. Benzoin (ZOIN) gave 3 negative responses and a single but confirmed positive response in the CHL assay. A partially consistent data set was observed for HMPA which gave a negative response in each of the 2 CHO assays while being active in the 3 other cytogenetic assays. This may reflect a general technical inadequacy of these assay protocols or represent a general failure of this cell line to metabolize HMPA to a genotoxic species. One of the 2 CHO SCE assays gave a positive response for this agent, and this weakens but does not destroy the metabolic deficiency rationale.

Marked divergences in assay responses were

evident for the remaining five chemicals (TOL, SAF, BEN, CAP and DES). An approximately equal incidence of positive and negative responses was observed for these agents and each was regarded as clastogenic in the assay work group 'consensus' results (TOL 3/5 +, SAF 2/4 +, BEN 3/6 +, CAP 2/6 + and DES 3/4 +). The extent to which such divergences can be rationalized in terms of identified protocol deficiencies of individual assays is discussed in the assay group report. These deficiencies were generally associated with the selection of inappropriate sampling times or dose-levels, and if they prove to be the critical contributors to 'false' negative assay responses, then the performance of these assays would be dramatically improved.

For the purpose of defining a complementary assay within the present context, the 4 classes of chromosomal-aberration assays considered herein cannot be considered as equivalent. For example, the Chinese hamster lung (CHL) assay has been extensively studied and reported on in the literature, but its use is centered mainly in a single laboratory. Assays involving human lymphocytes, especially if a rat-liver S9 mix is incorporated into the protocol, are few [30]. The present data using Chinese hamster-liver fibroblasts form the majority of the available database for this assay [assay data for the related rat-liver RL4 cell line (4.5.7) were omitted from the present discussion due to the observation of 9 negative responses]. In contrast, the CHO assay probably represents the one most widely used in industrial and academic laboratories. This assay can therefore be regarded as particularly pertinent to the present discussion as probably more data are generated using it than in any other when compounds are assessed prior to legal registration. Given that some of the negative responses observed in CHO cells may be corrected by appropriate modifications to the assay protocol, a single message remains, namely, that at present different responses may be observed in different laboratories using nominally the same assay. This is similar to the situation currently evident with the L5178Y gene mutation assays, and as in that case, urgent attention to protocol design and data interpretation is indicated.

Within this study the CHL assay (4.5.6), or perhaps the protocol by which it is conducted, was more sensitive than the others in this group. The partial database evident for the human lymphocyte assay was also encouraging by the same criteria and worthy of elaboration. The question confronting the authors on this and many other occasions in this overview, is the extent to which 'assays' may have been judged, or misjudged, by the test protocols adopted. This important problem demands resolution because of the real possibility that if more detailed or refined protocols were to be used, many assays would have appeared in a more favourable light.

An advantage of the chromosomal assays is the extent of the previous data available and the experience that exists with these tests. This should provide a stable starting place from which protocol modification can be developed and rapidly, and generally, adopted. A second advantage is that a range of supplementary and probably 'complementary' end-points can be easily assessed once the decision has been taken to conduct cytogenetic studies within a laboratory. These include the recording of polyploid cells, an assessment of aneuploidy, and the studying of sister-chromatid exchanges (SCE). Aneuploidy induction in mammalian cells is considered as a separate assay, and the induction of SCEs is discussed below. However, the induction of polyploidy is pertinent to the present discussion as it can be assessed either concomitant with, or in parallel to, chromosomal aberration assays, depending on the sampling time of the assay.

The chemical induction of polyploidy was discounted as a separate assay earlier in this discussion, but the data generated have not been neglected. Three complete sets of polyploidy data were presented [see Table 1; assays 4.8.1 (CHL), 4.8.2 (CH liver) and 4.8.3 (RL4, rat liver)] and each detected DES as positive. This activity is probably associated with the ability of DES to induce aneuploidy, spindle effects and chromosome aberrations (see elsewhere). The latter 2 assays (4.8.2 and 4.8.3) found the remaining 9 compounds negative while the CHL assay (4.8.1) also detected TOL and CAP as active. These responses indicate that this genetic endpoint is not prone to false positive responses, in fact, activity seems to be rare.

The mode of action of DES, BEN and DEHP as carcinogens is less clearly defined than with most other agents. Not only are they inactive in the Salmonella assay, but they were difficult to detect as positive in many of the eukaryotic assays employed in this study. Further, these agents do not appear as likely to interact covalently with DNA based on a consideration of their chemical structure and known or anticipated metabolism. It was therefore perceived by many investigators as particularly interesting that each of these agents was capable of inducing both aneuploidy and cell transformation in vitro. Further, if 'associated' chromosomal assay data are considered in conjunction with the aneuploidy findings a potentially useful pattern emerges, as shown in Table 4.

Reference to Table 4 indicates that diethylstilboestrol (DES) is clearly active in each of the 3 categories of assay and its mode of action in each may be closely related to its ability to damage the microtubules of the metaphase spindle. In contrast, DEHP was inactive in the 3 polyploidy assays as well as in the several chromosomal assays. The question is therefore posed of whether the cell transforming and aneuploidy inducing properties of DEHP are mechanistically unrelated to those produced by DES. If totally different mechanisms of action are involved then the relationship of these findings to the carcinogenicity of these agents may be equally different. In this connection, the difference between modification of spindle integrity and spindle function may be important (see Investigator report, assay 4.9.2).

During the preparation of this overview a paper appeared concerning the relationship between these several endpoints [31]. The data presented related to the ability of colchicine to induce aneuploidy and cell transformation in the absence of gene mutations or chromosomal aberrations. Colchicine is a compound of unknown carcinogenicity.

It is thus concluded that if an assay for chromosomal aberrations is to be used as a complementary test, then the determination of changes in ploidy may be a useful additional parameter to measure. In some assays it may not be possible to score both chromosomal aberrations and ploidy concurrently, due mainly to the need to progress to the second mitosis after treatment for polyploidy to become evident. However, the maintenance of a parallel culture after treatment for separate assessment of ploidy should present minimal difficulties.

4.6. Sister-chromatid exchange assays. The relationship between the chemical induction of SCEs and the production of chromosomal aberrations is not clear, and the mechanism of this phenomenon is obscure. The role of SCE assays in the detection of carcinogens has yet to be defined. These assays have been considered separate from the aberration assays in the present discussion for the above reasons and because their respective test protocols are quite distinct.

One set of data was omitted from Table 2 due to the recording of 6 of 6 negative responses (4.6.4) and in the remaining 5 SCE assays only 12 positive responses were observed out of 41 determinations. These positive responses were not randomly distributed among the compounds as evidenced by Table 3 and Fig. 7 (later). Two-thirds of the SCE assays made on HMPA, TOL, SAF and ACN gave positive responses, while only a single positive response was observed among the 14 determinations made on BEN, DEHP, DES or PB. This grouping of positive responses is very similar to that seen with the Drosophila mutation assays and to a lesser extent by the yeast gene mutation and UDS (scintillation) assays. This supports earlier suggestions that the induction of SCEs is more closely related to gene-mutagenicity than to clastogenicity. No activity was observed for the two non-carcinogens.

Given that the greatest number of positive responses were observed in this study for the first four 'genotoxic' carcinogens shown in Table 3, the SCE findings are unexceptional. Their joint inability to detect the remaining 4 carcinogens may

TABLE 4

COMPARATIVE PERFORMANCE OF DES, BEN AND DEHP IN THE CLASSES OF ASSAY SHOWN

Results are based on the concensus conclusions given by the workgroups. The spindle studies refer to assay 4.9.2.

	Cell trans	Aneu-ploidy	Clasto-genicity	Poly-ploidy	Spindle studies
DES	+	+	+	+	spindle damage
BEN	+	+	+	−	modified spindle function
DEHP	+	+	−	−	

provide clues (along with the Drosophila assays) regarding the mechanism of carcinogenic action of these agents. An observation of particular interest is that 3 carcinogens, BEN, DES and PB (but not DEHP) were each active in one or more of the other chromosome based assays — their inactivity in the SCE assays therefore becomes pointed.

The SCE assays therefore appear to offer limited advantage as a complementary assay. Along with several other assays they successfully detected the 'agreed' genotoxins in this study, and as such the assay seems to be more closely related to the Drosophila and yeast gene-mutation assays. The low detection rates evident for the four remaining carcinogens suggest that SCE assays are less generally useful as a complementary test than are the chromosomal aberration and mammalian cell gene-mutation assays (see Fig. 7, later).

4.7. Transformation assays. For the reasons displayed earlier, this class of assay was not considered in this Section as a source of a generally applicable complementary assay. However, their good overall performance in this study (see base of Table 3), coupled to the extent to which they have been discussed previously within the present context, demands that they be also discussed here.

The chemical induction in vitro of the transformed cell phenotype is appealing because the derived cell morphology and behaviour in vivo and in vitro bear a striking resemblance to the malignant phenotype. The many different definitions of the transformed cell phenotype [32], coupled to the technical difficulties that accompany the conduct of such assays have proven to be major factors contributing to the slow progress that has been made in the general utilization of these tests for the detection of possible new carcinogens. The initial promise shown by the BHK-21 cell transformation assay was due to the compatibility of these cells with S9 mix [33], but progress was impeded by the karyotypic instability of this cell line and inter-laboratory reproducibility problems encountered at an early stage [6]. The present study represents the first occasion when the mouse C3H $10T\frac{1}{2}$ assay and the Syrian hamster embryo (SHE) assay have been exposed to general study, yet each has an impressive history in a few laboratories. Although both of these classes of assay performed well in the present study, there was a marked reluctance among the assay investigators when it came to suggesting any of these tests as suitable for routine adoption as a complementary assay. The insensitivity to the present carcinogens of the Balb C assay [4.2.1] and the CHO assay [4.2.8] confirmed previous observations that established cell lines tend to become metabolically incompetent, yet the metabolically competent primary cell systems suffer from other problems associated with the criteria used to select clones of cells for study, and the selection of appropriate media.

Recent studies associated with the probable role of oncogene activation in the aetiology of cancer have invoked the transformed cell phenotype as a critical marker, thus the future of this class of assay in cancer research seems assured. Nonetheless, the practical problems that would probably be encountered by a new laboratory endeavouring to establish such an assay are probably still sufficient to warn against their use as a routinely employed complementary test.

Perhaps it is significant that the solution to the apparently insoluble phenomenon of chemically-induced carcinogenesis is recurringly presented by a class of assay that is generally deemed to be unsuitable for routine adoption as a screening test — 'No light, but rather darkness visible' (Milton, Paradise Lost).

Relative literature citation frequencies for different classes of in vitro assays

The degree to which individual assays are numerically represented in this study is not necessarily representative of their relative usage in general. Elsewhere in this discussion, and in the conclusion section, it is suggested that if one or more eukaryotic assays are to be refined for general adoption as complementary assays, then it would be prudent to concentrate these efforts on the development of assays that performed well in this study and are currently most used. In order to obtain a balanced perception of the relative usage of assays a search of Biological Abstracts was made for the period 1972–date, as shown in Table 5. The Salmonella sample should be representative of the complete literature on this assay given that it only came into general use about 1975. The comparative usage of mammalian cell gene-mu-

tation and chromosomal-aberration assays is of particular relevance to the conclusions outlined later. Inclusion of SCE data in the chromosomal assay count markedly affects the figures, but these have been parenthesized as it is the chromosomal-aberration assays that are of particular current interest. The hepatocyte UDS figures were included for comparative purposes, and as expected, papers describing this class of assay are not well represented in *Mutation Research*. The *Mutation Research* figures for the first 3 classes of assay are, however, probably the most representative of the relative usage of these tests.

Similar figures were not calculated for all of the assays because low figures could be very misleading of actual usage patterns. We chose the hepatocyte UDS test as a 'low' reference because the key words employed were unlikely to be misinterpreted during the search.

Selection of a preferred complementary assay

No single assay was defined by this study as giving high carcinogen sensitivity and specificity together with an established ability to perform optimally in several laboratories. We consider, however, that there is compelling evidence both from within and beyond this study, for the adoption of a chromosomal-aberration assay as the reference complementary test. This conclusion is based on the following data and reasoning:

(a) The foregoing discussion assessed the performance as complementary assays of 8 classes of tests, as listed in Table 3. Of these, the gene mutation assays in yeast (Fig. 2) and Drosophila (Fig. 3) were not favoured due to their repetition of genetic endpoint and their poor performance with the last 4 carcinogens (BEN, DES, PB and DEHP). Assays for aneuploidy were not recommended at this time due to their novelty which

TABLE 5

RESULTS OF INTERROGATING *Biological Abstracts*, USING BIOSIS ONLINE, FOR THE KEY-WORD COMBINATIONS SHOWN

The period covered in each case was 1972–date and therefore includes all Salmonella mutation data of relevance to this study. Additional reference to *Mutation Research* in isolation is made because the 'all paper' entry may include clinical data on chromosomal aberrations. The chromosomal aberration entry was expanded to include SCE papers (parentheses). The hepatocyte UDS figures are for comparison and were not expected to be selected by the *Mutation Research* sub-search. We are indebted to Mr. S. Kettle of Imperial Chemical Industries PLC for these data. ★ Ratio of citations taking that for mammalian cell gene-mutation assays as 1.

Interrogation key words (1972–date)	All papers abstracted by *Biological Abstracts*	Ratio ★	*Mutation Research* papers abstracted by *Biological Abstracts*	Ratio ★
Ames Salmonella + Mutagen- Mutat-	3971	17	869	11
Mammalian cell line Mouse lymphoma L5178Y + Mutagen- Mutat-	228	1	80	1
Chromosomal aberrat Chromosome aberrat Clastogen- + Mutagen- Mutat-	1282 (+SCE, 3222)	6 (14)	290 (+SCE, 674)	4 (8)
Hepatocyte + UDS unscheduled DNA	53		4	

necessarily prevented independent studies from being commissioned. The DNA repair assays showed poor sensitivity for the last four carcinogens, and occasionally dramatic differences in sensitivity between the single-strand breakage, and scintillation UDS tests were evident (Fig. 4). The database for the SCE assays suggested marked insensitivity to the last 4 carcinogens (Fig. 7). Several of the above assays were therefore defined as capable of acting as a *partial* complement to the Salmonella assay, but none can be recommended at this time for routine use as a generally sensitive complementary test.

(b) The transformation workgroup recommended that assays for cell transformation were not suitable for routine adoption at this time due to the technical difficulty of their conduct and the remaining uncertainty regarding the definition of the transformed phenotype. Nonetheless, 6 of these assays showed high sensitivity to the present 8 carcinogens, in fact, as a class they performed better than any other category of test (see lower part of Table 3). It is clear that both the C3H $10T\frac{1}{2}$ and the SHE assay are sensitive assays, and each performed similarly in this study in 2 different laboratories. Nonetheless, the advice of the assay group is endorsed here, namely, that transformation assays are not to be recommended at this time for routine adoption, especially by laboratories new to such techniques.

(c) Two classes of assay remain for consideration, each of which was well represented in this study; mammalian cell gene-mutation and chromosomal-aberration assays. Both of these classes of assay showed a similar level of carcinogen sensitivity when considering the reduced database (Tables 2 and 3). The apparent similar performance of these two classes of assay as complementary tests is shown in Figs. 5 and 6. The performance of the SCE and Drosophila assays is shown in Fig. 7 for purposes of comparison and to illustrate that some assays only *partially* complement the Salmonella assay. When considering the *total* database (Table 1) for the gene-mutation and chromosomal assays, the latter appear to be more sensitive yielding 22 positive responses out of 43 determinations made

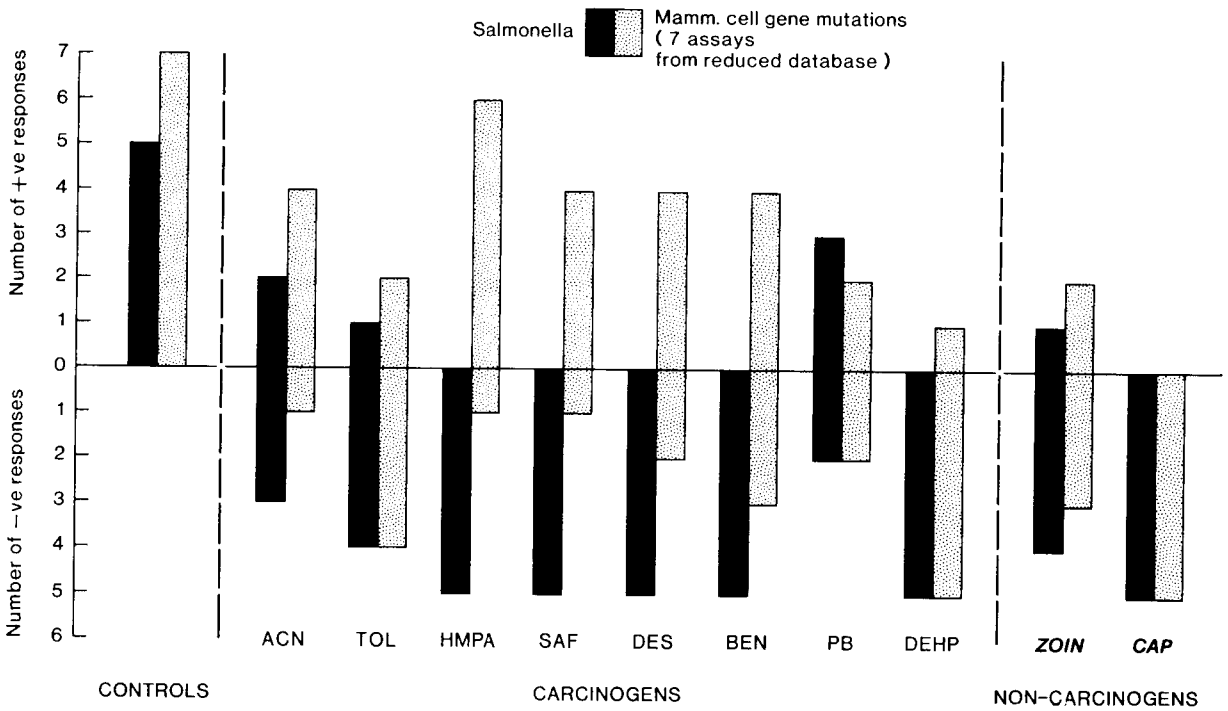

Fig. 5. Comparison of the performance of the Salmonella assay and the 7 mammalian cell gene-mutation assays listed in the reduced database (Table 2). Questionable responses have been ignored.

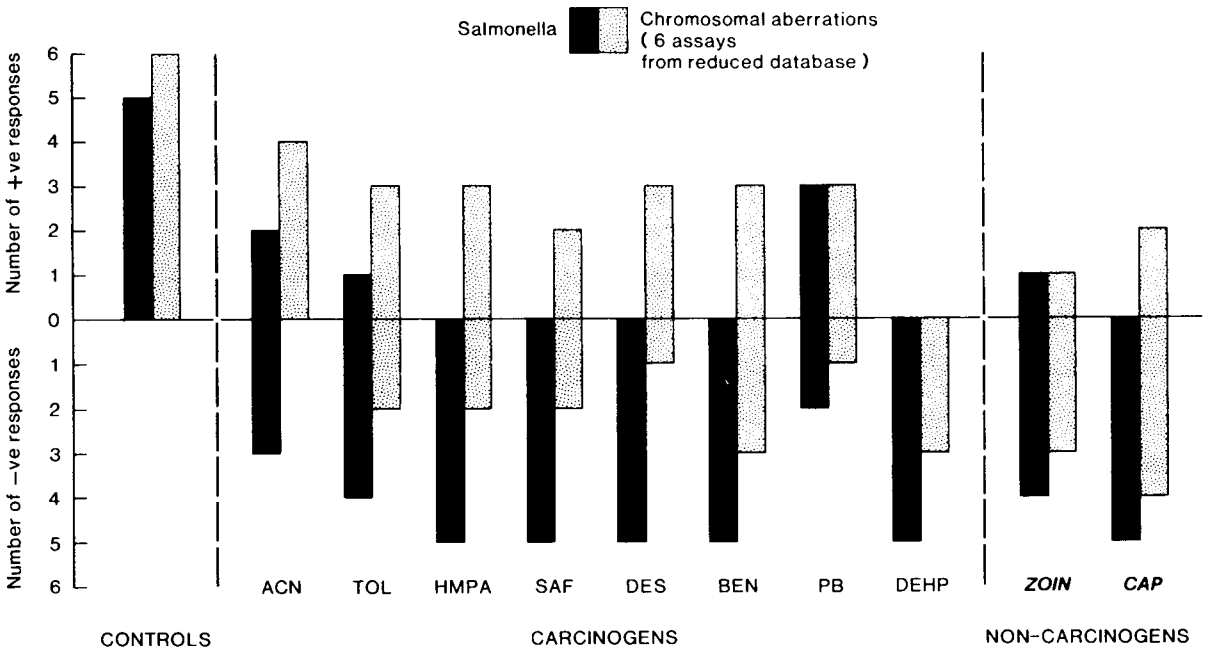

Fig. 6. Comparison of the performance of the Salmonella assay and the 6 chromosomal-aberration assays listed in the reduced database (Table 3). Questionable responses have been ignored.

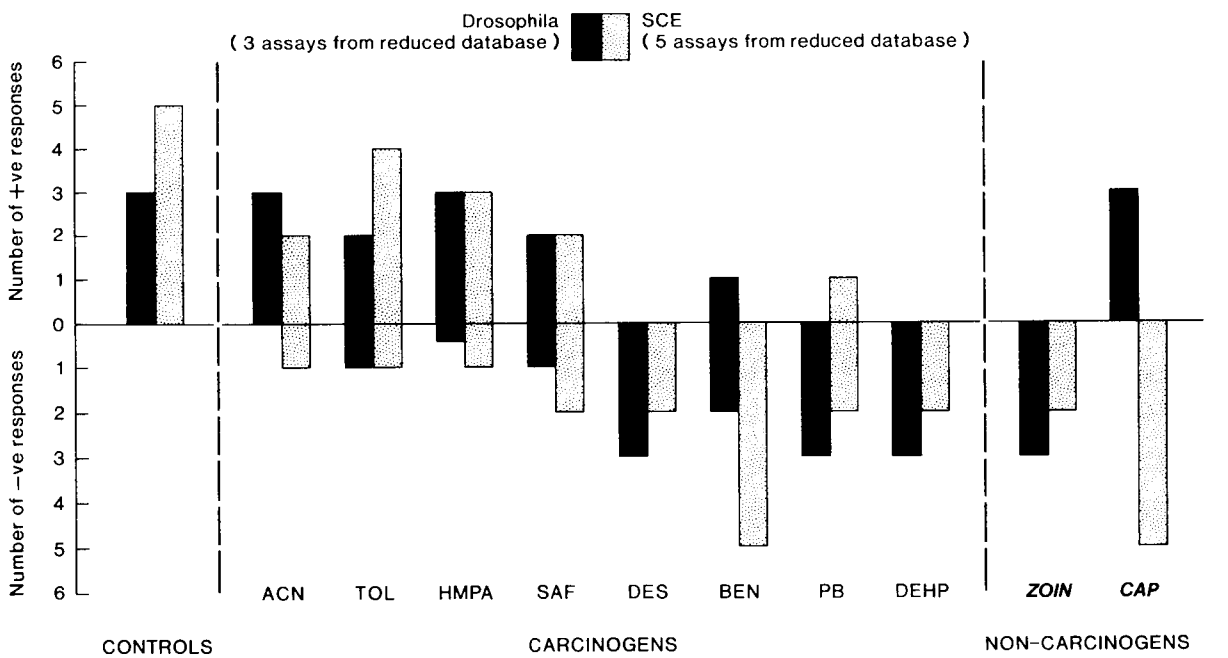

Fig. 7. Comparison of the relative performance of the 3 Drosophila and 5 SCE assays as listed in the reduced database (Table 2). Questionable responses have been ignored.

on the 8 carcinogens (51%) as opposed to only 40 of 103 for the mutation assays (39%). The low sensitivity of some of the mutation assays led to 6 of the 16 being eliminated in Table 3 as opposed to only 1 of the 7 cytogenetic assays. Fig. 8 compares the normalized (%) performance of these 2 classes of assay as complementary tests. Similar efficiencies are evident with the chromosomal assays proving marginally more sensitive to the carcinogens.

Figs. 5, 6 and 8 enable the problem of reproducibility of response to be visualized and it is clear that harmonization and/or extension of assay protocols is indicated for both classes of test in order that each should speak with a common voice. The effort required to produce a common and sensitive assay protocol, coupled to a realistic assessment of whether such efforts are likely to be rewarded constitutes the final factor in our recommendation of the chromosomal aberration assays. Four points appear to be particularly relevant:

(i) Chromosomal aberration assays offer an independent genetic endpoint from the Salmonella assay.

(ii) Chromosomal assays have a more extensive history than do the mammalian cell gene-mutation assays. This means that more laboratories have employed them for a longer period, thus, any protocol corrections required should be more clearly perceived and easier to institute. The frequency of literature citations for these 2 classes of assay support this contention as discussed earlier (Table 5).

(iii) The recommendations for assay protocol modification made by the chromosomal aberration workgroup were more clearly defined, less fundamental and seemingly easier to institute than were those made by the gene-mutation workgroup.

(iv) Once the decision is made to conduct a chromosomal aberration assay in a laboratory, the expertise and facilities required will serve equally for the assessment of aneuploidy, polyploidy and SCEs.

It is therefore recommended that major effort

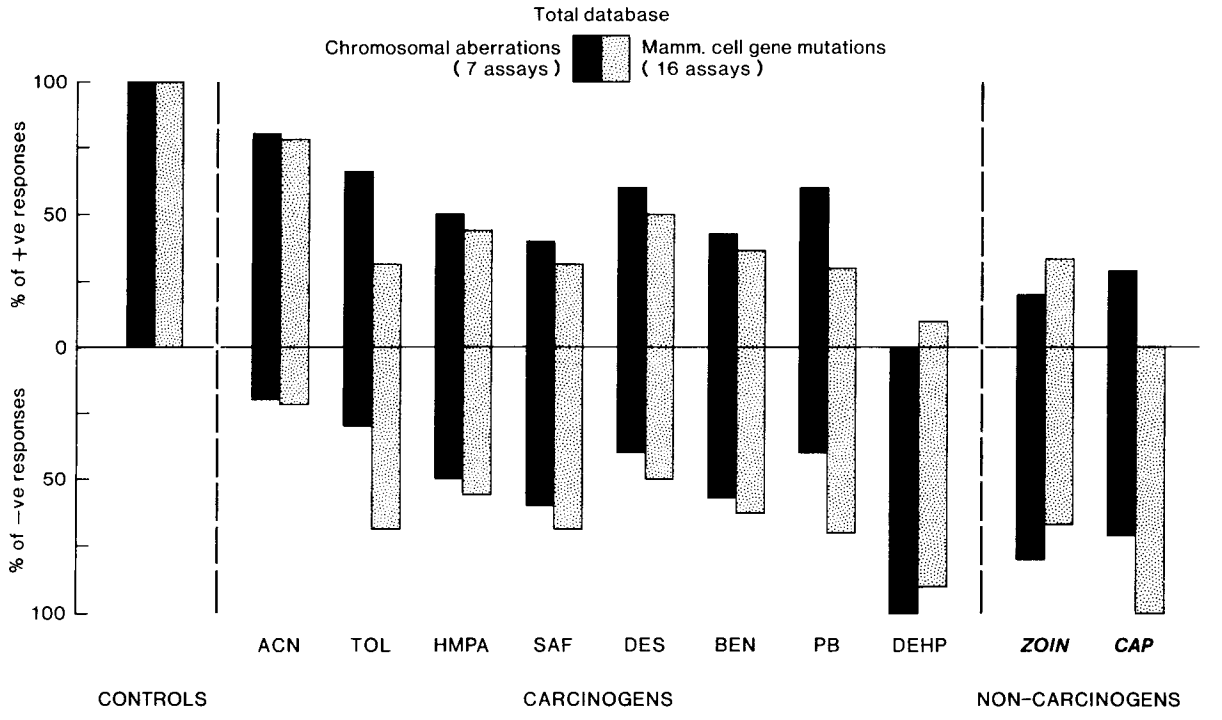

Fig. 8. Relative performance of the 16 mammalian cell gene-mutation assays and the 7 chromosomal-aberration assays, as listed in the total database (Table 1). Responses have been normalized (%) because of the different group sizes, and questionable responses have been ignored.

be focussed on designing a minimum effective protocol by which chromosomal-aberration assays should be conducted, and that this could constitute the assay with which to complement the Salmonella test. Recourse to other assays may be indicated in order to assess fully the in vitro activity of certain test chemicals. Acquisition of test data from a minimum of these 2 standard assays, if optimally performed, will enable a greater degree of comparability of data between both independent laboratories and different countries, and this can only help the efficient detection of possible new human carcinogens.

In many testing schemes, resources are spread across a large number of in vitro assays, and this may lead to assays being conducted sub-optimally on a new chemical. The alternative suggested here is that with two well-conducted and common assays there could be an enhanced comparability and reliability of data, together with an increase in carcinogen sensitivity. An addition to this advice is that the Salmonella assay should be conducted according to what is now regarded as the best current practice, i.e., employing strains TA1535, 1537, 1538, 98 and 100 in the presence and absence of an induced rat-liver S9 mix (10% [5,29]) and also using a preincubation protocol with strains TA98 and 100 in the case of initial negative responses. Use of a different source of S9 mix (e.g. hamster) and additional levels of rat-liver S9 mix (e.g. 4% and 30%) should also be considered [34]. In the case of volatile chemicals, an enclosed test system should be employed.

The particular cell line employed for cytogenetic assays may be critical but should not necessarily be restricted to the widely used Chinese hamster ovary (CHO) line. The Chinese hamster lung (CHL) line performed well in this study but is not currently commercially available, and while human lymphocytes present a sensitive and readily available source of primary euploid cells, further studies will be required to demonstrate the general utility of this system [30]. The Chinese hamster-liver system protocol was designed to measure both aberrations and aneuploidy and likewise will require further independent studies to confirm the promise shown in this study.

10. Biological activity profiles of the test chemicals

Consistent with the overview of the previous international study [6] the biological activity profiles of the test chemicals are presented as histograms in Figs. 9–18. The visual impact provided by exposition of the total database on each chemical may trigger useful speculations by the reader, but these have not been pursued here. This is because the purpose of this study was not to

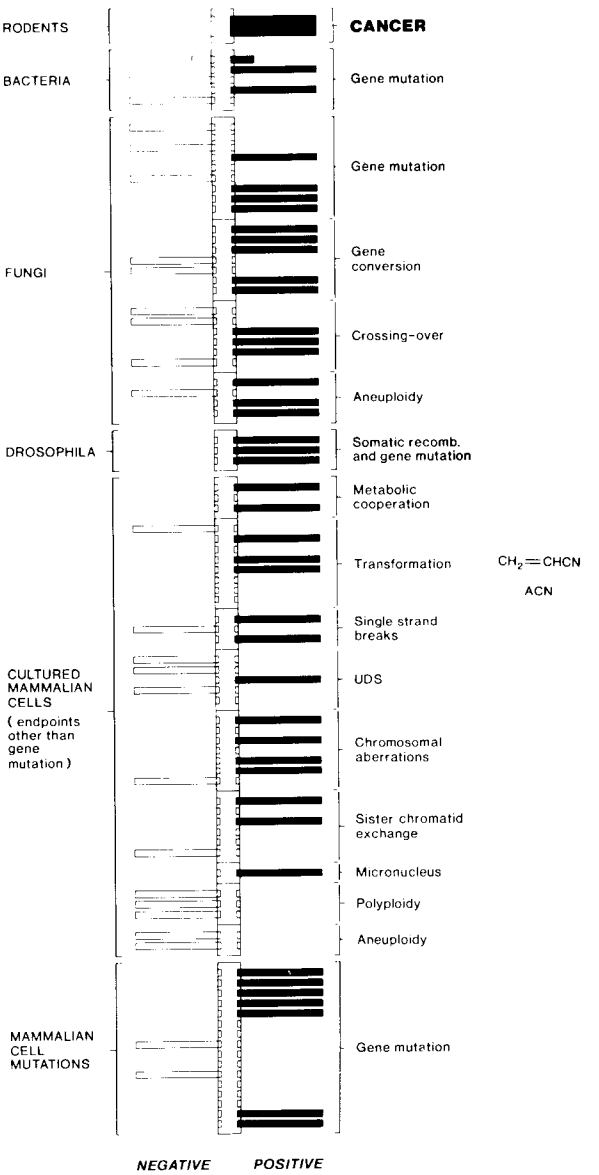

Fig. 9. Genotoxic activity histogram for ACN.

discuss the possible inter-relationship of different genetic end-points, nor to analyse the effect these may have on current theories regarding the mode of carcinogenic action of these agents. The few points made below are enlarged at various places elsewhere in this overview.

Activity profiles of HMPA, TOL, SAF and ACN. One of the criteria adopted earlier herein to enable an assessment of the present database to proceed was that because these four chemicals displayed a broad spectrum of genotoxic activities in eukaryotic cells they could usefully be considered as a group of 'classical' genotoxins. The four activity profiles (Figs. 9–12) are generally supportive of this conclusion. In the 3 cases where comparisons are possible (HMPA, TOL and SAF) similar profiles were observed in the previous study [6].

Activity profile of BEN. This chemical was not included in the group of 4 genotoxins discussed above because until the results of this study became known it was generally regarded as being clastogenic in vivo but non-mutagenic in vitro.

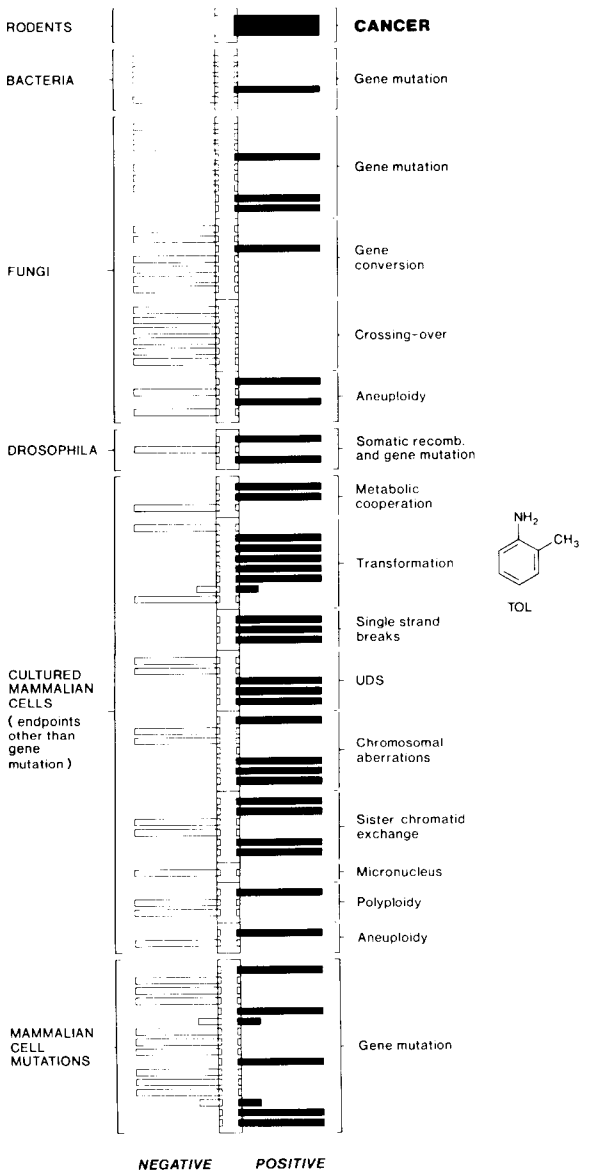

Fig. 10. Genotoxic activity histogram for TOL.

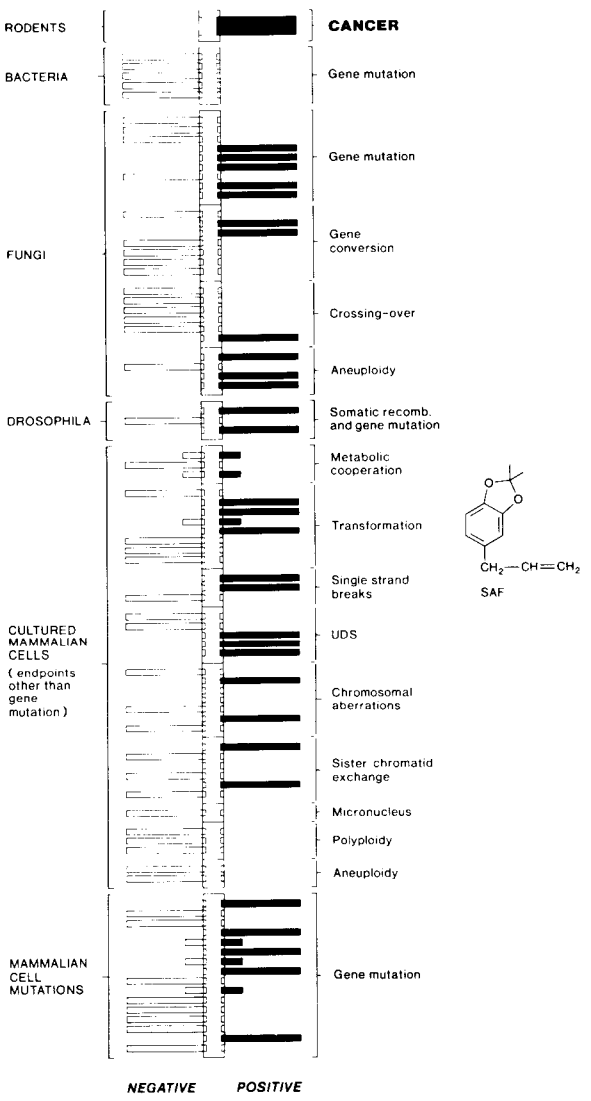

Fig. 11. Genotoxic activity histogram for SAF.

The present data (Fig. 13) suggest that this agent is equally placed between the genotoxic group and the remaining 3 carcinogens (DEHP, DES and PB). Benzene is clearly genotoxic, but its activities lie in certain constrained areas, further study of which may help to elucidate its mode of action as a human leukaemogen.

Activity profile of DES. Previous theories that the mode of action of this agent as a carcinogen is associated with its aneuploidy-inducing activities have been strengthened [35]. Its parallel ability to induce chromosomal aberrations, polyploidy and cell transformation may be consistent with this idea, but its apparent ability to induce primary damage in DNA is not (Fig. 14). These activities were unexpected and remain to be explained. The hormonal activities of this compound may also contribute to its carcinogenicity, but this could not be pursued in this study (cf. comments in previous study [6]).

Activity profile of PB. The range of activities observed for phenobarbital in this study was unex-

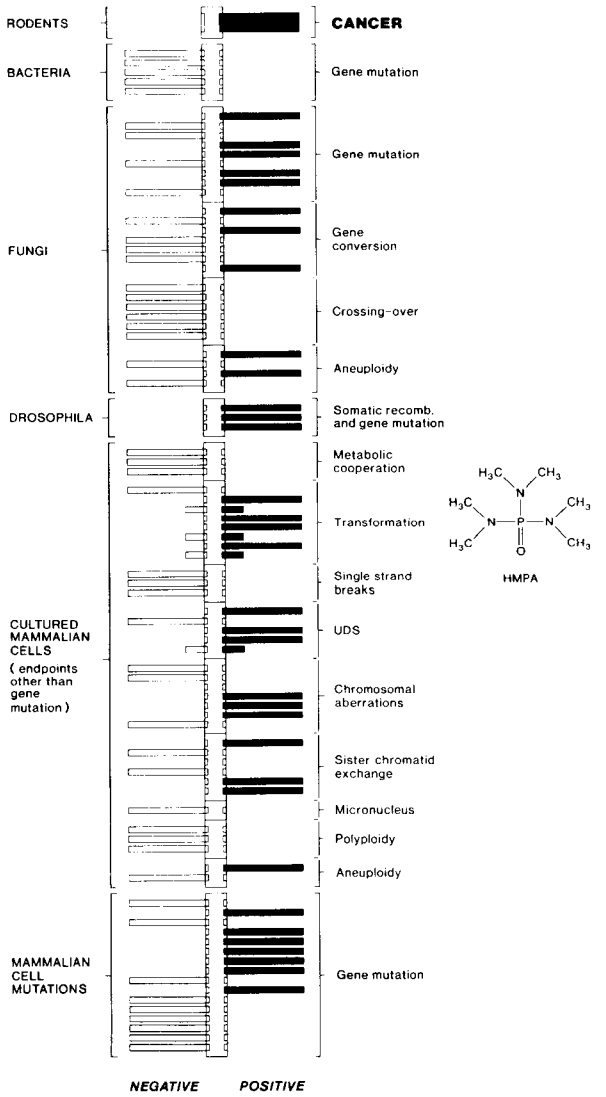

Fig. 12. Genotoxic activity histogram for HMPA.

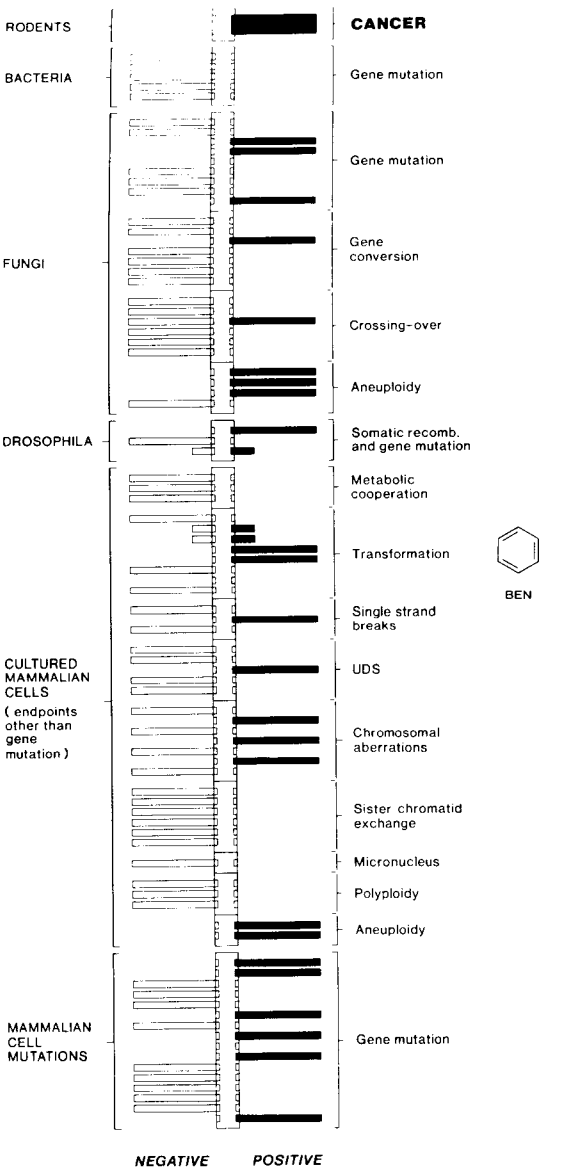

Fig. 13. Genotoxic activity histogram for BEN.

pected (Fig. 15), thus a need exists to confirm their reality. If PB is confirmed as a gene-mutagen and clastogen, then a re-appraisal of its several and complex biological activities in vivo would be indicated. It may be pertinent that PB is reported to induce dominant lethal mutations in mice [36] and that Cifone et al. recently reported the ability of certain organic acids (which PB is) to give positive results in assays for gene mutations and chromosomal aberrations in vitro simply by virtue of their ability to increase the acidity of the assay medium [37].

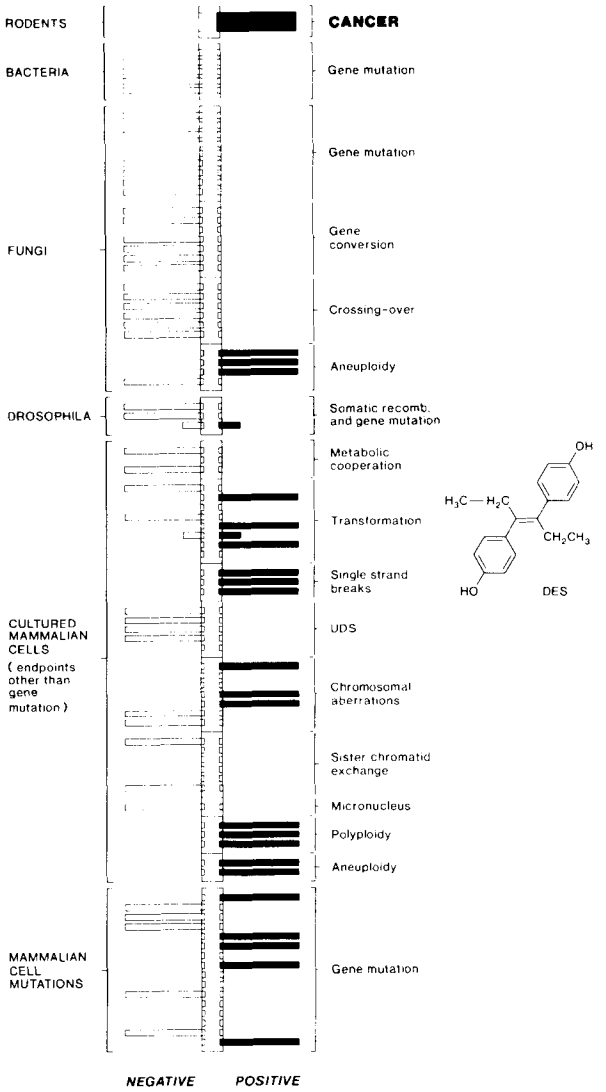

Fig. 14. Genotoxic activity histogram for DES.

Activity profile of the non-carcinogens ZOIN and CAP. The activities observed in vitro for these agents have been considered separately (below) as they present problems beyond their individual biological activity profile (see Figs. 17 and 19, later).

Activity profile for DEHP. This carcinogen has been selected for more detailed discussion because its overall level of genotoxic activity is similar to that of the two non-carcinogens. This is evident in Fig. 1 and in Table 3.

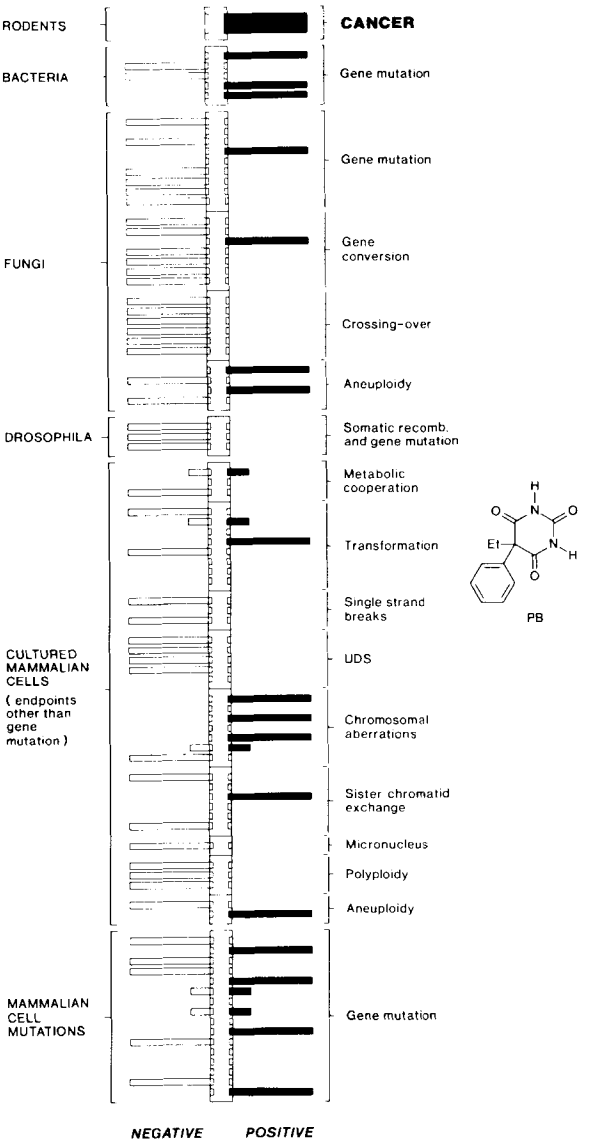

Fig. 15. Genotoxic activity histogram for PB.

11. Activity in vitro of diethylhexylphthalate (DEHP)

As outlined earlier in this Chapter, DEHP was studied primarily to investigate the hypothesis that it was not genotoxic per se, but rather, that the liver tumours induced by it in rodents were produced via a secondary biological response; the hepatic proliferation of peroxisome microbodies being a currently favoured mechanism [12,21]. The substantially negative experimental database observed (Tables 1, 2 and 3; and Fig. 1 earlier and Fig. 16) appears initially to be consistent with these ideas, but isolated activities were seen.

Before discussing these activities it is necessary to discuss the general implications of focussing on a few positive responses observed for a chemical within the context of a largely negative database. First there is the danger of sequentially testing a carcinogen in a series of in vitro assays until a positive response is finally produced. Apparently 'non-genotoxic' carcinogens can easily become the subject of such relentless attempts to force a correlation between carcinogenicity and activity in vitro, irrespective of whether the eventual correlation has any mechanistic significance. The second major consideration is that in the present study the two non-carcinogens each produced a similar range of positive responses in vitro, but in these cases a correlation with carcinogenicity could not be made (see Fig. 1 and Table 3, earlier).

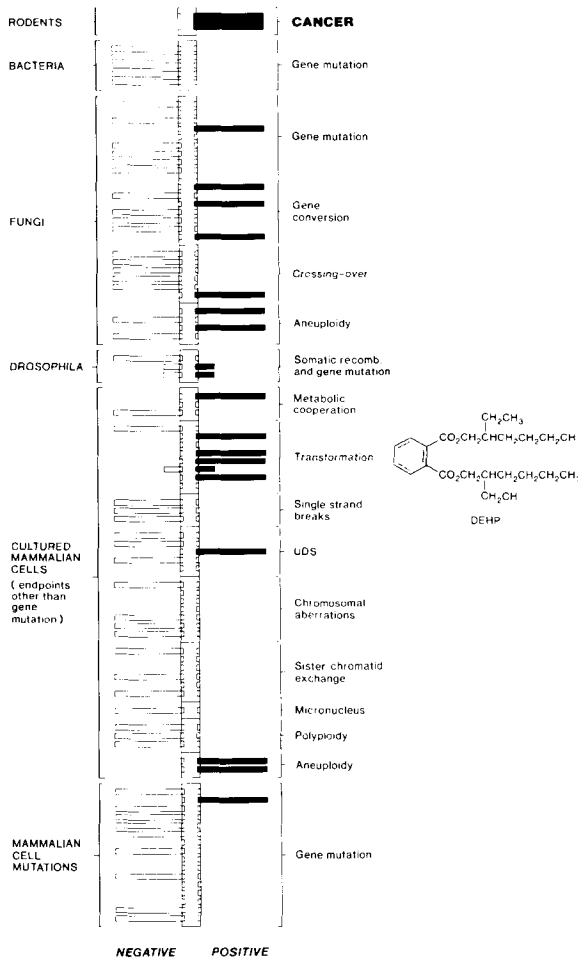

Fig. 16. Genotoxic activity histogram for DEHP.

The structure of DEHP does not alert one to potential carcinogenicity; the majority of in vitro findings observed prior to and in this study confirm its innate non-mutagenicity and non-reactivity to DNA; it is a confirmed mouse and rat hepatocarcinogen when evaluated at high dose-levels; it induces the proliferation of peroxisomes in the mouse and rat liver, microbodies which may lead to DNA damage via the overspill of radical species, and finally, it is a chemical of major commercial importance. In fact, it is probably one of the most ubiquitous of synthetic environmental chemicals. Thus, the significance to man of its rodent-liver carcinogenicity is of immediate importance. It was the prospect that the generation of a completely new genotoxicity database would contribute to a mechanistic understanding of its carcinogenicity that prompted its selection for this study.

At the empirical level it could be argued that the activities observed for DEHP in the present study define cell transformation and aneuploidy assays as useful complements to the Salmonella assay, however, the possibility that these phenotypic changes induced in vitro may have no relationship to the hepatocarcinogenicity of this agent is worthy of discussion. The pertinent points for consideration and future study are as follows:

(a) The issue of greatest interest is whether DEHP is a genotoxic or a non-genotoxic carcinogen. Many definitions of these terms exist, those adopted herein are that DEHP is confirmed as unable to demonstrate the classical phenotypes of direct interaction with DNA, i.e., gene mutagenic-

ity in bacteria and eukaryotic cells and clastogenicity and UDS-induction in mammalian cells. This is consistent with the inability of DEHP to interact covalently with DNA [38]. These properties separate it from the majority of other chemical carcinogens. Equally, it may be a non-genotoxic (epigenetic) carcinogen either by virtue of its cell-transforming and aneuploidy-inducing properties, or by virtue of the ability of its mono acid metabolite (MEHP) to induce peroxisome microbodies in vivo and in vitro. The first of these mechanisms fits the model proposed by Weisburger and Williams [9,10], i.e. the retention of an unmodified cellular genome, but the induction of cellular changes that lead to a modified expression of the genome. The second mechanism would be novel, i.e., the chemical induction of a stable biological change (peroxisome induction) that itself induces tumours *via* secondary genotoxic activity.

(b) The cell-transforming and aneuploidy-inducing properties reported herein for DEHP form a new and unexpected aspect of its biological activity in vitro. These activities were not associated with a single assay; they appear to be of general significance to eukaryotic cells treated in vitro.

(c) It is important to establish the degree to which hydrolysis of DEHP to the mono ester (MEHP) is occurring in the in vitro assays. MEHP has previously been defined as a clastogen in vitro under conditions of test where DEHP was non-clastogenic [22]. Also, (ω-1)-oxidized metabolites of MEHP are the proximate hepatocyte peroxisome-inducing agents both in vitro and in vivo [39]. In addition, two other acids, shikimic acid and arecaidine were recently established as cell-transforming agents devoid of activity as gene mutagens to Salmonella [40]. A precedent has therefore been established for carboxylic acids to act as cell-transforming agents, thus it should be ascertained if DEHP or MEHP was the active species in the present observations. In several of the assays that found DEHP active in these respects, auxilliary metabolism (S9 mix) was not required, therefore, evaluation of the extent of formation of MEHP in these cell lines should answer this question (e.g. via HPLC analysis).

(d) An issue of particular interest is the extreme specificity of the activities of DEHP in vitro. For example, both benzene and DES were similarly shown to be able to induce aneuploidy in vitro in this study, and more limited evidence for their ability to transform cells was presented. However, both of these agents were shown to possess a range of other genetic activities, in particular, each were also shown to be clastogenic and DES as capable of reproducibly inducing polyploidy. It therefore seems probable that the activities observed for DEHP are specifically associated with a chemical property not associated with classical interaction with DNA. Some unique interaction of this exceptionally lipophilic agent with the cytokinetic spindles, as opposed to the induction of spindle toxicity, may explain the aneuploidy effects, and these may lead to subsequent cell transformation. DEHP may, therefore, represent a case of specificity of 'genetic' action, and as such is worthy of further study.

(e) The pharmacokinetic studies of DEHP disposition in rodents undertaken by Albro et al. [38] and Kluwe [41] indicate that both DEHP and MEHP are present within the liver of rodents when administered at dose-levels equivalent to those employed in the cancer bioassay of DEHP. Either or both of these chemicals may therefore contribute to the induction of liver tumours in rodents. The fact that the conversion of DEHP to MEHP, the absorption of the former through the gut wall, and that the conjugative excretion of each from rats, mice, marmosets and humans are distinctly different, suggest that inter-species extrapolations of the rodent carcinogenicity of DEHP should initially be cautious.

Further studies are therefore required to ascertain the exact significance of the biological effects of DEHP demonstrated in the present study. It may be too simplistic to assume that its carcinogenicity to the rodent liver can now be easily explained in terms of its in vitro cell-transforming and aneuploidy-inducing properties, rather, these unexpected activities should focus efforts to explain its true mode of action as a rodent carcinogen. Whatever the outcome of such studies, these observations add credibility to the concept that there may be more ways of modifying normal gene expression than by the test chemical interacting directly (chemically) with the constituent genes of the genome (see calculations of relative sensitivity

at the foot of Table 3, and Fig. 1). As such, fundamental studies on the possible association between the induction of aneuploidy and perhaps the concomitant induction of a transformed cell phenotype are indicated.

12. Activity in vitro of the two non-carcinogens

Two non-carcinogens were included in the present study specifically to assess if the short-term tests under study could distinguish between carcinogens and non-carcinogens i.e., to assess if a particular test was sensitive to carcinogens as well as specific to them (it is recognised that the use of only two non-carcinogens offers a limited assessment of specificity). This approach has three potential flaws. First, there is an implicit assumption that the possible mutagenicity of a chemical in vivo is of no consequence so long as it is not a carcinogen. Second, there is the assumption that the negative cancer bioassay data are adequate when in fact they may actually reflect a weak carcinogenic effect that is beyond the resolving power of the test, and finally, the fact that activity observed in vitro may not be expressed in vivo because of factors such as non-absorption, rapid enzymic detoxification or hydrolysis. Such questions come to the fore whenever activity is observed for a non-carcinogen in an in vitro genotoxicity assay, especially if the assay measures a mutagenic event.

As can be seen by reference to Table 1, both caprolactam (CAP) and benzoin (ZOIN) showed activity in a minority of the in vitro assays conducted. It is now generally accepted that a weak positive response that cannot be repeated is occasionally observed for chemicals in short-term tests. These are of little significance. Attempts are currently being made to confirm apparent positive responses for these two agents; for the present purposes, all positive responses in this database are regarded as valid (Figs. 17 and 18).

One of the more interesting aspects of the database shown in Figs. 17 and 18 is that they focus attention on the problems inherent in using multiple short-term tests. Thus, were an investigator to select the appropriate assays, caprolactam would be defined as a confirmed genotoxin. Alternatively, chance selection of any of the negative results would acquit this chemical of any possible suspicion of genotoxic activity. Viewed as a single database the results actually define caprolactam as a somatic cell mutagen and clastogen that fails to elicit activity from the majority of assays. Similar comments apply to benzoin, and each database is discussed below.

Caprolactam (CAP. Several of the positive responses for CAP were weak, unconfirmed and only observed at high dose-levels, for example the C3H10T½ transformation data (4.2.2). However, two of the positive data sets clearly indicated mutagenic activity; the Drosophila data in which

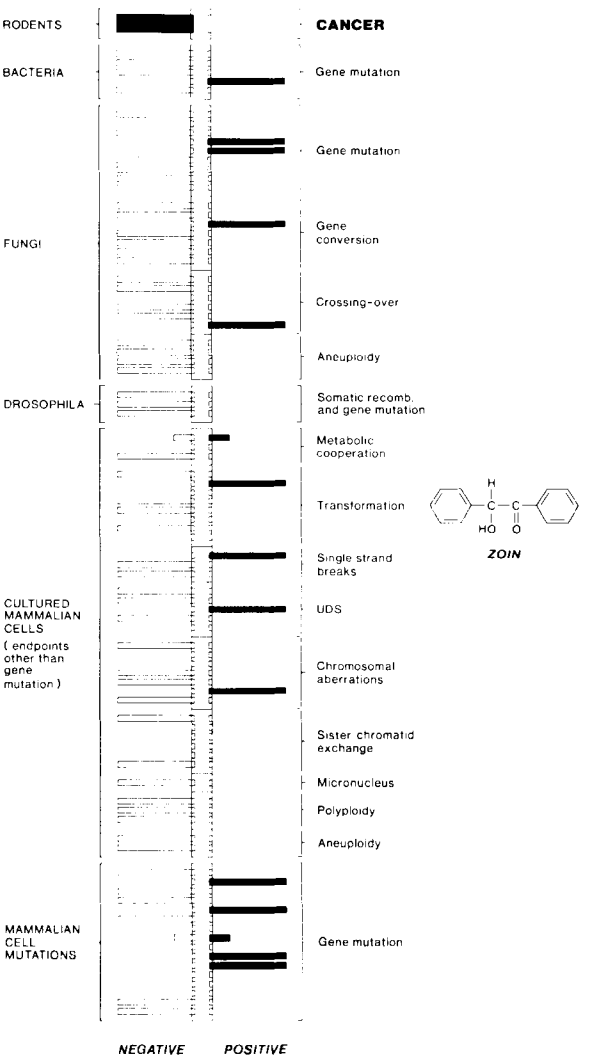

Fig. 17. Genotoxic activity histogram for ZOIN.

somatic cell mutation was demonstrated in 3 separate assay systems, and the human lymphocyte data (4.5.4) where the production of chromatid gaps and breaks was observed in the lymphocytes of 2 separate human donors. Caprolactam is therefore a clastogenic somatic cell mutagen that fails to elicit tumours when administered *via* gavage to male and female rats and mice at dose-levels up to the maximum tolerated dose (MTD). The multiplicity of possible factors that could explain this non-correlation, coupled to the equal number of constructions that could be devised to determine its general significance, have led to the following:

(a) The cancer bioassay of this agent was devoid of any suggestion of carcinogenicity. This result is consistent with the complete non-mutagenicity of this agent to Salmonella and the absence within its structure of actual or potentially DNA-reactive substructures. The non-carcinogenicity of CAP to rodents is therefore assumed as an established fact.

(b) The positive responses observed for CAP in vitro will be confirmed in independent laboratories; in particular, the positive human lymphocyte, Drosophila and yeast data.

(c) Assuming that these in vitro observations are confirmed, the possible clastogenicity and/or mutagenicity of CAP to rodents in vivo will be assessed. These studies are hoped to include a mouse micronucleus test, mouse and rat bone marrow cytogenetic assays and a mouse coat colour spot test.

(d) If activity is evident for CAP in vivo, its position as a non-carcinogenic mutagen/clastogen will have been confirmed. If negative data are observed in vivo an explanation for its complete genotoxic inactivity in vivo will be sought via (e), below.

(e) CAP is a very simple saturated cyclic lactam. It should be prone to enzymic ring opening to yield ω-aminocaproic acid, a compound that should be rapidly excreted from mammals either in the free form or as a conjugate. Similarly, oxidation of the methylene ring should lead to its similar detoxification and excretion as water-soluble metabolites. If negative mutagenic effects are seen for CAP in vivo, it may be due to its rapid detoxification and excretion in vivo. If this happens, appropriate pharmacokinetic studies in vivo should be sought to confirm this hypothesis.

The above approach will not explain the mutagenic effects seen for CAP in vitro. A possible avenue for explaining these may be to investigate the possible formation of ω-aminocaproic acid in vitro; several carboxylic acid derivatives having recently been shown to possess mutagenic/clastogenic activity. These include lacchaic acid, sodium benzoate, secalonic acid, ethylenediaminetetracetic acid (EDTA), the *mono* acid derivative of diethylhexylphthalate (MEHP) and possibly related, sodium sacchrin (reviewed in [12]). The clastogenic/mutagenic effects of some of these agents in vivo as well as in vitro made it prudent to proceed as described above rather than simply to conduct the metabolic studies outlined in (e).

The isolated but significant genotoxic effects observed for caprolactam in this study have therefore provided an intriguing area of future research. Nonetheless, in the absence of further data, it is necessary to regard the few positive responses observed in vitro as clear examples of incorrect predictions of the mammalian carcinogenicity of CAP made by the assays in question. It may be more appropriate to regard these incorrect predictions as representing deficiencies of in vitro assays per se rather than as deficiencies of the specific assays in question.

Benzoin (ZOIN). Analysis of the biological activity of this chemical is more difficult than that of CAP. As with CAP, the majority of the in vitro assays studied found ZOIN inactive and only limited activity was seen for it in the bank of Salmonella assays. The positive responses seen (Fig. 18) were less clearcut than with CAP, were generally only very weak and no clear genetic pattern was evident. For example, while negative results were observed in most of the UDS assays, two of the three hepatocyte assays recorded a clear positive response. Similarly, weak activity was seen in only 4 of the 11 mammalian cell gene-mutation assays, etc. The spread and magnitude of the positive responses seen seem consistent with ZOIN having a general but very weak ability to interact with DNA. It is possible that low levels of radical species are being formed in some assays. These could be associated with the phenylketone moiety (perhaps *via* reduction to benzil) and lead to active oxygen radicals. Formation of these radicals would

probably be dependent upon specific cytochromes, slight changes in the level of which between cell lines and assays could possibly lead to the equivocal and weakly positive database. A possible role for light-activated radical formation has not been eliminated. This explanation is speculative and is not supported by the inactivity of ZOIN in strain TA102 of Salmonella (assays 1.1.1 and 1.1.3).

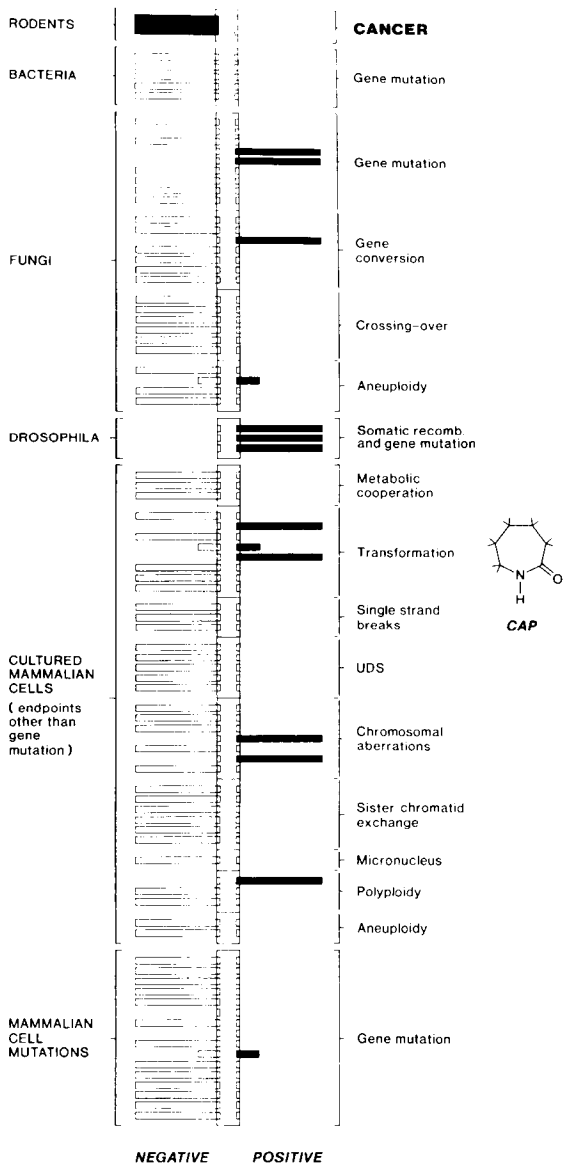

Fig. 18. Genotoxic activity histogram for CAP.

Although ZOIN was reported as a non-carcinogen in the NTP bioassay report the result was not as clearcut as with CAP. In male mice a dose-related increase in lymphomas was seen, and in female mice a non-dose-related increase in leukaemia was recorded. These effects were not considered as evidence of its carcinogenicity to mice, and no effects were seen in rats. Having selected ZOIN as a reference non-carcinogen for this study it would be inappropriate to question the overall conclusion arrived at by the NTP investigators; thus, the activities seen for it in this study must be regarded as false predictions of mammalian carcinogenicity by the assay in question. However, as with CAP, certain further studies are planned in an attempt to clarify its genotoxic activity, as follows.

(a) Selected activities observed in vitro will be independently confirmed or refuted.

(b) Given the suggestive evidence of an increase in lymphoma/leukaemia reported in the cancer bioassay of ZOIN attempts will be made to evaluate it for clastogenic activity in the bone marrow of mice and rats. Possible activity of this compound in the mouse coat spot test will also be evaluated.

(c) If activity is observed in the bone marrow in vivo it will be legitimate to recommend re-evalution of benzoin in a study designed to maximize the possibility of detecting leukaemogenic activity. If it is devoid of clastogenic activity in vivo the original negative carcinogenicity finding will have been endorsed.

The possible role of mutagenic impurities in the biological activity profile of a chemical cannot ever be fully excluded, but their presence within a test chemical generally leads to a consistent and general level of activity in in vitro assays. The few positive effects observed in vitro with CAP and ZOIN are therefore not consistent with an impurity being responsible. Further, no evidence for such was found using the analytical criteria listed in Appendix 1. Other more sensitive techniques would doubtless lead to the identification of trace contaminants but these should be the same or fewer than in the batches employed in the cancer bioassays.

13. The role of rat-liver S9 mix in eukaryotic in vitro assay responses

When employing the Salmonella mutation assay it is generally necessary to include an homogenate of induced rat liver (S9 mix) in order to obtain a positive response for carcinogens that are not overtly electrophilic in nature. This precaution appears not to be so important in assays employing mammalian cells. This is consistent with these cells having been derived from mammals possessing a wide spectrum of endogenous metabolic capacity. Fungal cells also appear to possess a superior metabolic competence when undergoing active growth. Time restraints precluded a detailed analysis of this topic in this Volume, but the primary data are available herein for study. As an illustration, S9 mix was omitted from the hepatocyte UDS scintillation assay (4.4.3) where 7 positive responses were observed, and all of the positive responses observed in the human lymphocyte cytogenetic assay (4.5.4) were S9-independent. Both of these cell types were primary, but many cases of established cell lines giving a positive response in the absence of auxiliary enzymes were recorded. This topic is worthy of detailed evaluation; the present database will provide a useful starting point.

14. Criteria for defining positive and negative assay responses: the problem of 'false' results

At the beginning of this discussion it is worth reiterating that the potency of a genotoxic response in vitro is not obviously related to the potency of the same agent as a mammalian carcinogen. Lacking a quantitative correlation, one must therefore rely on a qualitative correlation as a basis for comparing activities among test systems. Therefore, reproducible mutagenic responses, whether weak or strong, need be weighed equally as potentially predictive of the carcinogenicity of a chemical. Not surprisingly, given the chemicals tested, weakly mutagenic responses and interlaboratory disagreements in results were very common in this study. The latter is probably a consequence of the former given that few investigators provided evidence of the sensitivity of their test protocol; i.e., subtle variations from one protocol to another may assume a heightened importance in terms of their influence on the ability of an individual investigator to detect a weak mutagenic effect.

As a result of this need to contend with weakly mutagenic responses, one is led to a major conclusion of the present study: urgent attention needs to be given to the question of what constitutes a positive or negative response in in vitro assays. This problem is a general one and is not new. Although it is extremely unlikely that a single solution will be found, some of the major considerations are of widespread significance. It was considered inappropriate to discuss herein the varied test response criteria used by investigators for the many assays entered into this study. Instead, it was decided to discuss the general principles and issues, and to illustrate these with examples selected from the present database. Future detailed analysis of this database may provide clearer advice for individual investigators, but the time required for such studies precluded their incorporation here.

A fundamental question in the determination of what constitutes a positive or negative response is the extent to which historical control data should influence the interpretation of results generated in an experiment that contains a concurrent control group. For example, when describing a cytogenetic study on ZOIN conducted after the joint meeting, Ishidate qualified the repeat positive response he had observed in the following form:

'Based on our cumulative historical control data for aberrations in CHL cells, a compound is labeled positive only when the incidence of aberrant cells exceeds 10% for at least one dose. It seems highly questionable to arrive at one's final decision solely on the basis of a comparison between the test compound results and a limited number of concurrent control cells.'

This viewpoint is reflected in the UKEMS guidelines (1983) [29], where one of the criteria for the evaluation of cytogenetic data was given as 'A minimal size of response may be required to avoid modulations within the historical control range being classified as positive responses'.

These two statements epitomize the dilemma of historical *versus* concurrent controls, a dilemma that is particularly acute when the compound in question has generated a weak response. That this

concern transcends short-term tests is evidenced by the fact that the weak leukaemogenic effects seen in the cancer bioassay of benzoin were significant when compared with the concurrent controls, but not when considered against the historical level of leukaemia in the strain of rodent employed in the laboratory that conducted the study.

Two apparently antagonistic concerns exist regarding the role of historical control data vis-à-vis a concurrent control. Some individuals worry that a total disregard of relevant historical control data may lead to a breed of mutagens whose regulatory incrimination is solely attributable to concurrent controls that are by chance depressed relative to historical levels. Others worry that disregard or diminution of the role of the concurrent control may lead to a breed of non-mutagens whose regulatory acquittal is attributable to what may be irrelevant historical controls. These two concerns are not as antagonistic as they seem; contexts exist in the evaluation of short-term test data in which each is a defensible position. Which treatment of historical controls is appropriate in any one context can be determined empirically, provided certain supplemental data to be discussed later have been gathered over time. Failure to distinguish between these contexts has muddled the discussion of the usefulness of historical control data and confused the science immeasurably.

The simplest assay data to interpret would be from an assay in which it was known a priori that the practical spontaneous incidence of the parameter under study was zero. No assay in use today is in that enviable position. A zero observed control incidence for any discernible genetic change is inextricably linked to the extent to which it has been sought in the control population, i.e. the aggregate number of control observations made. The Syrian hamster embryo (SHE) cell transformation assay, for example, was considered by some to have a zero incidence of spontaneous transformation, but this was not the case in the present study. For practical purposes, then, one must assume that all genotoxicity data have arisen from an assay with a positive, albeit possibly very low, true spontaneous or background incidence. Were this true spontaneous level known in any laboratory on a given day of experimentation, then it would be against this level that one would need to examine observations from treated groups for evidence of chemically induced changes. Of course, this true level of spontaneous events is never known.

Two options present themselves immediately, use of the observed concurrent control as a surrogate for the unknown true level, or alternatively, use of the aggregated laboratory control history for this purpose. When this choice is phrased explicitly, it rapidly becomes apparent that the key consideration is the extent to which the true level has varied over time within the given laboratory. Although the variation of the true level over time is unknown, one can obtain an empirical answer to this question by studying intra-day and inter-day variability in observed levels of spontaneous incidence. Any compelling evidence for inter-day variability is then indicative of a true spontaneous level that is changing over time, possibly systematically. It is the magnitude of the inter-day relative to the intra-day variability that speaks directly to the usefulness of the historical control data. If the inter-day variability is minimal relative to the intra-day variabiity, then one can infer that the true underlying spontaneous level of the event being monitored is effectively constant over time. This is precisely the situation in which current statistical practice envisions historical control data playing their most important role in the evaluation of a single experiment [42,43]. A good example of such a situation is provided by the mouse specific-locus assay of Russell; here inter-laboratory variability in observed control rates, which perforce must be larger than inter-day variability within any one laboratory, is minimal, and the treatment response is evaluated against the aggregated historical control data via a Fisher exact test [44].

At the other extreme, the inter-day variability may be substantial when compared with the intra-day variability in observed control levels, implying that the true underlying spontaneous level of genetic change is varying substantially over time. In this case, the historical control data are of limited value in the evaluation of a single experiment; here the concurrent control retains paramount importance as the yardstick for assessing chemically induced change.

It is unclear whether many or few of the potentially complementary in vitro assays in this study

exhibit high inter-day variability in control levels because this issue has rarely been addressed in the literature; one exception is the investigation by Margolin et al. [45] of sources of variability in the Salmonella test. In this the authors reported sufficient inter-day variability to give pause to anyone wanting to employ historical control reversion frequency to evaluate the evidence for induced genetic change. There was, however, an assay in the present study that does exhibit minimal inter-day variability — at least for one endpoint. Richardson [assay 4.5.4] has observed neither an interchange nor a dicentric chromosome in 10000 control human lymphocytes collected over 6 years (personal communication 1983), so both his inter- and intra-day variability in control measurements have been zero. This low/zero incidence is partially due to the selection and repeat sampling of human donors exhibiting low overall spontaneous aberration rates. Assessment of interchanges or dicentrics occurring in treated populations should then be analyzed, as the morphological mouse specific-locus test data are, against the aggregated historical control, here 0/10000. In this extreme case a single such event occurring in a treated population of 100 cells would be associated with a p-value of 0.01 using Fisher's exact test. Despite this level of statistical significance, prudence would dictate a repeat study, possibly with varied experimental conditions (see UKEMS guidelines 1983 [29]), so as to avoid determining a chemical's clastogenicity by reference to a single datum. Note, as well, that this situation is altered considerably within the same assay if one is considering chromatid gaps and breaks as the response of interest; here low but significant control incidences are normal.

Few assays, even those with relatively rare responses, have a control history as extreme as that experienced with chromosomal interchanges and dicentrics. In the $10T\frac{1}{2}$ transformation assay, for example, the occurrence of type III transformed foci is a relatively rare but observed event exhibiting minimum inter-day variability. Thus, in a recent paper by Scribner et al. [3] a single plate containing such a colony occurred within the 39 untreated control plates. No such colonies occurred in the remaining 133 treated and water control plates. The *historical* incidence of spontaneous type III colonies in the laboratory in question is 4 in 1879, i.e. 1 in 469. Thus, by chance, an effect was seen using 39 control plates that should, on average,have required 469 plates to detect. Had this colony occurred in one of the 20 top dose treated plates, with none in the concurrent 39 controls, it would have triggered statistical significance with a p-value of 0.06 by the Fisher exact test, thereby indicating marginal evidence for concern. Contrast this with the effect of a single event in Richardson's case, and one begins to appreciate quantitatively the critical role that the aggregated historical control incidence of an assay can play in the inferential process.

As opposed to the two assay endpoints discussed immediately above, the majority of in vitro assays under discussion employ as endpoints genetic events that occur spontaneously in control cells with a substantial frequency. In these cases, unlike the earlier two, one is unable to make a judgement about the relevance of historical control data by simple inspection. With this in mind, it becomes imperative that the intra- and inter-day variability in spontaneous incidences for an assay should be well established in a laboratory before that assay is used routinely for testing new chemicals. The fact that this precaution is not generally taken was illustrated recently by the decision of the Gene-Tox review group to consider only results from the mouse lymphoma mutation assay as positive if the induced effects exceeded 3 times the concurrent control mutation frequency [46]. This arbitrary criterion was intended to encompass the range of variations of the spontaneous mutation rate likely to be encountered, and was an increse on the earlier, but equally arbitrary factor of 2 times the spontaneous rate [46]. Criteria such as these, both of which are devoid of any statistical justification because they reflect no quantitative assessment of observational variability, tend to be extremely conservative and thereby increase the chance of missing weak mutagens. These n-fold criteria, in turn, are ultimately exceeded in conservatism by the criterion that requires an observed treatment response to be classified as negative if the treatment response falls within the historical control range; as the control database grows, this range, of necessity, will increase and eventually make it impossible for any weak mutagen to be detected.

The simple criterion that a 2-fold or greater increase in the spontaneous mutation rate constitutes evidence for an induced effect first became of general importance to this science when it was selected by Ames and his colleagues for use with the Salmonella reverse mutation assay [5]. For this reason it is appropriate to consider this assay in detail first. The 2-fold criterion is much older than the Salmonella assay and is probably related to the concept that a dose of ionizing radiation that leads to a doubling of the cancer incidence in rodents constitutes a significant radiation hazard. Early 'validations' of the Salmonella assay, which themselves led to the current stability of this science, employed this single criterion for a positive response, although the need for a dose-related effect had been emphasized by Ames et al. [5]. By the time of the first international study [6] additional criteria were discussed, but not considered mandatory. These included the requirement for a dose-related effect and the need for responses to be repeated successfully before being finally accepted. The 2-fold rule is not currently employed in its earliest form, although some investigators remain uneasy at the prospect of classifying a chemical as a mutagen based on induced effects that do not exceed twice the concurrent spontaneous rate. Published accounts of the weak bacterial mutagenicity of acrylonitrile [19,20] and 4-*N*-piperidinylazobenzene [47–50] and certain surfactants [51,52] illustrate well these uncertainties. Fortunately, the present database provides a perfect example in the form of the unexpected and weak mutagenicity of PB to Salmonella.

It is appropriate to discuss the Salmonella data on PB at this stage in the discussion because it illustrates many of the statistical considerations mentioned so far. First, and of extreme importance, is the overall Table of results prepared by the Assay workgroup and summarized in Tables 1 and 7 of this section. These represent the findings of 5 laboratories employing 8 strains of Salmonella in the presence and absence of S9 mix and a variety of technically distinct assay protocols including a pre-incubation test. The overwhelmingly negative nature of the database indicates the extent to which reproducible and interlaboratory consistent determinations can now be obtained using this assay.

Phenobarbital (PB) was detected as a reproducible but weak bacterial mutagen during the course of the joint meeting, and two issues are worthy of study in relation to this weak mutagenic response. First is the fact that although the positive responses observed in strains TA1535 and TA100 were reproducible and dose-related, none of them exceeded twice the maximum spontaneous mutation rate observed in the individual laboratories during the course of the study — in short, PB is a very weak mutagen. In such cases, appropriate statistical and biological criteria by which to assess such responses become of importance. Second, having established a statistically significant positive response when assessed against concurrent control levels, how should historically observed changes in the spontaneous mutation frequencies for these strains affect this conclusion?

The available mutagenicity data on PB in strain TA1535 are shown in Fig. 19. Employing the same 'mean revertants per plate' axis, this figure also exhibits the frequency distribution of the spontaneous control rates observed for this strain by the appropriate investigators in the course of the present study [1.1.4 and 1.1.5]. This figure provides a perfect database for future use when discussing weak mutagenic responses; a few of the most pertinent points are discussed below.

First, consider the concerns expressed by the Gene-Tox committee that reviewed the mouse lymphoma mutation assays ([46], see earlier), namely, that given the apparently inherent variability of the spontaneous mutation rate among different clones of the same cell line, a minimum of a 3-fold increase in this rate would be required to label a chemical as a mutagen. Whatever the rights or wrongs of that decision, and some investigators and statisticians are not convinced by its underlying logic, it represents a concern that is not usually considered within the context of Salmonella assay data. The variable spontaneous mutation frequencies evident for TA1535 in Fig. 19 suggest that if the Gene-Tox concerns are legitimate, they should also be applied to these Salmonella assay data, in which case PB would be regarded as non-mutagenic. This debate cannot be concluded definitively here, but these weak positive responses recorded for PB could acquire a quite different significance depending on how this issue were to

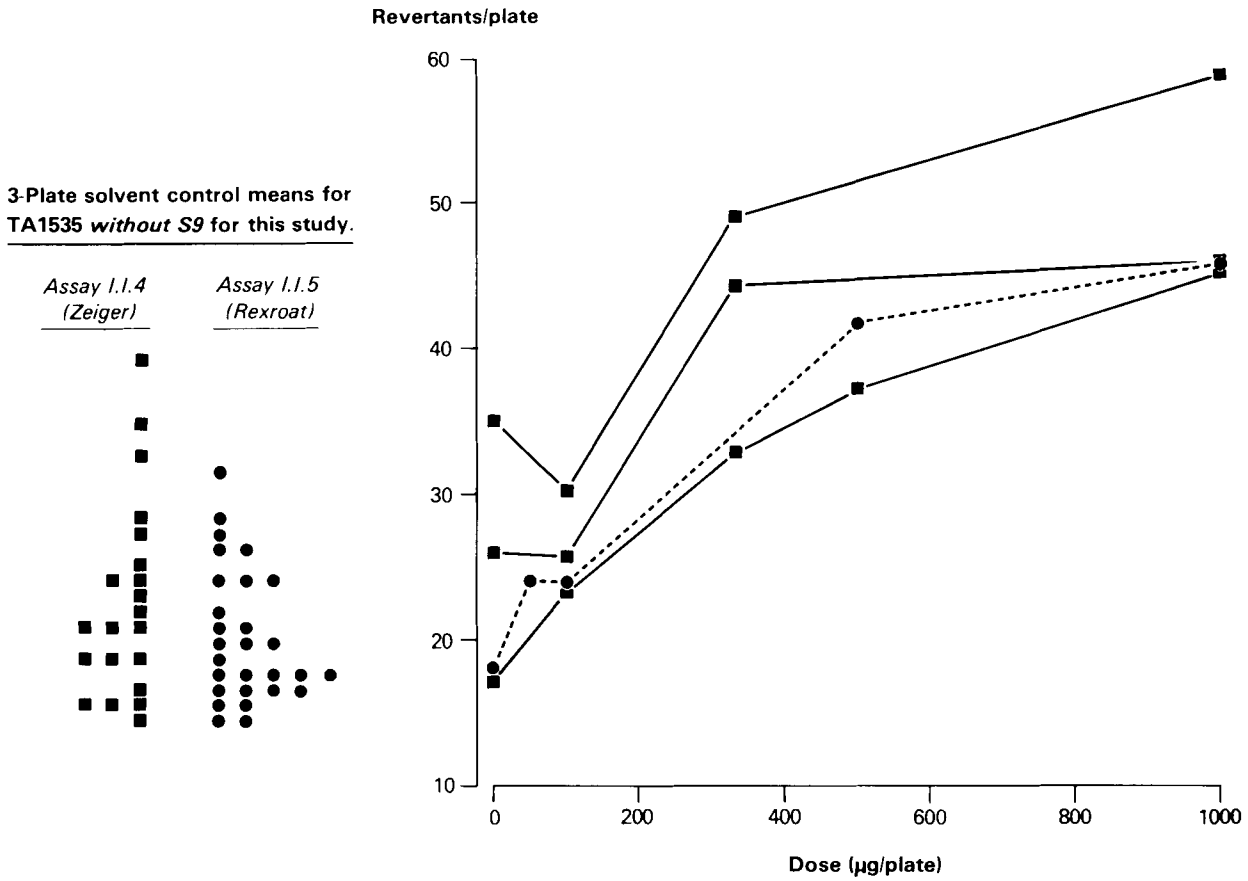

Fig. 19. Mutagenicity to Salmonella (TA1535; − S9) observed for PB in assays 1.1.4 and 1.1.5. The data points on the left-hand side of the Figure relate to control observations made in the laboratories in question during this study, and are plotted against the same central axis.

be resolved. A fundamental concern underlying the above discussion is that the number of revertant colonies on a test plate may not represent the true mutation frequency. Thus, x mutations may occur in reality, yet only a fraction of these mutant cells may mature to become countable colonies during the course of the experiment. The prospect therefore exists that some latent spontaneous colonies may be encouraged into growth by the test chemical due to purely physical influences exerted by it on the growth environment. The increased number of countable revertants per plate observed could therefore represent an 'optimization' by the test chemical of expression of the endogenous mutants as colonies, as opposed to the chemical induction of new revertant cells/colonies. This mechanism would not be expected to produce a strong 'mutagenic response' e.g. in excess of the highest naturally expressed mutation frequency for the strain; this criterion is amply met by the PB data shown in Fig. 19. When attempting to rationalize how a chemical could produce such growth-modulating effects one would initially look for physico-chemical properties that could modulate the pH/dielectric, etc., properties of the growth environment. The fact that PB is a moderately strong acid could therefore be significant as Cifone et al. [37] and Brusick et al. [53] recently reported mutagenic and clastogenic changes induced by changes in the pH of the in vitro assay

medium. Likewise, Ishidate et al. [54] have shown that changes induced by ionic test chemicals in the osmotic pressure of an assay medium may lead to clastogenic effects, and both Ishidate et al. [52] and Trueman et al. [51] have reported on the weak genotoxic activity of certain surfactants such as propylene glycol.

To summarize, the two concerns generated by the weak dose responses for PB, as shown in Fig. 19 are:

(i) that most of the observed treatment responses are essentially within the range of spontaneous frequencies observed for this strain in the current study, and surely within those observed historically; and

(ii) that other than genetic phenomena may have caused the weak responses observed with TA1535.

Two components of the Salmonella database speak to these concerns. First, the investigators using assay 1.1.5 reported 28 spontaneous or solvent control means for 3 plates in TA1535 because they included two independent control groups in each experiment, one instituted at the beginning of plating, and another at its conclusion. Fig. 19 gives no indication of how the 28 control means are paired. When these data are paired appropriately, as in Table 6, one can see that the pairs tend to rise and fall together. A formal analysis of variance [55] on the 84 individual plate counts underlying these 28 control means indicates that the estimated proportion of intra-laboratory variability for these control data attributable to inter-day sources is 0.42, a value that is in excellent agreement with the laboratory control average of 0.46 reported by Margolin et al. [45] for this strain in 38 laboratories. It follows that computations of variability based on inter-day comparisons of control data *overstate* the variability of an intra-day comparison by approximately 70%, and substantially weaken one's ability to detect weak effects (the data for benzo[*a*]pyrene (BP), pyrene (PYR), 2-acetylaminofluorene (2AAF) and 4-acetylaminofluorene (4AAF) shown in Table 6 are anticipated from the second IPCS study [28]).

Rather than comparing the PB responses in TA1535 observed in assay 1.1.5 to the range of their corresponding historical control means, it is far more meaningful to compare the observed induction of 24, 28 and 32 revertants/plate at 500, 1000 and 5000 μg/plate, respectively (the last not in Fig. 19) with the difference between paired controls. The largest difference in Table 6 among the 14 pairs of independent control means was 8, leading one to the conclusion that the revertants on PB-treated plates did not occur due to random fluctuations. A formal statistical analysis follows shortly (Table 7 and text).

This still leaves open the possibility that the effect of PB in TA1535 is due to physico-chemical factors. The mutagenicity results for PB in TA100 (−S9) seemingly eliminate this from consideration. Fig. 20 contains the results for six experiments from four laboratories. Unlike Fig. 19, however, the vertical axis here presents the average number of *induced* his^+ revertants/plate, while the legend contains the corresponding mean solvent control values. One striking feature of Fig. 20 is the degree of agreement among the six experiments in terms of the induction of his^+ revertants at 1000 μg/ml. Even though the mean solvent control levels vary across these 4 laboratories from

TABLE 6

A STATISTICAL ANALYSIS OF THE SALMONELLA CONTROL DATA (TA1535; −S9) FOR ASSAY 1.1.5 IS MADE IN THE TEXT BASED ON THE DATA SHOWN IN THIS TABLE

Control	HMPA	SAF	TOL	BEN	DEHP	ACN	DES	PB	CAP	ZOIN	PYR	BP	4AAF	2AAF
Start of Expt.	27	25	18	32	21	19	22	19	27	25	28	17	23	19
End of Expt.	19	21	18	29	18	21	16	17	20	22	25	16	19	18
Difference	8	4	0	3	3	−2	6	2	7	3	3	1	4	1

84.7 to 138.0 revertants/plate for the six experiments, the mean induction of his^+ revertants/plate by PB at the 1000 μg/ml dose-level varies only from 25.5 to 33.7. The inescapable conclusion is that a weak but highly reproducible effect was seen for PB in TA100 without S9 in all four laboratories doing the Salmonella plate test. Any physico-chemical explanation put forth for this weak result would need to account for the specificity of effect within the base-pair substitution strains and for the apparent near constant rather than proportional induction of revertant colonies across the six experiments despite the substantial range of mean solvent control levels. Alternatively, one might be inclined to attribute this weak, but reproducible induction to the possibility that PB is actually a much stronger mutagen whose response in TA1535 and TA100 is being moderated by concurrent toxicity; this position is given little support by the relevant toxicity assays conducted after the 'Meeting of Investigators' by McGregor using the strains of Waleh et al. [56]. The conclusion at this time remains that PB is a weak bacterial mutagen, but the possible effects of induced changes in the pH or other aspects of the assay medium, remain to be evaluated [12,53,54].

The formation of a computerized database for data from this study created an opportunity to apply a common statistical analysis to all data generated from the same assay, and to investigate whether there was evidence of arbitrariness in the workgroup conclusions. As an illustration, the Salmonella data from each of 611 experiments were analyzed via a statistical procedure based upon a slightly modified version of the method described in Margolin et al. [57]. Each experiment

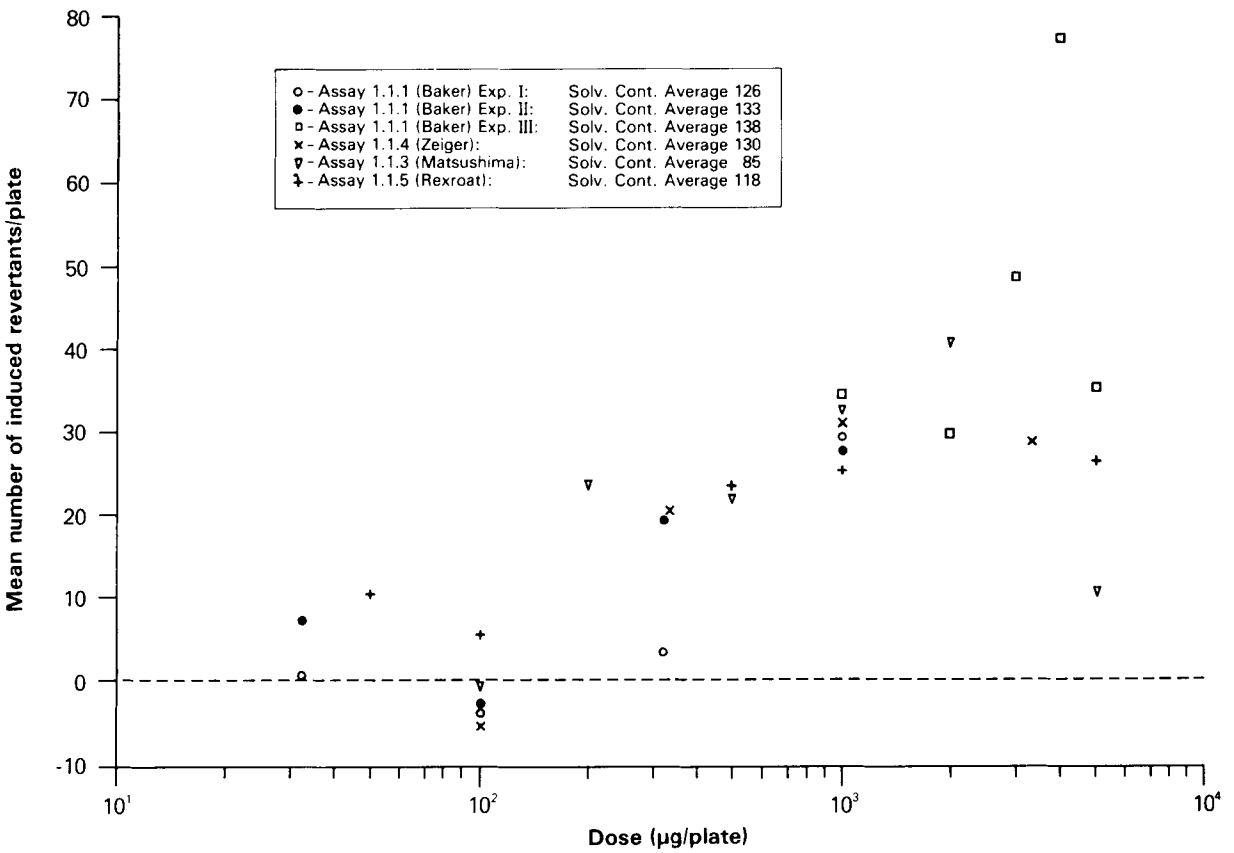

Fig. 20. Mutagenicity to Salmonella (TA100; −S9) for PB in the assays shown in the boxed area of the Figure. The datapoints represent the number of *induced* mutants, i.e. plate counts minus the average background count for that experiment (see text).

TABLE 7

STATISTICAL ANALYSIS OF THE SALMONELLA DATABASE

Raw data were reassessed using a slight modification of the procedure described by Margolin et al. [57]. U represents data unsuitable for analysis due to insufficient number of dose-levels, +, $P < 0.01$ and −, $P > 0.01$. In the S9 column R = induced rat-liver S9 and H = hamster S9. In all cases where the investigator work group concluded that a positive or questionable response was produced, the statistical procedure was in agreement. Isolated cases of unrepeated or unrepeatable weak yet statistically significant responses also occurred, but their failure to be replicated led to a negative conclusion. The unreplicated and isolated positive responses seen for PB in strain TA100 of assay 1.1.5 led the investigator to record this as a questionable response, presumably because it was observed in the presence and absence of S9. Each of these unreplicated responses were deemed to be statistically significant. 17 isolated positive responses were seen in the TA97/TA102 database. These were either non-reproducible, or not reproduced, thus agreement exists with the assay workgroup report which records only one questionable response in these strains.

Laboratory code (see master table)	Strain	S9	HMPA	TOL	SAF	BEN	CAP	ACN	DEHP	ZOIN	DES	PB
1.1.1	98	−	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −
		R	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −
	100	−	− −	− −	− −	− −	− −	− −	− −	− −	− −	+ + +
				− −	− −	− −	− −	− −	− −	− −	− −	− − − −
1.1.3	98	−	−	−	− U	−	−	−	−	−	−	−
		R	−	−	U	−	−	−	−	−	−	−
	100	−	−	−	− U	−	−	−	−	−	−	+
		R	−	+	U	−	−	−	−	−	−	+ −
1.1.4	98	−	− −	− −	− −	− −	− −	−	− −	−	− −	−
		R	− −	− −	− −	− −	− −	−	− −	−	− −	−
		H	− −	− −	+ −	− −	− −	−	− −	−	− −	−
	100	−	− −	− −	− −	− −	− −	−	− −	+	− −	+
		R	− −	− −	− −	− −	− −	− − + − − −		−	− −	−
		H	− −	+ + +	− −	− −	− −	− + + + − −		−	− −	−
	1535	−	− −	− −	− −	− −	− −	−	− −	+ + +	− −	+ + +
		R	− −	− −	− −	− −	− −	+ + + + − −		− −	− −	− − −
		H	− −	+ + −	− −	− −	− −	+ + + + − −		− −	− −	− − +
	98	−	−	−	−	−	−	−	−	−	−	−
		R	−	−	−	−	−	−	−	−	−	−
	100	−	−	−	−	−	−	−	−	−	−	+
		R	−	+	+	−	−	−	−	−	−	+
1.1.5	1535	−	−	+	−	−	−	−	−	+	−	+
		R	−	−	−	−	−	−	−	−	−	−
	1537	−	−	−	−	−	−	−	−	−	−	−
		R	−	−	−	−	−	−	−	−	−	−
	1538	−	−	−	−	−	−	−	−	−	−	−
		R	−	−	−	−	−	−	−	−	−	−
1.1.1	97	−	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −
		P	− −	− −	− −	− −	− −	− −	− −	− −	− +	− −
	102	−	− −	− −	− −	− −	− + +	− +	− −	− −	− −	− −
		R	− +	− −	− −	− −	− −	− − +	− +	− −	− −	− −
1.1.3	97	−	−	−	− U	+	−	−	+	−	−	−
		R	−	+	U	−	−	−	−	+	−	−
	102	−	−	−	− U	−	−	−	−	−	−	+
		R	−	+	−	−	−	+	−	−	+	+
1.1.4	97	−	− − − −	+ − −	+ − −	− −	− − −	−	− − −	−	− −	−
		R	− − − −	− − −	− − −	+ −	− − −	−	− + −	−	− − −	−
		H	− − −	− − −	− − −	− −	− −	−	− − −	−	− −	−

was labelled + if the observed significance level was smaller than 0.01; otherwise it was labelled −. These symbolic summaries are reported for each of the experiments with the standard Salmonella strains and separately for those with the recently introduced strains TA97 and TA102 in Table 7. For example, Table 7 indicates that 3 of 4 experiments using assay 1.1.4 for ACN in TA100 with hamster S9 were labelled +. This Table also demonstrates that there is clear statistical support, frequently based on good reproducible results, for every positive finding of the Salmonella workgroup. Moreover, this analysis indicates that the PB results obtained in assays 1.1.3 and 1.1.4 using TA100 without S9 are statistically significant, thereby supporting the acknowledged positive findings of assays 1.1.1 and 1.1.5. While the workgroup did not judge the PB results of assays 1.1.3 or 1.1.4 positive, Table 7 testifies eloquently to the credibility of a positive conclusion. The totally negative statistical findings for the 160 experiments with HMPA, BEN, CAP and DEHP and the essentially negative findings for the 78 experiments with DES and SAF clearly refute the claim that statistical analysis will produce many falsely proclaimed mutagens.

Observations made using TA97 and TA102 present an entirely different picture. The two laboratories that utilized these strains and employed replicate experiments exhibited a significantly increased inter-replicate rate of disagreement relative to their results with the 5 standard strains. While a biological explanation for this phenomenon is lacking it may be associated with the increased 'fragility' of these strains.

When attempting to discuss appropriate statistical criteria for assays other than the Salmonella test, one is confronted by a range of complex biological criteria in addition to statistical ones. How these will eventually mesh is an intriguing problem. Reference to the range of different criteria employed in the cell transformation and yeast assays illustrates this point. Given that a slightly different statistical analysis might be required for each of these assays, it was decided to focus the discussion on the SCE and chromosomal aberration assays. As with the Salmonella assay, application of a common statistical analysis can shed light on whether there is substantial arbitrariness in an investigator's or workgroup's conclusions. The analyses adopted here are those discussed in Galloway et al. [58]. In brief, the authors propose a statistical analysis for SCE data that is simply a linear regression of the observed SCE/chromosome response on the logarithm of the chemical dose. This analysis is predicated on an assumption of random Poisson sampling for the number of SCE's scored in cells under comparable control conditions within a particular trial whether in a common flask or not. Galloway et al. [58] report that 'extensive analysis of SCE cell counts from a common control flask and from replicate control flasks within the same trial strongly support the Poisson model'. This statement gives another illustration of the benefit of employing independent control groups. The total SCE database from this study has been reassessed according to the analysis advocated by Galloway et al. [58], where (+) or (−) responses are recorded according to significance at the 0.01 level. As with the Salmonella data, statistical support for the workgroup conclusions was substantial and extensive (data not shown). One obvious exception was the set of three experiments with DEHP conducted in assay 4.6.1 (−S9). Fig. 21 contains the three dose–response curves; each was considered negative by the investigators, but all were highly statistically significant by the analytic method employed here. The paucity of other SCE data for DEHP in this study precludes a clear adjudication of this discrepancy. Further experimentation is clearly in order to establish the SCE-inducing capacity of this chemical. This example provides an elegant illustration of the interface between the statistical significance of data and the reluctance of an investigator to consider the effect induced to be biologically significant.

Another pair of experiments conducted using assay 4.6.1 serves to highlight an important but often unaddressed point. Fig. 22 contains replicate experiments with BEN; no protocol variations are available to explain the major inconsistency observed. In one experiment, there is no suggestion of an induced effect, however, a repeat experiment produced one of the cleanest dose–response curves obtained in this study. Anyone who has reviewed a sufficient number of replicate experiments knows that this inconsistency, while some-

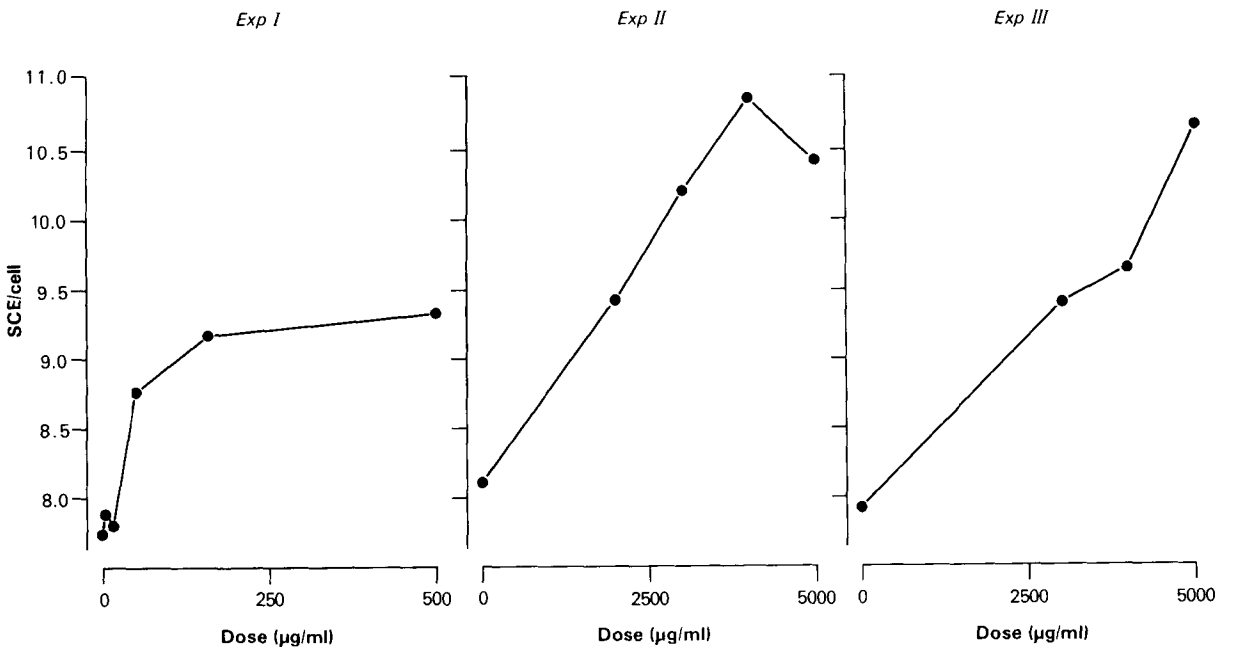

Fig. 21. Three assay responses observed for DEHP in SCE assay (4.6.1). These responses are discussed in the text.

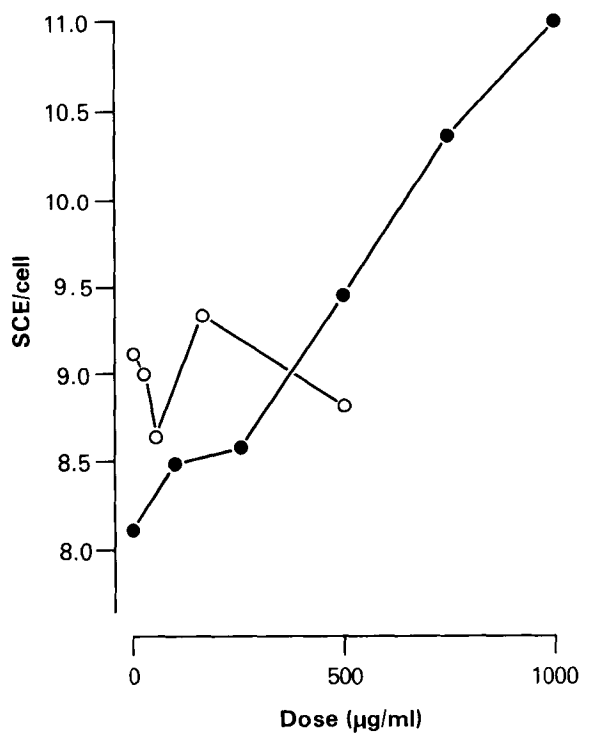

Fig. 22. Two responses observed for BEN (−S9) in an SCE assay using CHO cells (4.6.1).

what extreme by choice, is not uncommon. Had the positive response been the only data available to a regulator considering the genotoxicity of this compound, he would of necessity have concluded that BEN was an SCE-inducing agent. The negative experiment would have given the regulator pause, and further experimentation, as done here by the other five laboratories, could well have indicated the possibly misleading nature of the clear dose response. What better argument is there for confirmatory experimentation especially as Morimoto et al. [59] have reported this agent to be a weak and *S9-dependent* SCE-inducing agent.

The term 'false positive response' has been avoided in the discussion of BEN above. Aside from being overworked in discussions of short-term tests, the phrase is overly vague. At least four different 'false positive' contexts exist. The first is illustrated by the BEN/SCE example, where a positive result was obtained that was not reproduced upon repeat testing. Hypothetical discussions of whether chance or laboratory error are at work here are largely irrelevant, since the truth can rarely be determined. The second context is where a short-term test clearly and reproducibly gives a

positive response for a compound, but in fact, the compound is causing the result by growth modulating (physico-chemical) means. An example of this may be provided by PB herein. Third, there is the situation where a truly mutagenic response observed in vitro does not correlate with mutagenicity or carcinogenicity in an intact mammal because of pharmacokinetic or DNA-repair factors. Lastly, there is the context that permeates this entire search for a complementary assay, a true in vivo mammalian mutagen that fails to induce tumours in rodents during a chronic carcinogenicity bioassay. The term 'false positive' gives the illusion of communicating a great deal of information succinctly, but the context of its use needs to be explicit to avoid misunderstanding.

The term 'false negative' is similarly afflicted with vagueness. For in vitro assays, parallels exist for the four contexts in which 'false positives' were considered: (i) a non-reproducible negative result is obtained experimentally, but adequate repeat testing would disclose a true mutagen in the assay system being considered; (ii) reproducible negative results are obtained in all laboratories performing the assay, but the compound is genotoxic in similar in vitro assays; the mutagenicity is merely hidden by some specific characteristic of the assay protocol; (iii) a reproducible, non-mutagenic response is obtained for a compound in vitro, but a mutagenic response is observed in an intact mammal because of the uniqueness of the experimental environment afforded by in vivo systems; (iv) a reproducible negative in vitro result is obtained for a compound judged tumorigenic in a chronic rodent carcinogenicity bioassay.

There is one other context for a false negative response that apparently lacks a parallel when considering false positive responses; that is, where specific protocol considerations in a particular laboratory elevate the threshold for detecting a weak mutagenic response relative to that evident in other laboratories conducting the same assay. It is well documented that even when investigators indicate that they have used the same standardized protocol, interlaboratory differences in the ability to detect a given mutagen can be considerable [6,45]. Not all disagreements in results among laboratories conducting a given assay are attributable to differing protocol sensitivities, but it is interesting to speculate to what extent this phenomenon was operative in the current study. The repeated demonstration by a laboratory of a clear positive response for an elevated dose-level of a strong positive control chemical, while necessary to establish credibility of negative test results, is not sufficient to delineate the limits of the assay for detecting weak mutagens. The need seems clear to calibrate quantitatively the sensitivity of an assay for mutagenicity (etc.) in much the way any instrument of detection should be calibrated — in the hands of the ultimate user.

The question of what constitutes an adequate level of sensitivity is a complex question. While it is easy to increase sensitivity by employing or assessing a greater number of treated cells, etc., costs rapidly escalate. Also, to modulate a test protocol in order to detect optimally agent A may be to desensitize the assay to agent B. In considering this issue, therefore, distinction should be made between the ultimate goal of improving an assay's general sensitivity in all laboratories, and *assessing* the sensitivity of a particular assay protocol as

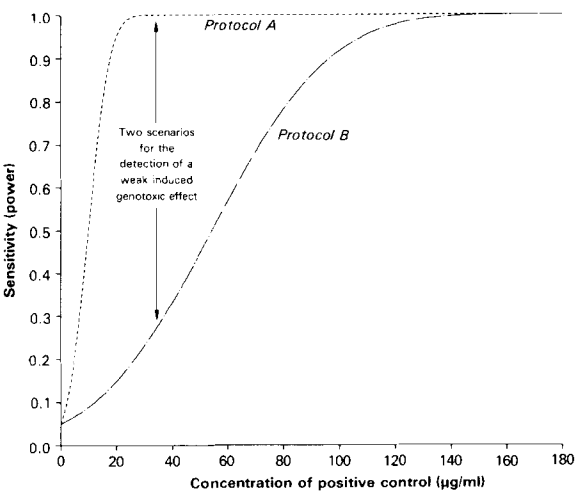

Fig. 23. The sensitivity curves for 2 hypothetical in vitro genotoxicity assays. Protocol A is superior to protocol B when weak induced genotoxic events are under study. Both protocols would detect the positive control agent dosed at 200 μg/ml, but only the former could confidently be expected to detect it at the lower dose-level of 20 μg/ml. The general failure of investigators to derive such sensitivity curves for their assays is suggested to represent one of the major factors leading to divergent assay responses in the present study (see text).

operated in a particular laboratory. An example of the potential importance of calibrating the sensitivity of an assay is represented in Fig. 23. This illustrates a hypothetical comparison for a chosen level of significance of 0.05 using two different assay protocols each of which has a 100% chance of detecting 200 μg/ml of the positive control chemical. However, based on the sensitivity curves shown, only protocol A would be likely to detect 20 μg/ml of the same control chemical, protocol B showing a $> 80\%$ chance of finding it negative. The chance of detecting a weak mutagenic response in this hypothetical laboratory would clearly be dependent upon which of the 2 assay protocols were used. This example, although hypothetical, is based upon an extensive sensitivity calibration curve observed for 2 chemicals in an SCE assay (Margolin, unpublished, 1984). Reference to the assay workgroup reports reveals that many of the protocol recommendations made are associated with this issue, e.g. the mammalian cell gene-mutation group recommendation that an adequate number of cells be employed, etc.

Finally, perhaps the greatest stimulus for improving test protocols and adopting an appropriate statistical method for data assessment is to enable responses to be interpreted with consistency and confidence, i.e., that they give information regarding the activity of a chemical as measured against a given genetic endpoint, rather than merely providing evidence concerning the sensitivity or otherwise of the assay protocol employed. In cases where the genetic activity profile of a chemical is to be considered and interpreted, this distinction becomes of paramount importance.

15. Conclusions

The dilemma that preceeded the formalization of final conclusions is simple to state but was difficult to resolve. Several carcinogens of uncertain genotoxicity were selected for this study in order to ascertain which among the many available eukaryotic in vitro assays for carcinogens would be most efficient and best suited for adoption as complements to the generally available Salmonella mutation assay by laboratories engaged in the routine screening of chemicals for potential carcinogens. The organizing committee was aware of the impact any conclusions derived from this study might have on legislative guidelines, but was primarily interested in the optimal utilization of the resources of laboratories that may be attempting to identify possible new human carcinogens. In order to provide a comprehensive study, the number or types of assays included were not limited. This non-restrictive method of collecting assays presented a unique problem; by implication, some investigators had been invited to submit their assay for scrutiny for a purpose they had not originally intended it. For example, many oncologists conduct cell transformation, aneuploidy, UDS, etc., assays for research purposes in order to study the relations of genetic end-points and the subtle nuances of the cancer process. Such research studies may have supported the selective use of a particular assay that was not claimed to be of general utility for the present purposes, and which was not being recommended as such by the investigators. It is therefore important to emphasize that the failure of an assay to qualify as a complementary assay does not preclude its use for research into the intricate processes of cancer initiation/promotion. This caution is most important to remember when whole classes of assay such as 'cell transformation', 'metabolic cooperation', etc., are dismissed from consideration herein. On the one hand is the immediate and worldwide need for efficient and reliable means for screening new chemicals leading to their registration as safe new products [60]; on the other, the need to utilize selected in vitro assays to probe the aetiology of chemically induced cancer. The present book is aimed solely at the former endeavour, but its findings might expedite the achievement of the latter. Ancillary findings of this study are first, that certain classes of in vitro assays appear to be insensitive to the cancer-related activities of certain chemical carcinogens. Second, it is suggested that a clear distinction should be made between the utilization of eukaryotic in vitro assays to confirm genotoxic activity seen previously for a chemical in bacteria, and their use to complement bacterial mutation assays, i.e. to detect genotoxins found inactive in bacteria — the choice of assay and the interpretation of data are influenced by the need originally perceived.

(1) Significant differences exist among individ-

ual investigators conducting nominally identical assays. A direct result of this study is that many recommended protocol changes were identified for all assays and some have been instituted already by some investigators. Inadequate test protocols clearly contributed to some assays appearing to be insensitive. It is likely that the need currently perceived to conduct a range of genotoxicity assays on a new chemical has been stimulated, in part, by the inadequacy of some test protocols.

(2) Urgent attention should be given to the definition of criteria for judging positive and negative test responses in all assays. Methods by which weak responses can be interpreted with confidence were identified, including the adoption of appropriately sensitive test protocols, the acquisition of a sufficiently large control database, and the use of appropriate statistical methods for data assessment.

(3) Carcinogens that are inactive or difficult to detect in the Salmonella assay fall into two distinct groups in this study. The first are genotoxins that are probably only non-mutagenic to Salmonella because of protocol deficiencies associated with the overall metabolic capacity of the assay system. These agents, HMPA, TOL, SAF and ACN, were detected by most of the eukaryotic assays studied. Thus, a range of assays can act as *limited* complements to the Salmonella mutation assay. The other group of carcinogens, BEN, DEHP, DES and PB, possessed a more selective range of genotoxic activities, and none of the assays was selectively sensitive to them. Of the assays studied, only the induction of chromosomal aberrations, transformation, gene mutations in mammalian cells and aneuploidy in yeast gave an encouraging overall performance for the 8 carcinogens (see Conclusion 9).

(4) A surprising finding of this study was the apparent mutagenicity observed for the three carcinogens BEN, DES and PB in some of the mammalian cell gene-mutation assays. These activities were not seen by the majority of investigators in this assay group and were not generally confirmed in either the Drosophila or fungal gene-mutation assays. If they prove repeatable, 3 cell-specific gene-mutagens will have been defined. If they are not repeatable this will emphasize the eed already perceived by the assay workgroup to improve the protocol and data assessment processes for this class of assay. The concomitant monitoring of two gene loci in a single cell line, and the development of appropriate and common data assessment procedures represent two areas for research with mammalian cell gene-mutation assays. The precise role of these assays in routine screening programmes requires clarification; in particular, whether they are to be used to anticipate possible mammalian carcinogens or possible mammalian mutagens, or both. The use of negative results in a mammalian cell gene-mutation assay to negate positive observations made in the Salmonella assay may be premature.

(5) A range of cell-transformation assays were studied, and the overall performance of several of them was impressive. The duplicated studies with the Syrian hamster embryo (SHE) assay and the mouse C3H10T$\frac{1}{2}$ assay gave particularly encouraging results. Nonetheless, these assays are not recommended for routine-screening purposes at this time due to the expertise required for their conduct and the remaining problems with clone selection and the judging of when chemically-induced transformation has occurred.

(6) Both of the non-carcinogens showed a low incidence of activities in vitro, several of which were confirmed and most of which were weak. The clastogenicity of caprolactam to human lymphocytes and its somatic cell mutagenicity to Drosophila were particularly clear cut. The activities observed for these agents in certain in vitro assays do not, therefore, correlate with their rodent carcinogenicity, and they thus represent false positive predictions of carcinogenicity. This underlines the fact that the utility of in vitro assays is for *predicting*, rather than *defining* carcinogenic activity.

(7) Three of the carcinogens, DES, PB and DEHP, were chosen to evaluate the hypothesis that some chemicals can induce tumours in rodents without first modifying the integrity of nuclear DNA. Although these chemicals were generally less active in the tests than the other carcinogens, they each displayed a range of genotoxic activities in vitro. DEHP showed least activity with levels similar to those observed for the two non-carcinogens. Nonetheless, it induced significant aneuploidy and cell-transformation effects in

an almost total absence of evidence for a direct interaction with DNA. The data generated for DES confirmed earlier reports that it is an aneuploidy inducing and cell-transforming agent, and the present study also defined it as a clastogen and capable of producing primary damage to DNA; it cannot, therefore, be regarded as non-genotoxic. PB was perhaps the previously best documented non-genotoxin of those studied, but it has been defined herein as a gene mutagen, a clastogen and an aneuploidy inducing agent. It is concluded that the phrase 'non-genotoxic' should only be used when a sufficiently large genotoxicity database has been collected.

(8) Many of the assays evaluated may have an important research role. However, if their main function is to act as confirmatory or complementary assays in conjunction with the Salmonella test, then substantial redundancy among in vitro eukaryotic genotoxicity assays is evident.

(9) In relation to the stated aim of this study, it is recommended that resources be applied to the definition of a generally acceptable and applicable protocol for the conduct of chromosomal aberration assays, preferably in an agreed cell type. Use of this class of assay in conjunction with an adequate assessment of the mutagenicity of a chemical to Salmonella should provide an efficient primary screen for possible new carcinogens. Certain chemicals that induce tumours in rodents at elevated dose-levels will remain undetected by these tests, as exemplified by DEHP in the present study. The 'detection' in vitro of such agents may be achieved by the incorporation of additional assays, but the price in terms of a reduction in overall specificity and the increased burden on technical resources may be high. Consideration of whether the joint inactivity of a chemical in an adequate Salmonella assay and an adequate in vitro cytogenetic assay provides evidence of its inactivity to DNA, and thus its probable non-carcinogenicity to mammals at low dose-levels, is indicated. The adoption of a chromosomal aberration assay as a common complementary test would have the additional advantages of allowing easy comparison of data, ready access to supplementary cytogenetic assays and the provision of test data derived using an independent genetic endpoint from that of the Salmonella (gene-mutation) assay.

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PART II

INVESTIGATORS' REPORTS

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11

Tests with the Salmonella plate-incorporation assay

Robert S.U. Baker and Antonio M. Bonin

Cell Biology Laboratory, Commonwealth Institute of Health, University of Sydney, New South Wales (Australia)

Summary

Using Salmonella strains TA97, TA98, TA100 and TA102, phenobarbitone was detected as weakly mutagenic in plate-incorporation assays; a statistically significant dose-related increase in revertant colonies occurred with TA100.

Although a satisfactory dose-relationship could not be established with acrylonitrile (ACN), this chemical also provided some evidence of mutagenicity; a weak though statistically significant increase in revertant colonies occurred with strain TA102.

Other carcinogens tested in this series of assays, hexamethylphosphoramide (HMPA), safrole (SAF), *o*-toluidine (TOL), diethylstilboestrol (DES), benzene (BEN) and diethylhexylphthalate (DEHP) were negative as were the presumed non-carcinogens, caprolactam (CAP) and benzoin (ZOIN).

Salmonella pour-plate, or plate-incorporation, assays were conducted as part of the Collaborative Study on Short-Term Tests for Genotoxicity and Carcinogenicity. The purpose of the present investigation was to determine the nonmutagenic or mutagenic status in Salmonella assays of test chemical batches employed in the Collaborative Study.

Compounds selected for investigation include eight known carcinogens and two noncarcinogens.

Criteria for selection of test substances are discussed elsewhere in this volume, but include complete or near-complete lack of activity in a standard Salmonella assay such as that used in the present investigation.

Materials and methods

The Salmonella plate-incorporation assay utilizing strains TA97, TA98, TA100 and TA102 was conducted according to Maron and Ames (1983) with various modifications listed below.

(1) Bacterial stock cultures were stored frozen at −70 °C, and characterized by established criteria except that diagnostic mutagens Dexon, sodium azide, ICR-191, 2,4,7-trinitro-9-fluorenone, 4-nitro-*o*-phenylenediamine and 2-aminofluorene were not used. In their place 9-aminoacridine, 2-acetylaminofluorene and aflatoxin B_1 were employed.
(2) Fresh overnight broth cultures were incubated at 37 °C in a stationary position, and shaken for 2 h on the day of each assay.
(3) S9 was stored at −70 °C for 6–20 days before use in S9 mix at a concentration of 10% v/v. Other cofactors were as given by Maron and Ames (1983).
(4) Plates containing 25 ml of minimal agar and additional top agar incorporating bacteria, test chemical with or without S9 mix were incubated at 37 °C for 72 h.
(5) Solutions and dilutions of test chemicals were prepared freshly for each test from stock chemicals (stored frozen in the case of ampuled liquids, and at room temperature in the case of solids). Four half-log dilutions were prepared in DMSO for incorporation as a 100-μl aliquot in each top agar, the maximum

test concentration having been chosen by a preliminary toxicity prescreen with strain TA100.

(6) Data was statistically evaluated using an AMES FIT program on an Apple II microcomputer (Moore and Felton, 1983). This program uses least-squares linear regression to determine the adequacy of fit of data and also assesses significance of the slope. In addition to satisfying tests for statistical significance, other criteria used to determine a response as positive in Salmonella included reproducibility of a dose-related increase in revertant colony numbers.

Results and discussion

Of test compounds clearly negative in this investigation (Tables 1 and 2), HMPA, SAF, TOL and DES had been previously assayed with TA1535, TA1537, TA1538, TA98 and TA100 and shown to be nonmutagenic in the previous International Collaborative Program (Baker and Bonin, 1981). Other compounds not detected here and reported elsewhere to be nonmutagenic in Salmonella include BEN (Dean, 1978) and DEHP (Simmon et al., 1977). Finally, the nonmutagens CAP and ZOIN are also noncarcinogens according to recent NCI Bioassay results.

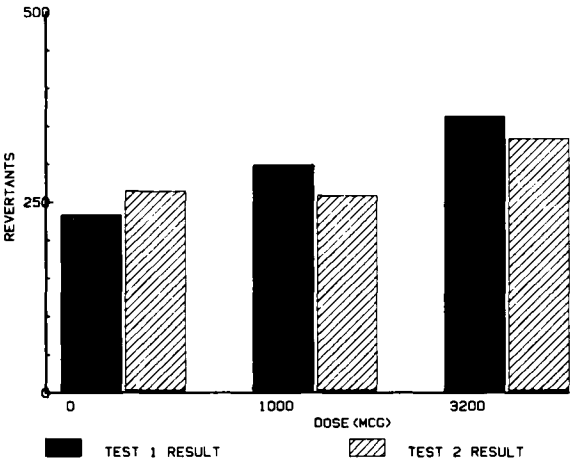

Fig. 1. Acrylonitrile in two separate tests with TA102 in the absence of S9 mix.

ACN (Fig. 1) and PB (Fig. 2) alone provide any evidence of mutagenic activity. For ACN, the effect was marginal and confirmed by statistical evaluation in two out of three tests, though a dose-related increase occurred in only one assay. ACN, therefore, does not fulfill the above criteria for positive results in Salmonella assays.

Although ACN was previously reported to be mutagenic in Salmonella (Milvy and Wolff, 1977), certain reservations were recorded against these

TABLE 1

SUMMARY OF TEST COMPOUND ACTIVITY IN SALMONELLA POUR-PLATE ASSAYS [a]

	−S9				+S9			
	TA97	TA98	TA100	TA102	TA97	TA98	TA100	TA102
HMPA	–	–	–	–	–	–	–	–
SAF	–	–	–	–	–	–	–	–
TOL	–	–	–	–	–	–	–	–
BEN	–	–	–	–	–	–	–	–
DEHP	–	–	–	–	–	–	–	–
ACN	–	–	–	? [b]	–	–	–	–
DES	–	–	–	–	–	–	–	–
PB	–	–	w+ [c]	–	–	–	–	–
CAP	–	–	–	–	–	–	–	–
ZOIN	–	–	–	–	–	–	–	–

[a] Data represents at least two separate assays of each compound. Additional tests were performed with ACN and PB.

[b] Clear dose-related increase in revertant colonies in one assay only.

[c] Positive in two out of three assays.

TABLE 2

NEGATIVE MUTAGENICITY ASSAY DATA [a]

Compound	Dose (μg/plate)	−S9				+S9			
		TA97	TA98	TA100	TA102	TA97	TA98	TA100	TA102
HMPA	0	129	31	118	237	172	42	124	358
	320	121	34	102	260	142	38	108	360
	1000	126	31	112	256	144	42	111	334
	3200	130	29	110	246	146	44	116	332
	10000	129	34	112	239	142	46	121	337
SAF	0	129	31	118	237	172	42	124	358
	3.2	142	28	108	244	139	44	112	318
	10	134	26	120	232	146	45	110	344
	32	122	25	111	249	150	43	104	318
	100	117	28	108	255	160	42	116	326
TOL	0	122	31	132	272	180	45	111	375
	320	124	36	138	278	183	40	131	332
	1000	134	35	138	260	193	40	121	380
	3200	126	30	138	262	166	46	139	368
	10000	121	32	123	261	142	44	114	335
BEN	0	122	31	132	272	180	45	111	375
	320	132	28	118	292	133	36	104	363
	1000	129	31	114	274	136	36	132	368
	3200	130	28	116	259	143	34	114	370
	10000	122	26	118	246	138	40	104	344
DEHP	0	122	31	132	272	180	45	111	375
	320	128	31	125	262	117	40	125	346
	1000	127	40	128	276	130	42	120	364
	3200	118	33	114	264	132	38	118	326
	10000	145	34	132	280	126	46	130	392
DES	0	126	35	133	269	178	37	129	366
	3.2	134	31	114	256	145	42	120	356
	10	139	35	115	251	162	40	122	360
	32	130	30	104	222	159	32	126	352
	100	122	26	98	220	136	40	112	306
CAP	0	131	40	126	252	146	49	125	368
	32	114	44	113	290	130	50	118	356
	100	120	45	126	282	122	52	122	348
	320	120	45	112	274	122	48	116	369
	1000	120	46	116	285	130	52	122	372
ZOIN	0	129	31	118	237	172	42	124	358
	32	117	32	112	225	155	36	114	338
	100	126	32	118	232	170	43	114	310
	320	137	32	106	228	168	34	114	338
	1000	132	26	118	238	190	32	111	304
S9 positive controls									
B[a]P	5					1257 ±114	670 ±98	1526 ±206	754 ±89
2-AAF	100					1559 ±118	3434 ±206	2287 ±157	502 ±38

[a] Data from the second test is presented here as the means of two separate plate counts, except for negative controls, where three plates were used. S9 positive control data for benzo[*a*]pyrene (B[a]P) and 2-acetylaminofluorene (2-AAF) represent means ±1 standard deviation ($n = 10$ plates from 5 assays).

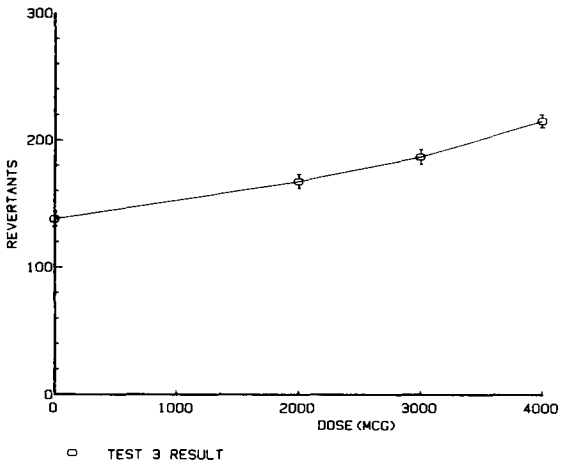

Fig. 2. Phenobarbital assay with TA100 in the absence of S9. Data represent means and standard deviations for one test using 3 plates per dose point.

results (Venitt, 1978). Moreover, Venitt et al. (1977) had tested ACN in TA1535, TA1538, TA100, *his*G46 and *his*D3052 and only detected mutagenicity in *his*G46 fluctuation assays, the results of which were reported to be erratic and to lack statistical significance (Venitt et al., 1977).

PB was also confirmed as mutagenic in two out of three tests with TA100 in the absence of S9, though without resulting in a doubling of spontaneous revertant colony numbers, even at high concentrations of test compound (Fig. 2). PB was previously reported negative in strains TA1535, TA1537, TA98 and TA100 (McCann and Ames, 1976), though other data presented in the present Collaborative Study convincingly demonstrate the genotoxicity of PB with Salmonella.

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Mutation tests on *Salmonella typhimurium* by the preincubation method

Taijiro Matsushima, Megumi Muramatsu and Mitsuru Haresaku

Department of Molecular Oncology, Institute of Medical Science, University of Tokyo (Japan)

Summary

None of the compounds tested were mutagenic on *S. typhimurium* TA98, TA100, TA97 and TA102 by the preincubation method with and without a metabolic activation system.

Mutagenicity of hexamethylphosphoramide (HMPA), safrole (SAF), *o*-toluidine (TOL), benzene (BEN), acrylonitrile (ACN), diethylhexylphthalate (DEHP), diethylstilbestrol (DES), phenobarbital (PB), caprolactam (CAP), and benzoin (ZOIN) was tested on *Salmonella typhimurium* TA98, TA100, TA97 and TA102 by the preincubation method (30 °C, 30 min) with and without a metabolic activation system (sodium phenobarbital and 5,6-benzoflavone pretreated rat-liver S9). All ten compounds tested proved to be non-mutagenic under these test conditions.

Materials and methods

Tester strains

S. typhimurium TA98, TA100, TA97 and TA102 were provided by B.N. Ames, Biochemistry Department, University of California, Berkeley, CA (U.S.A.). The stock cultures of these tester strains were stored at −80 °C in nutrient broth containing 8% DMSO. They were grown overnight (14 h) in nutrient broth (Difco) with shaking, before each test.

S9 mix

Male Sprague–Dawley rats (6 weeks old) were pretreated by an intraperitoneal injection of sodium phenobarbital and 5,6-benzoflavone (Matsushima et al., 1976). Liver homogenate (S9) was prepared by a slight modification of the Ames method (Ames et al., 1975) without starving the animals before killing. Livers were perfused with chilled saline through the portal vein and homogenized with three volumes of 0.154 M KCl using a Potter–Elvehjem homogenizer with a Teflon-pestle. The homogenate was centrifuged at 9000 g for 10 min in a refrigerated centrifuge. The S9 was stored at −80 °C until used.

The S9 mix contained 10% S9 (50 μl S9 per plate), 4 mM each NADPH and NADH, 5 mM glucose 6-phosphate, 8 mM $MgCl_2$, 33 mM KCl and 100 mM Na-phosphate buffer (pH 7.4). S9 mix was freshly prepared for each test.

Mutation tests

Mutation tests were carried out by the preincubation method (30 °C, 30 min) (Matsushima et al., 1980) with and without the metabolic activation system.

The test compound, dissolved in 0.05 ml of DMSO, was added with 0.5 ml of S9 mix or Na-phosphate buffer pH 7.4, and 0.1 ml of the tester strain. After the mixture was incubated at 30 °C for 30 min with shaking, it was rapidly mixed with 2 ml of molten top-agar containing 0.1 μmole of L-histidine and biotin, and poured onto 30 ml of Vogel–Bonner minimal glucose agar in a γ-ray sterilized 9-cm plate. After 48 h incubation at 37 °C, the number of revertant colonies was scored. For the determination of the toxicity of the test compounds, the growth of the background

TABLE 1

MUTATION TEST ON *S. typhimurium* TA98

S9 mix	Dose (μg/plate)	Experiment number											
		1		2		3		4		5			6
		HMPA	SAF	TOL	BEN	ACN	DEHP	DES	PB	CAP	ZOIN	Dose (μg/plate)	SAF
–	0	31		35		27		28		30		0	28
	20	–	–	–	–	–	–	20	–	–	–	1	27
	50	–	k	–	–	–	–	21	–	–	–	2.5	31
	100	27	k	31	39	27	29	22	27	30	16	5	23
	200	20	k	35	28	26	24	20	29	25	31	10	32
	500	34	k	32	39	23	28	23	24	32	20	25	24
	1000	50	k	40	36	22	26	19	21	40	30	50	k
	2000	36	–	42	32	16	27	–	21	37	27		
	5000	35	–	k	k	18	20	–	14	26	26		
	AF-2 (0.1)	483		618		531		508		395			455
+	0	52		55		43		41		47			
	20	–	–	–	–	–	–	55	–	–	–		
	50	–	45	–	–	–	–	64	–	–	–		
	100	45	39	62	68	55	52	46	52	37	53		
	200	57	k	69	58	38	59	52	46	58	41		
	500	50	k	68	55	43	49	41	45	42	53		
	1000	37	k	66	51	43	38	46	39	49	38		
	2000	48	–	54	53	41	53	–	42	50	49		
	5000	58	–	k	k	k	44	–	33	48	38		
	2AA (0.5)	268		343		313		175		294			

k, organism killed; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; 2AA, 2-aminoanthracene.

TABLE 2

MUTATION TEST ON *S. typhimurium* TA100

S9 mix	Dose (μg/plate)	Experiment number											
		1		2		3		4		5			6
		HMPA	SAF	TOL	BEN	ACN	DEHP	DES	PB	CAP	ZOIN	Dose (μg/plate)	SAF
–	0	122		114		101		82		114		0	130
	20	–	–	–	–	–	–	67	–	–	–	1	104
	50	–	k	–	–	–	–	57	–	–	–	2.5	103
	100	112	k	116	122	81	94	55	84	96	111	5	95
	200	100	k	123	116	76	94	k	109	116	102	10	94
	500	96	k	130	106	93	103	k	107	102	128	25	k
	1000	120	k	125	124	76	116	k	118	117	101	50	k
	2000	142	–	125	112	67	106	–	126	108	124		
	5000	105	–	k	k	57	104	–	96	109	110		
	AF-2 (0.01)	562		657		566		339		550			540
+	0	106		84		83		71		104			
	20	–	–	–	–	–	–	89	–	–	–		
	50	–	109	–	–	–	–	77	–	–	–		
	100	96	99	71	86	71	80	84	49	92	86		
	200	112	k	95	79	77	102	65	62	105	86		
	500	99	k	112	80	65	91	k	61	90	91		
	1000	106	k	115	99	85	85	k	72	93	74		
	2000	108	–	140	k	81	95	–	58	106	112		
	5000	97	–	k	k	k	76	–	52	90	114		
	2AA (0.5)	407		292		277		232		350			

k, organism killed; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; 2AA, 2-aminoanthracene.

TABLE 3

MUTATION TEST ON *S. typhimurium* TA97

S9 mix	Dose (μg/plate)	Experiment number											
		1		2		3		4		5			6
		HMPA	SAF	TOL	BEN	ACN	DEHP	DES	PB	CAP	ZOIN	Dose (μg/plate)	SAF
–	0	205		173		116		129		167		0	140
	20	–	–	–	–	–	–	111	–	–	–	1	124
	50	–	k	–	–	–	–	112	–	–	–	2.5	113
	100	163	k	158	151	131	93	91	112	144	131	5	121
	200	165	k	171	182	118	105	k	142	155	139	10	128
	500	187	k	168	215	128	145	k	135	164	143	25	k
	1000	177	k	174	194	137	145	k	121	163	129	50	k
	2000	171	–	187	176	120	131	–	112	159	154		
	5000	149	–	k	k	97	175	–	114	138	124		
	AF-2 (0.01)	413		711		392		334		388			626
+	0	250		200		161		170		188			
	20	–	–	–	–	–	–	166	–	–	–		
	50	–	196	–	–	–	–	189	–	–	–		
	100	241	194	245	175	159	158	159	181	209	198		
	200	234	k	263	181	166	172	170	179	211	206		
	500	258	k	254	173	170	162	96	191	191	194		
	1000	255	k	245	180	164	172	k	187	226	201		
	2000	266	–	240	k	k	166	–	172	226	235		
	5000	245	–	k	k	k	180	–	157	220	248		
	B(a)P (1)	507		479		506		323		440			

k, organism killed; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; B(a)P, benzo[*a*]pyrene.

TABLE 4

MUTATION TEST ON *S. typhimurium* TA102

S9 mix	Dose (μg/plate)	Experiment number											
		1		2		3		4		5			6
		HMPA	SAF	TOL	BEN	ACN	DEHP	DES	PB	CAP	ZOIN	Dose (μg/plate)	SAF
–	0	255		280		209		177		222		0	200
	20	–	–	–	–	–	–	131	–	–	–	1	215
	50	–	k	–	–	–	–	79	–	–	–	2.5	193
	100	249	k	309	320	278	220	62	216	221	212	5	196
	200	270	k	282	315	263	187	k	193	201	196	10	190
	500	227	k	289	301	219	192	k	199	215	196	25	194
	1000	259	k	265	298	207	198	k	200	216	161	50	174
	2000	256	–	236	289	203	227	–	183	222	193		
	5000	262	–	k	k	252	243	–	139	217	100		
	BLM (2)	1254		1093		816		791		899			705
+	0	353		241		270		221		320			
	20	–	–	–	–	–	–	280	–	–	–		
	50	–	380	–	–	–	–	266	–	–	–		
	100	403	358	253	223	252	292	199	295	350	325		
	200	381	255	311	212	245	272	105	265	339	303		
	500	409	k	290	215	265	280	k	286	329	282		
	1000	375	k	306	230	293	246	k	252	316	262		
	2000	370	–	289	k	257	309	–	252	312	325		
	5000	386	–	k	k	311	267	–	201	306	284		
	B(a)P (1)	611		558		269		333		–			
	B(a)P (2)	–		–		–		–		1195			
	2AA (5)	–		–		–		–		804			

k, organism killed; BLM, bleomycin; B(a)P, benzo[*a*]pyrene; 2AA, 2-aminoanthracene.

lawn of tester strain on each plate was examined with a dissecting microscope. All test compounds were tested in duplicate and the solvent control in quadruplicate.

Results

Results of mutation tests on *S. typhimurium* TA98, TA100, TA97 and TA102 by the preincubation method are shown on Tables 1, 2, 3 and 4, respectively. All 10 compounds were not mutagenic on *S. typhimurium* TA98, TA100, TA97 and TA102. Data on each dose level of test compounds and positive controls were mean of duplicate plates and those on solvent control (dose: 0) were mean of quadruplicate plates. In each experiment, two compounds were tested on the same day using all four tester strains with and without the S9 mix.

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Tests with a preincubation modification of the Salmonella/microsome assay

Errol Zeiger and Steve Haworth [a]

Cellular and Genetic Toxicology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, NC and [a] Genetic Toxicology Department, Microbiological Associates, Bethesda, MD (U.S.A.)

Summary

Eight carcinogens and two non-carcinogens were tested in Salmonella strains TA97, TA98, TA100 and TA1535 in a preincubation modification of the Salmonella/mammalian microsome assay without metabolic activation and with activation by Aroclor-1254-induced rat and hamster S9s. The carcinogens acrylonitrile, phenobarbital and *o*-toluidine were mutagenic, as was the non-carcinogen benzoin. The carcinogens benzene, di(2-ethylhexyl)phthalate, diethylstilbestrol, hexamethylphosphoramide and safrole, and the non-carcinogen, caprolactam, were not mutagenic.

Ten chemicals — eight carcinogens and two noncarcinogens — were tested in a preincubation modification of the Salmonella/mammalian microsome assay. All chemicals were tested without metabolic activation and activation by Aroclor-1254-induced rat and hamster S9, and were coded prior to testing. The codes were not broken until after the data had been evaluated and a decision made as to the chemical's mutagenicity or non-mutagenicity. The carcinogens acrylonitrile, phenobarbital, and *o*-toluidine were mutagenic, as was the noncarcinogen benzoin. The other carcinogens — benzene, di(2-ethylhexyl)phthalate, diethylstilbestrol, hexamethylphosphoramide, and safrole — and the noncarcinogen caprolactam were not mutagenic.

Materials and methods

All test chemicals were obtained from John Ashby (Table 1). The details of the protocol used are outlined in Venitt (this volume) and described, in part, in Haworth et al. (1983). Briefly, *Salmonella typhimurium* strains TA97, TA98, TA100 and TA1535 (obtained from B.N. Ames, Berkeley, CA) were used in a (20 min) preincubation procedure (Yahagi et al., 1975; Maron and Ames, 1983) without metabolic activation and with Aroclor-1254-induced S9 from male Sprague–Dawley rats and Syrian hamsters. All chemicals were tested, under code, at a minimum of five doses, in half-log intervals, up to a toxic dose or the limit of solubility, to a maximum dose of 10 mg per plate; triplicate plates were used. In the first experiment, the concentration of S9 in the S9 mix was 10%. If all tests (nonactivation [NA], rat S9 [RLI], and hamster S9 [HLI]) were negative, they were re-

TABLE 1

CHEMICALS TESTED

	CAS RN [a]	Solvent
Acrylonitrile	107-13-1	DMSO [b]
Benzene	71-43-2	DMSO
Benzoin	119-53-9	DMSO
Caprolactam	105-60-2	Water
Di(2-ethylhexyl)phthalate	117-81-7	95% Ethanol
Diethylstilbestrol	56-53-1	95% Ethanol
Hexamethylphosphoramide	680-31-9	Water
Phenobarbital	50-06-6	DMSO
Safrole	94-59-7	DMSO
o-Toluidine	95-53-4	DMSO

[a] Chemical Abstracts Service Registry Number.
[b] Dimethyl sulfoxide.

TABLE 2

POSITIVE CONTROLS

Strain	NA [a]	HLI	RLI
TA97	9-Aminoacridine 4.0 [b]	2-Aminoanthracene 0.75, 0.75, 2.0 [c]	2-Aminoanthracene 1.5, 1.5, 2.0
TA98	4-Nitro-*o*-phenylene-diamine 12.0	2-Aminoanthracene 0.75, 0.75, 2.0	2-Aminoanthracene 1.5, 1.5, 2.0
TA100	Sodium azide 2.5	2-Aminoanthracene 0.75, 0.75, 2.0	2-Aminoanthracene 1.5, 1.5, 2.0
TA1535	Sodium azide 2.5	2-Aminoanthracene 0.75, 0.75, 2.0	2-Aminoanthracene 1.5, 1.5, 2.0

[a] NA, not activated; HLI, hamster-liver S9; RLI, rat-liver S9.
[b] μg/plate.
[c] μg/plate for 5, 10, 30% S9.

peated, with the S9 concentrations at 30% instead of the original 10%. If the repeat tests were also negative, the chemical was declared nonmutagenic. If a positive result was seen, only the strain-activation combination giving the positive was repeated. If an equivocal or weak response was seen, only that strain-activation combination was repeated; if S9 was used, the repeat test concentrations were 5, 10 and 30%. On occasion, the dose levels used were changed in the repeat test based upon the response obtained in the original test. Concurrent positive and negative controls were run; the posi-

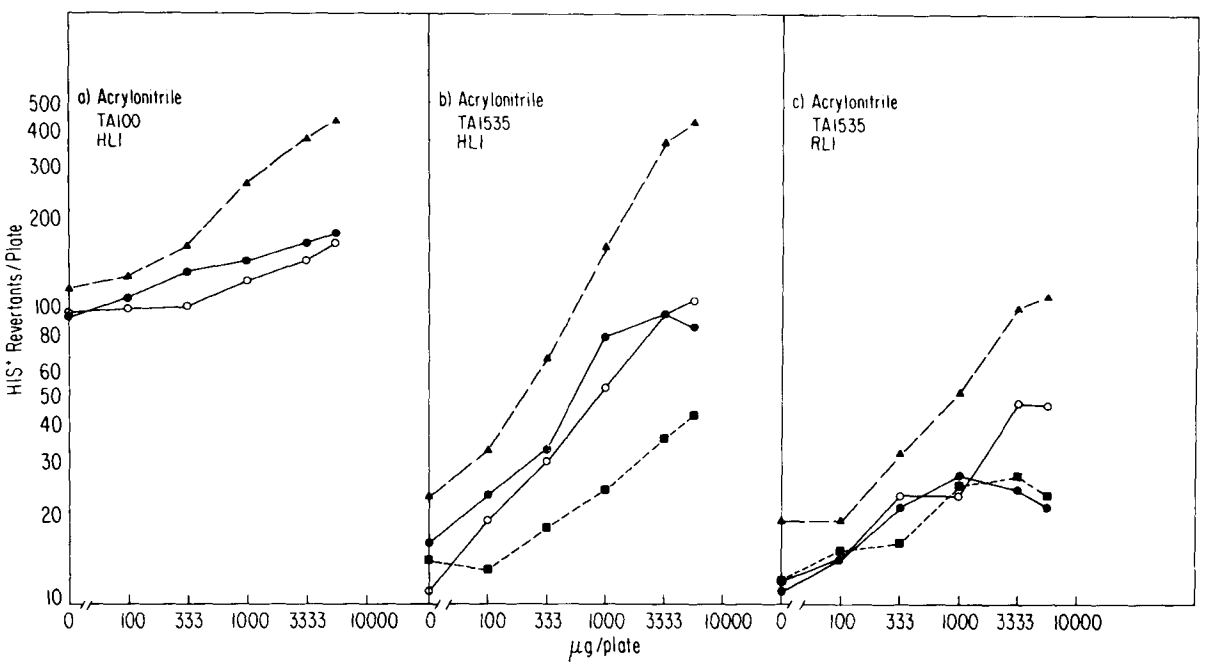

Fig. 1. Mutagenicity of acrylonitrile in: (a) TA100 with 10% (●, ○), and 30% (▲) HLI; (b) TA1535 with 5% (■), 10% (●, ○) and 30% (▲) HLI; (c) TA1535 with 5% (■), 10% (●, ○), and 30% (▲) RLI. Solid figures, first experiment; open figures, second experiment.

TABLE 3

MUTAGENIC EFFECT OF BENZENE IN SALMONELLA

Dose [a]	NA [b]	10% HLI	30% HLI	10% RLI	30% RLI	NA	10% HLI	30% HLI	10% RLI	30% RLI
	TA1535					*TA100*				
0	25 ± 3.2 [c]	10 ± 1.5	9 ± 0.3	16 ± 2.3	12 ± 2.4	105 ± 10.5	89 ± 4.2	97 ± 4.1	95 ± 2.8	109 ± 6.0
10.000	19 ± 0.9	9 ± 1.2	9 ± 2.3	10 ± 0.9	10 ± 3.5	74 ± 4.9	84 ± 5.8	103 ± 5.9	93 ± 8.7	105 ± 3.1
33.000	23 ± 3.8	11 ± 1.9	9 ± 1.3	10 ± 2.1	14 ± 0.9	87 ± 4.9	76 ± 4.3	107 ± 12.4	88 ± 6.1	92 ± 7.3
100.000	22 ± 2.2	6 ± 1.2	11 ± 0.9	16 ± 0.7	13 ± 3.2	100 ± 8.5	90 ± 8.1	101 ± 5.0	82 ± 4.3	97 ± 2.7
333.000	18 ± 2.1 [e]	12 ± 1.3	9 ± 1.8	11 ± 2.1	11 ± 1.8	102 ± 2.6	82 ± 3.2	91 ± 6.8	87 ± 2.0	94 ± 4.6
1000.000	20 ± 0.6 [e]	9 ± 0.3 [e]	9 ± 1.7 [e]	12 ± 1.2 [e]	9 ± 1.2 [e]	100 ± 4.4 [e]	73 ± 3.8 [e]	89 ± 4.0	99 ± 22.9 [e]	92 ± 6.7
Pos. [d]	898 ± 25.9	106 ± 7.7	153 ± 16.7	149 ± 4.4	100 ± 6.4	991 ± 19.8	938 ± 25.9	1316 ± 49.4	2151 ± 63.2	533 ± 33.1
	TA97					*TA98*				
0	102 ± 7.5	81 ± 3.8	140 ± 5.6	127 ± 6.7	138 ± 19.6	17 ± 1.5	27 ± 1.8	30 ± 1.2	27 ± 3.4	29 ± 1.5
10.000	104 ± 4.9	82 ± 8.8	129 ± 14.3	130 ± 6.4	173 ± 10.2	15 ± 2.7	35 ± 2.7	30 ± 3.0	28 ± 3.7	28 ± 1.2
33.000	78 ± 9.7	98 ± 6.6	117 ± 6.2	136 ± 11.6	176 ± 4.4	16 ± 1.2	31 ± 3.0	30 ± 5.0	32 ± 4.2	27 ± 3.5
100.000	97 ± 1.2	74 ± 7.8	125 ± 5.0	148 ± 6.8	181 ± 8.2	16 ± 1.3	38 ± 4.9	24 ± 1.5	30 ± 2.7	30 ± 1.7
333.000	87 ± 4.5	50 ± 8.3	114 ± 5.0	134 ± 6.9	190 ± 3.3	16 ± 2.3	32 ± 2.5	26 ± 2.4	24 ± 5.0	30 ± 1.7
1000.000	92 ± 3.2 [e]	87 ± 3.2	122 ± 4.2 [e]	117 ± 6.2 [e]	159 ± 4.5 [e]	16 ± 2.3	29 ± 1.7 [e]	33 ± 2.7	26 ± 0.6 [e]	25 ± 1.7
Pos.	513 ± 44.0	880 ± 16.0	725 ± 28.8	1167 ± 16.7	361 ± 18.8	1174 ± 8.6	760 ± 23.6	1420 ± 110.9	1579 ± 31.2	391 ± 17.6

[a] μg/plate.

[b] NA, not activated; HLI, hamster-liver S9; RLI, rat-liver S9.

[c] Mean ± S.E.

[d] Positive control.

[e] Partial clearing of background lawn.

TABLE 4

MUTAGENIC EFFECT OF DI(2-ETHYLHEXYL)PHTHALATE IN SALMONELLA

Dose [a]	NA [b]	10% HLI	30% HLI	10% RLI	30% RLI	NA	10% HLI	30% HLI	10% RLI	30% RLI
	TA1535					*TA100*				
0	20 ± 3.2 [c]	20 ± 1.2	22 ± 2.9	28 ± 3.5	24 ± 0.7	90 ± 1.7	106 ± 7.3	95 ± 5.2	119 ± 12.7	103 ± 6.3
100.000	17 ± 1.0	18 ± 1.7	14 ± 4.0	26 ± 2.1	20 ± 2.7	87 ± 2.3	113 ± 12.7	95 ± 4.8	123 ± 10.7	90 ± 11.3
333.000	18 ± 2.6	23 ± 0.7	26 ± 0.6	32 ± 2.1	20 ± 3.6	77 ± 5.5	106 ± 6.7	88 ± 6.2	116 ± 7.5	103 ± 10.5
1000.000	15 ± 3.8	21 ± 1.9	18 ± 1.0	21 ± 2.8	26 ± 2.6	74 ± 0.9	97 ± 4.6	97 ± 3.4	118 ± 5.8	108 ± 3.8
3333.000	14 ± 3.5	17 ± 3.5	19 ± 3.5	23 ± 4.9	20 ± 4.1	91 ± 5.0	105 ± 7.9	95 ± 4.7	124 ± 2.6	106 ± 3.8
10000.000	17 ± 2.1	24 ± 4.7	16 ± 0.9	22 ± 4.6	21 ± 2.7	89 ± 2.2	122 ± 4.1	99 ± 9.5	114 ± 11.5	105 ± 4.9
Pos. [d]	1031 ± 13.9	88 ± 5.5	203 ± 10.2	153 ± 7.2	130 ± 11.0	1247 ± 44.0	829 ± 28.6	1109 ± 22.4	2003 ± 7.5	525 ± 15.6
	TA97					*TA98*				
0	103 ± 4.3	132 ± 10.1	140 ± 4.4	142 ± 7.9	164 ± 9.0	16 ± 2.3	37 ± 4.7	28 ± 1.5	26 ± 1.5	30 ± 4.0
100.000	97 ± 4.3	122 ± 7.8	136 ± 3.2	137 ± 3.8	146 ± 2.4	18 ± 1.9	32 ± 0.9	25 ± 4.1	32 ± 2.1	31 ± 5.5
333.000	95 ± 4.0	111 ± 9.3	132 ± 6.1	129 ± 12.5	159 ± 5.8	18 ± 0.7	29 ± 2.7	26 ± 2.0	34 ± 3.7	36 ± 4.1
1000.000	102 ± 7.3	126 ± 10.7	144 ± 9.3	140 ± 8.1	159 ± 0.7	19 ± 2.6	32 ± 2.6	36 ± 4.3	33 ± 1.7	32 ± 1.9
3333.000	99 ± 4.1	111 ± 10.9	153 ± 7.9	139 ± 5.0	160 ± 4.1	18 ± 1.5	33 ± 4.8	36 ± 2.2	30 ± 1.3	39 ± 7.2
10000.000	104 ± 3.8	109 ± 12.7	155 ± 2.5	145 ± 6.4	163 ± 7.5	16 ± 1.8	34 ± 0.9	35 ± 3.8	30 ± 2.0	30 ± 4.5
Pos.	983 ± 23.5	768 ± 27.1	941 ± 54.5	1070 ± 49.2	491 ± 45.7	1748 ± 17.0	751 ± 23.7	540 ± 25.2	1780 ± 26.3	345 ± 30.6

[a] μg/plate.

[b] NA, not activated; HLI, hamster-liver S9; RLI, rat-liver S9.

[c] Mean ± S.E.

[d] Positive control.

TABLE 5

MUTAGENIC EFFECT OF DIETHYLSTILBESTROL IN SALMONELLA

Dose [a]	NA [b]	10% HLI	30% HLI	10% RLI	30% RLI	NA	10% HLI	30% HLI	10% RLI	30% RLI
	TA1535					*TA100*				
0	22 ± 2.6 [c]	34 ± 1.5	19 ± 3.5	34 ± 3.2	24 ± 2.7	81 ± 19.0	125 ± 6.3	106 ± 3.7	129 ± 4.4	121 ± 13.6
0.030	24 ± 1.7					92 ± 0.9				
0.100	18 ± 4.6					93 ± 4.7				
0.300	20 ± 1.9		24 ± 2.3		22 ± 3.5	91 ± 5.2		110 ± 2.6		108 ± 1.9
1.000	24 ± 0.6	34 ± 5.0	25 ± 2.1	36 ± 4.0	23 ± 3.4	94 ± 5.6	117 ± 6.4	97 ± 2.2	119 ± 8.1	107 ± 4.4
3.300	16 ± 2.4 [e]	31 ± 2.6	24 ± 1.0	32 ± 6.5	22 ± 2.6	74 ± 1.5 [e]	129 ± 3.3	117 ± 2.3	126 ± 10.5	98 ± 9.3
10.000		37 ± 4.3	25 ± 0.9	31 ± 6.3	22 ± 6.7		112 ± 5.9	101 ± 5.9	113 ± 6.8	101 ± 12.9
33.000		32 ± 4.4 [e]	21 ± 0.3	30 ± 4.4 [e]	23 ± 4.5		122 ± 10.4 [e]	114 ± 10.3	117 ± 7.1 [e]	113 ± 5.8
66.000		22 ± 2.0 [e]		26 ± 0.0 [e]			111 ± 3.7 [e]		92 ± 2.3 [e]	
Pos. [d]	1056 ± 41.9	77 ± 2.0	221 ± 15.8	101 ± 3.3	154 ± 5.4	1096 ± 23.1	919 ± 29.9	1173 ± 71.7	1124 ± 40.8	481 ± 22.5
	TA97					*TA98*				
0	106 ± 2.9	134 ± 8.3	134 ± 0.7	140 ± 9.3	141 ± 4.2	24 ± 0.9	34 ± 2.9	32 ± 1.5	43 ± 6.4	26 ± 3.8
0.030	100 ± 8.8					26 ± 2.3				
0.100	106 ± 4.9					23 ± 3.2				
0.300	100 ± 3.5		132 ± 6.4		127 ± 5.5	25 ± 0.9		35 ± 3.7		33 ± 2.3
1.000	107 ± 5.2	128 ± 5.2	129 ± 6.1	120 ± 9.0	116 ± 8.3	26 ± 1.7	40 ± 2.9	34 ± 4.3	38 ± 1.2	44 ± 2.0
3.300	86 ± 3.5 [e]	120 ± 2.2	145 ± 6.4	140 ± 7.9	110 ± 14.2	19 ± 2.1 [e]	40 ± 2.7	31 ± 1.3	42 ± 6.4	35 ± 2.0
10.000		116 ± 5.7	134 ± 5.4	142 ± 3.2	113 ± 9.9		38 ± 1.5	34 ± 4.4	44 ± 3.8	37 ± 4.4
33.000		112 ± 6.2 [e]	135 ± 5.8	121 ± 8.1 [e]	106 ± 8.1		41 ± 3.2	33 ± 2.9	42 ± 1.2	36 ± 4.9
66.000		119 ± 2.4 [e]		104 ± 2.3 [e]			34 ± 2.7 [e]		42 ± 4.2 [e]	
Pos.	739 ± 18.0	694 ± 4.3	521 ± 26.2	936 ± 37.6	432 ± 36.3	1736 ± 91.7	718 ± 22.8	1208 ± 42.8	958 ± 34.4	412 ± 18.9

[a] μg/plate.

[b] NA, not activated; HLI, hamster-liver S9; RLI, rat-liver S9.

[c] Mean ± S.E.

[d] Positive control.

[e] Partial clearing of background lawn.

TABLE 6

MUTAGENIC EFFECT OF HEXAMETHYLPHOSPHORAMIDE IN SALMONELLA

Dose [a]	NA [b]	10% HLI	30% HLI	10% RLI	30% RLI	NA	10% HLI	30% HLI	10% RLI	30% RLI
	TA1535					*TA100*				
0	20 ± 1.2 [c]	12 ± 1.3	8 ± 1.5	7 ± 4.1	11 ± 2.7	109 ± 1.5	101 ± 3.0	105 ± 8.9	118 ± 7.1	108 ± 9.3
100.000	22 ± 3.2	9 ± 1.9	9 ± 1.7	8 ± 2.2	11 ± 2.0	100 ± 2.5	97 ± 2.5	102 ± 1.0	114 ± 8.8	112 ± 2.1
333.000	29 ± 5.2	10 ± 2.3	11 ± 1.0	8 ± 1.0	9 ± 2.3	100 ± 0.3	102 ± 8.4	104 ± 7.3	94 ± 1.9	110 ± 0.9
1000.000	22 ± 4.4	12 ± 0.3	9 ± 0.6	9 ± 1.0	14 ± 1.5	89 ± 2.3	109 ± 10.8	100 ± 11.0	109 ± 11.1	114 ± 3.8
3333.000	27 ± 2.3	10 ± 3.7	15 ± 1.2	9 ± 1.2	11 ± 1.2	102 ± 8.4	95 ± 5.7	99 ± 4.2	102 ± 2.8	110 ± 3.2
10000.000	23 ± 3.4	11 ± 2.2	10 ± 2.5	10 ± 0.3	15 ± 1.3	114 ± 2.3	99 ± 3.7	89 ± 8.3	99 ± 1.8	106 ± 1.7
Pos. [d]	865 ± 20.5	117 ± 6.9	254 ± 11.6	128 ± 1.9	149 ± 4.2	1086 ± 14.5	1177 ± 31.7	1258 ± 30.8	1667 ± 50.6	621 ± 23.0
	TA97					*TA98*				
0	60 ± 1.2	112 ± 5.8	90 ± 5.2	130 ± 6.4	155 ± 2.9	14 ± 2.0	27 ± 5.9	32 ± 5.0	25 ± 2.3	31 ± 4.4
100.000	80 ± 1.5	127 ± 6.1	110 ± 6.1	139 ± 8.1	156 ± 4.9	15 ± 1.2	28 ± 0.6	30 ± 2.1	26 ± 1.7	28 ± 1.9
333.000	31 ± 4.2	109 ± 11.6	120 ± 2.3	105 ± 3.0	148 ± 7.6	18 ± 2.2	23 ± 0.9	27 ± 2.6	29 ± 3.6	29 ± 2.1
1000.000	56 ± 8.3	126 ± 9.4	114 ± 9.6	148 ± 1.9	169 ± 4.3	13 ± 1.2	27 ± 0.6	27 ± 2.9	27 ± 1.7	32 ± 0.9
3333.000	69 ± 5.0	124 ± 9.1	97 ± 6.7	138 ± 9.1	149 ± 7.8	17 ± 1.8	25 ± 2.2	23 ± 4.2	27 ± 2.3	27 ± 2.0
10000.000	64 ± 7.4	120 ± 2.7	122 ± 8.3	141 ± 4.4	172 ± 9.4	18 ± 1.3	23 ± 2.6	34 ± 3.8	25 ± 4.7	26 ± 2.3
Pos.	944 ± 22.6	530 ± 1.8	654 ± 2.8	868 ± 24.0	511 ± 13.5	2099 ± 71.7	994 ± 4.0	1210 ± 54.1	1247 ± 18.5	565 ± 22.2

[a] μg/plate.

[b] NA, not activated; HLI, hamster-liver S9; RLI, rat-liver S9.

[c] Mean ± S.E.

[d] Positive control.

TABLE 7

MUTAGENIC EFFECT OF SAFROLE IN SALMONELLA

Dose [a]	NA [b]	10% HLI	30% HLI	10% RLI	30% RLI	NA	10% HLI	30% HLI	10% RLI	30% RLI
	TA1535					*TA100*				
0	17 ± 1.5 [c]	11 ± 2.1	8 ± 2.1	12 ± 1.5	12 ± 1.8	83 ± 7.1	101 ± 9.2	83 ± 5.5	111 ± 5.0	90 ± 7.0
1.000	19 ± 1.5		8 ± 0.6		6 ± 1.3	94 ± 4.9		72 ± 2.0		101 ± 2.0
3.300	18 ± 3.4	9 ± 0.7	13 ± 2.7	18 ± 1.7	8 ± 1.9	82 ± 4.7	86 ± 4.4	92 ± 6.6	116 ± 4.5	98 ± 2.3
10.000	14 ± 1.9	16 ± 1.2	7 ± 1.0	16 ± 1.8	11 ± 2.0	96 ± 7.3	106 ± 14.1	86 ± 2.8	103 ± 10.4	101 ± 7.2
33.000	15 ± 1.8	16 ± 0.9	7 ± 2.4	18 ± 0.6	9 ± 3.0	83 ± 3.8	102 ± 0.9	90 ± 6.5	114 ± 6.0	90 ± 4.4
67.000	17 ± 1.7 [e]					65 ± 4.2 [e]				
100.000		13 ± 1.2	11 ± 1.8	13 ± 1.9	13 ± 1.7		115 ± 3.5	93 ± 8.2	109 ± 9.3	95 ± 6.3
200.000		12 ± 1.2 [e]		13 ± 2.1 [e]			83 ± 5.5 [e]		108 ± 10.7 [e]	
Pos. [d]	957 ± 29.5	132 ± 4.5	245 ± 6.0	132 ± 2.0	141 ± 5.5	1153 ± 22.8	1020 ± 44.7	1253 ± 29.1	1376 ± 33.9	593 ± 20.1
	TA97					*TA98*				
0	63 ± 4.3	105 ± 8.6	99 ± 4.8	138 ± 6.1	152 ± 2.1	11 ± 3.8	32 ± 1.9	24 ± 3.2	32 ± 2.3	21 ± 1.8
1.000	80 ± 10.1		111 ± 2.9		141 ± 4.4	14 ± 1.7		23 ± 2.4		24 ± 5.8
3.300	74 ± 0.6	99 ± 3.2	108 ± 6.1	134 ± 6.4	154 ± 4.6	17 ± 3.8	30 ± 1.2	27 ± 1.7	33 ± 3.8	24 ± 1.0
10.000	72 ± 3.1	109 ± 9.6	99 ± 9.3	92 ± 5.8	143 ± 5.2	11 ± 1.0	36 ± 3.8	28 ± 3.1	32 ± 3.8	24 ± 0.7
33.000	68 ± 11.9	125 ± 6.7	108 ± 6.6	143 ± 6.7	106 ± 8.3	16 ± 2.1	39 ± 2.6	20 ± 0.0	31 ± 2.6	28 ± 5.2
67.000	56 ± 2.7 [e]					8 ± 2.3 [e]				
100.000		120 ± 12.2 [e]	113 ± 0.9	113 ± 5.8	103 ± 6.7		47 ± 4.9	29 ± 1.5	37 ± 2.5	33 ± 2.9
200.000		101 ± 9.8 [e]		113 ± 14.9 [e]			t [f]		31 ± 2.1 [e]	
Pos.	594 ± 93.3	957 ± 19.5	581 ± 10.4	1266 ± 52.5	545 ± 13.6	2292 ± 42.4	702 ± 30.9	1557 ± 30.3	932 ± 22.4	654 ± 3.8

[a] μg/plate.

[b] NA, not activated; HLI, hamster-liver S9; RLI, rat-liver S9.

[c] Mean ± S.E.

[d] Positive control.

[e] Partial clearing of background lawn.

[f] Toxic effect.

TABLE 8

MUTAGENIC EFFECT OF CAPROLACTAM IN SALMONELLA

Dose [a]	NA [b]	10% HLI	30% HLI	10% RLI	30% RLI	NA	10% HLI	30% HLI	10% RLI	30% RLI
	TA1535					*TA100*				
0	20 ± 3.5 [c]	12 ± 1.8	8 ± 1.0	11 ± 1.0	13 ± 2.1	106 ± 4.4	93 ± 5.4	92 ± 9.9	87 ± 1.2	107 ± 1.9
100.000	20 ± 6.2	11 ± 1.2	14 ± 2.3	12 ± 3.4	14 ± 1.2	86 ± 2.6	74 ± 4.5	102 ± 5.9	93 ± 2.0	100 ± 3.6
333.000	16 ± 2.9	10 ± 0.9	12 ± 1.7	11 ± 2.3	14 ± 1.7	94 ± 1.3	78 ± 5.4	88 ± 4.4	81 ± 4.3	102 ± 0.7
1000.000	20 ± 1.3	14 ± 1.3	14 ± 4.4	11 ± 1.9	15 ± 1.2	85 ± 8.0	84 ± 5.8	92 ± 3.9	85 ± 9.1	106 ± 8.1
3333.000	23 ± 2.6	12 ± 3.3	10 ± 2.5	12 ± 4.3	16 ± 0.3	86 ± 3.4	87 ± 4.3	95 ± 7.7	84 ± 7.0	100 ± 5.0
10000.000	25 ± 5.2	13 ± 1.2	12 ± 2.9	12 ± 1.8	16 ± 1.8	101 ± 7.8	86 ± 5.6	94 ± 6.4	77 ± 2.6	101 ± 3.2
Pos. [d]	1068 ± 35.9	199 ± 9.8	298 ± 15.2	163 ± 4.5	155 ± 4.3	1406 ± 32.0	1446 ± 21.5	1262 ± 28.5	2102 ± 57.6	559 ± 18.8
	TA97					*TA98*				
0	108 ± 2.0	131 ± 6.5	182 ± 3.7	132 ± 3.3	207 ± 3.5	25 ± 7.5	50 ± 2.4	29 ± 1.3	49 ± 3.5	31 ± 2.8
100.000	104 ± 3.2	124 ± 7.0	186 ± 7.1	132 ± 6.2	192 ± 7.6	18 ± 0.9	51 ± 7.5	29 ± 2.3	53 ± 4.9	27 ± 5.5
333.000	110 ± 10.9	124 ± 9.1	178 ± 13.4	129 ± 3.3	201 ± 9.5	17 ± 2.2	39 ± 2.1	29 ± 1.0	27 ± 1.8	28 ± 1.2
1000.000	102 ± 7.0	137 ± 1.5	175 ± 6.7	134 ± 2.3	185 ± 11.7	18 ± 3.2	38 ± 2.8	31 ± 3.5	33 ± 2.1	36 ± 5.7
3333.000	87 ± 7.2	140 ± 7.9	184 ± 6.6	120 ± 8.6	195 ± 16.5	20 ± 3.2	30 ± 2.7	30 ± 1.5	29 ± 2.5	27 ± 2.1
10000.000	96 ± 5.2	135 ± 10.5	164 ± 13.0	113 ± 8.5	171 ± 2.6	17 ± 3.5	30 ± 3.0	28 ± 4.7	33 ± 2.1	24 ± 5.5
Pos.	336 ± 28.4	1268 ± 13.5	850 ± 11.0	1058 ± 48.1	461 ± 22.6	1772 ± 30.4	1517 ± 30.4	1232 ± 66.7	1944 ± 97.0	411 ± 6.5

[a] μg/plate.

[b] NA: not activated; HLI: hamster liver S9; RLI: rat liver S9.

[c] Mean ± S.E.

[d] Positive control.

TABLE 9

MUTAGENIC EFFECT OF BENZOIN IN SALMONELLA

Dose [a]	NA [b]	NA	NA	5% HLI	10% HLI	5% RLI	10% RLI	NA	10% HLI	10% RLI
	TA1535							*TA100*		
0	29 ± 2.1 [c]	16 ± 3.2	22 ± 2.2	14 ± 2.0	12 ± 0.7	18 ± 0.3	12 ± 3.2	97 ± 1.5	87 ± 4.2	101 ± 6.2
33.000	29 ± 2.8							95 ± 2.4		
100.000	33 ± 2.9	26 ± 2.1	24 ± 2.3	24 ± 2.5	14 ± 3.7	16 ± 4.6	12 ± 1.7	109 ± 4.3	77 ± 1.2	103 ± 2.4
333.000	34 ± 4.9	24 ± 4.0	32 ± 6.6	18 ± 1.5	12 ± 1.3	18 ± 0.3	9 ± 2.0	118 ± 5.2	74 ± 5.0	92 ± 2.3
500.000		36 ± 3.8	31 ± 0.6	17 ± 3.3		21 ± 1.3				
750.000		28 ± 2.0 [e]	45 ± 1.7	18 ± 2.2		21 ± 0.9				
1000.000	62 ± 5.9	31 ± 3.2 [e]	37 ± 1.8 [e]	20 ± 1.2	18 ± 2.0	21 ± 1.5	13 ± 2.1	141 ± 4.2	71 ± 7.8	99 ± 6.4
2000.000	45 ± 5.2 [c]	24 ± 4.0 [e]	26 ± 0.9 [c]	5 ± 1.7 [c]		19 ± 0.7 [e]		102 ± 6.6 [e]		
3333.000					10 ± 0.3 [e]		11 ± 2.3 [e]		66 ± 6.6 [e]	89 ± 10.1 [e]
10000.000					10 ± 1.2 [e]		9 ± 1.3 [e]		68 ± 3.6 [e]	85 ± 5.3 [e]
Pos. [d]	1019 ± 16.5	964 ± 25.2	854 ± 37.3	123 ± 6.7	134 ± 11.3	157 ± 11.0	157 ± 10.1	1243 ± 34.3	1203 ± 11.2	1638 ± 17.2

Dose	NA	10% HLI	10% RLI	NA	10% HLI	10% RLI
	TA97			*TA98*		
0	105 ± 10.7	131 ± 6.4	141 ± 14.4	19 ± 4.1	36 ± 2.7	35 ± 2.7
33.000	97 ± 3.8			14 ± 1.2		
100.000	97 ± 3.1	129 ± 5.6	133 ± 3.2	19 ± 1.2	25 ± 3.0	25 ± 3.8
333.000	104 ± 6.4	127 ± 10.5	119 ± 4.2	17 ± 1.2	22 ± 2.7	31 ± 4.2
1000.000	95 ± 1.5	139 ± 8.3	138 ± 4.9	10 ± 1.5	19 ± 1.9	33 ± 2.7
2000.000	85 ± 4.2 [c]			18 ± 2.8 [e]		
3333.000		119 ± 10.9 [e]	114 ± 9.2 [e]		20 ± 2.0 [e]	26 ± 2.0 [e]
10000.000		93 ± 4.1 [e]	94 ± 2.4 [e]		17 ± 3.0 [e]	20 ± 3.8 [e]
Pos.	513 ± 18.4	797 ± 36.7	1076 ± 15.6	2349 ± 116.2	1408 ± 55.9	1820 ± 24.3

[a] μg/plate.

[b] NA, not activated; HLI, hamster-liver S9; RLI, rat-liver S9.

[c] Mean ± S.E.

[d] Positive control.

[e] Partial clearing of background lawn.

tive controls used and their concentrations are given in Table 2.

A positive response was defined as a dose-related increase over the control, regardless of its magnitude; an equivocal response was a nondose-related increase over the control or an elevated response at only a single dose. A chemical was declared mutagenic if it gave a reproducible, dose-related increase in any strain-activation combination.

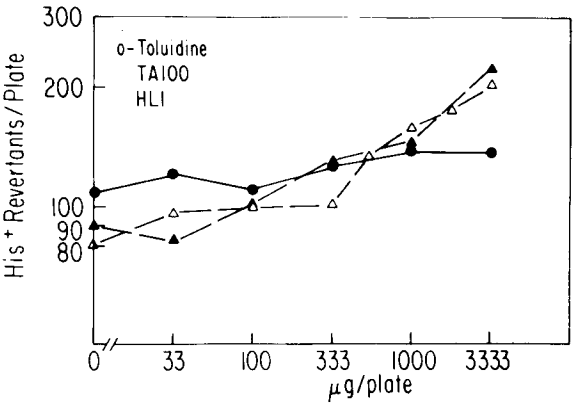

Fig. 2. Mutagenicity of *o*-toluidine in TA100 with 10% (●) and 30% (▲, △) HLI. Solid figures, first experiment; open figures, second experiment.

TABLE 10

MUTAGENIC EFFECT OF ACRYLONITRILE IN SALMONELLA

Dose [a]	NA [b]	5% HLI	10% HLI	30% HLI	5% RLI	10% RLI	30% RLI
	TA1535						
0	25 ± 1.0 [c]	14 ± 1.2	11 ± 2.3	23 ± 4.2	12 ± 3.4	12 ± 3.5	19 ± 1.2
100.000	25 ± 1.8	13 ± 1.2	19 ± 2.6	33 ± 2.1	15 ± 0.9	14 ± 3.1	19 ± 1.2
333.000	23 ± 0.6	18 ± 3.2	30 ± 2.1	67 ± 4.2	16 ± 3.2	23 ± 4.4	32 ± 1.9
1000.000	18 ± 0.3	24 ± 2.1	53 ± 3.2	161 ± 7.2	25 ± 2.1	23 ± 1.2	51 ± 3.8
3333.000	10 ± 2.5	36 ± 3.5	95 ± 5.2	364 ± 19.7	27 ± 2.7	47 ± 6.4	98 ± 8.6
6666.000		43 ± 7.5 [e]	105 ± 3.9	432 ± 13.0	23 ± 1.5	46 ± 3.2 [c]	107 ± 3.5
10000.000	6 ± 0.6 [e]						
Pos. [d]	829 ± 35.5	161 ± 5.9	118 ± 9.0	229 ± 14.4	173 ± 10.7	134 ± 2.7	137 ± 7.2
	TA100						
0	99 ± 5.6	94 ± 1.5	95 ± 2.1	115 ± 9.3	90 ± 10.2	93 ± 4.6	93 ± 3.1
100.000	96 ± 4.9	89 ± 7.4	98 ± 7.1	126 ± 7.2	97 ± 6.0	100 ± 4.3	100 ± 5.7
333.000	89 ± 0.9	75 ± 8.7	100 ± 6.3	161 ± 4.2	95 ± 7.1	104 ± 2.6	109 ± 4.4
1000.000	71 ± 4.7	102 ± 7.8	122 ± 12.5	260 ± 9.1	94 ± 2.4	106 ± 6.9	125 ± 2.0
3333.000	60 ± 6.9	100 ± 5.5	144 ± 7.3	373 ± 8.1	96 ± 6.0	109 ± 9.2	140 ± 1.5
6666.000		101 ± 4.3	165 ± 7.6	435 ± 8.2	108 ± 3.8	104 ± 7.0	124 ± 5.1
10000.000	61 ± 4.4 [e]						
Pos.	1188 ± 67.7	3138 ± 158.3	1197 ± 90.7	1115 ± 18.3	2871 ± 62.8	1523 ± 24.1	509 ± 5.6

Dose	NA	10% HLI	10% RLI	NA	10% HLI	10% RLI
	TA97			*TA98*		
0	65 ± 3.3	102 ± 1.8	124 ± 8.0	15 ± 0.3	33 ± 2.7	30 ± 2.5
100.000	82 ± 3.5	101 ± 4.0	120 ± 8.3	16 ± 3.5	27 ± 1.2	26 ± 2.7
333.000	81 ± 6.5	104 ± 1.7	110 ± 8.4	18 ± 0.9	24 ± 1.2	28 ± 3.9
1000.000	59 ± 8.5	112 ± 8.9	111 ± 14.6	17 ± 3.8	24 ± 1.5	20 ± 4.1
3333.000	51 ± 9.0	96 ± 11.3	95 ± 6.0	15 ± 0.7	27 ± 7.4	27 ± 5.2
6666.000		83 ± 3.5 [e]	100 ± 10.9 [e]		11 ± 2.6	24 ± 0.6
10000.000	25 ± 1.5 [e]			6 ± 1.5 [e]		
Pos.	583 ± 103.1	476 ± 36.2	578 ± 15.9	1374 ± 46.6	846 ± 19.8	1128 ± 54.0

[a] μg/plate.
[b] NA, not activated; HLI, hamster-liver S9; RLI, rat-liver S9.
[c] Mean ± S.E.
[d] Positive control.
[e] Partial clearing of background lawn.

TABLE 11

MUTAGENIC EFFECT OF PHENOBARBITAL IN SALMONELLA

Dose [a]	NA [b]	NA	NA	5% HLI	5% HLI	10% HLI	5% RLI	10% RLI	
	TA1535								
0	35 ± 1.2 [c]	17 ± 1.5	26 ± 2.5	9 ± 0.6	11 ± 2.3	11 ± 1.2	12 ± 3.5	16 ± 1.5	
100.000	30 ± 2.7	23 ± 0.7	26 ± 2.0	11 ± 0.9	14 ± 2.0	11 ± 2.7	14 ± 1.2	15 ± 5.2	
333.000	49 ± 7.0	33 ± 3.2	44 ± 5.0	15 ± 2.6	18 ± 1.9	9 ± 1.7	14 ± 3.2	14 ± 1.8	
500.000		37 ± 2.4		19 ± 2.3					
1000.000	59 ± 7.0	45 ± 0.9	46 ± 10.2	14 ± 2.4	19 ± 1.7	16 ± 0.7	14 ± 0.3	18 ± 1.3	
3333.000	47 ± 4.6	37 ± 3.2	40 ± 2.0 [e]	16 ± 0.9	21 ± 3.1	10 ± 0.3	17 ± 3.5	14 ± 1.8	
6666.000	28 ± 3.7 [e]	39 ± 7.0 [e]	40 ± 0.7 [e]	18 ± 1.5 [e]	t [f]	4 ± 1.3 [e]	14 ± 0.0 [e]	t	
Pos. [d]	831 ± 16.6	1014 ± 37.9	909 ± 21.7	126 ± 9.2	137 ± 14.0	105 ± 3.5	121 ± 11.3	119 ± 7.3	
Dose	NA	10% HLI	10% RLI	NA	10% HLI	10% RLI	NA	10% HLI	10% RLI
	TA100			*TA97*			*TA98*		
0	130 ± 17.1	98 ± 4.1	105 ± 8.7	106 ± 8.8	97 ± 4.1	129 ± 8.0	15 ± 1.8	29 ± 0.9	27 ± 1.5
100.000	126 ± 7.7	84 ± 2.2	97 ± 3.8	110 ± 13.5	102 ± 2.9	118 ± 9.0	16 ± 1.5	26 ± 1.5	28 ± 3.4
333.000	150 ± 3.1	83 ± 0.6	99 ± 6.8	116 ± 4.5	108 ± 5.5	126 ± 12.0	12 ± 0.6	28 ± 3.6	29 ± 1.5
1000.000	162 ± 4.3	92 ± 6.2	95 ± 6.1	95 ± 6.0	98 ± 5.4	120 ± 2.0	18 ± 0.7	27 ± 3.5	33 ± 2.0
3333.000	159 ± 5.5	88 ± 3.1	95 ± 3.8	18 ± 2.9	99 ± 8.3	85 ± 17.7	13 ± 1.5	30 ± 1.9	36 ± 2.6
6666.000	78 ± 5.5 [e]	60 ± 7.5 [e]	t	1 ± 0.3 [e]	t	46 ± 18.5 [e]	12 ± 2.1 [e]	21 ± 7.2 [e]	18 ± 1.9 [e]
Pos.	1039 ± 14.6	923 ± 24.3	1216 ± 18.9	936 ± 42.4	510 ± 12.7	631 ± 40.0	1455 ± 48.1	880 ± 25.6	1100 ± 53.7

[a] μg/plate.

[b] NA, not activated; HLI, hamster-liver S9; RLI, rat-liver S9.

[c] Mean ± S.E.

[d] Positive control.

[e] Partial clearing of background lawn.

[f] Toxic effect.

TABLE 12

MUTAGENIC EFFECT OF *o*-TOLUIDINE IN SALMONELLA

Dose [a]	NA [b]	10% HLI	30% HLI	30% HLI	10% RLI	30% RLI	NA	10% HLI	30% HLI	10% RLI	30% RLI
	TA1535						*TA100*				
0	22 ± 0.6 [c]	12 ± 1.5	9 ± 1.5	17 ± 1.9	14 ± 1.5	16 ± 1.8	84 ± 5.0	109 ± 9.9	81 ± 1.7	114 ± 10.1	86 ± 8.7
33.000	21 ± 1.0	11 ± 1.2	11 ± 1.2	9 ± 2.6	7 ± 1.5	12 ± 0.7	71 ± 2.2	121 ± 3.0	97 ± 8.6	111 ± 5.9	87 ± 7.2
100.000	24 ± 2.3	11 ± 1.2	11 ± 2.2	16 ± 1.2	11 ± 0.9	11 ± 1.5	71 ± 2.0	111 ± 6.1	100 ± 2.7	109 ± 7.1	88 ± 4.0
333.000	19 ± 3.0	10 ± 2.0	12 ± 2.1	17 ± 1.2	13 ± 2.9	10 ± 1.2	84 ± 5.0	127 ± 4.0	102 ± 2.7	120 ± 6.4	91 ± 2.0
500.000				15 ± 1.3					135 ± 8.2		
1 000.000	20 ± 2.4	13 ± 2.5 [e]	18 ± 1.8	18 ± 2.9	13 ± 1.5 [e]	11 ± 0.9 [e]	91 ± 3.8	139 ± 5.1	159 ± 6.0	117 ± 6.9	87 ± 2.9
2 000.000				30 ± 1.9					176 ± 6.7		
3 333.000	20 ± 2.8 [e]	20 ± 2.9 [e]	21 ± 1.3 [e]	21 ± 2.1 [e]	15 ± 2.3 [e]	11 ± 4.6 [e]	68 ± 12.0 [e]	137 ± 8.4 [e]	204 ± 14.7 [e]	114 ± 9.5 [e]	88 ± 0.9 [e]
Pos. [d]	761 ± 10.4	107 ± 0.9	175 ± 4.4	314 ± 15.9	128 ± 3.6	139 ± 8.7	1 159 ± 11.7	1 052 ± 30.7	1 339 ± 32.5	1 519 ± 30.1	484 ± 7.0
Dose		NA	10% HLI	30% HLI	10% RLI	30% RLI	NA	10% HLI	30% HLI	10% RLI	30% RLI
		TA97					*TA98*				
0		70 ± 2.0	112 ± 7.5	106 ± 6.2	122 ± 9.1	141 ± 10.8	12 ± 2.6	38 ± 4.6	29 ± 3.2	35 ± 0.3	28 ± 4.1
33.000		72 ± 3.5	107 ± 13.5	108 ± 8.2	112 ± 5.8	135 ± 12.1	13 ± 1.2	47 ± 4.3	28 ± 5.6	39 ± 4.6	26 ± 5.5
100.000		64 ± 5.5	111 ± 11.5	108 ± 7.5	113 ± 9.2	105 ± 4.6	13 ± 0.9	46 ± 1.5	31 ± 1.8	35 ± 3.0	30 ± 1.0
333.000		53 ± 3.2	126 ± 3.4	104 ± 7.8	127 ± 9.8	106 ± 6.2	14 ± 1.3	29 ± 4.1	26 ± 3.0	31 ± 2.1	35 ± 3.2
1 000.000		46 ± 2.4 [e]	110 ± 6.7	105 ± 1.2 [e]	114 ± 8.9	111 ± 3.5	12 ± 2.9 [e]	36 ± 0.6	39 ± 7.5	40 ± 2.1	28 ± 0.7
3 333.000		14 ± 6.3 [e]	106 ± 4.9 [e]	101 ± 1.7 [e]	100 ± 3.5 [e]	87 ± 7.0 [e]	10 ± 0.6 [e]	28 ± 5.1	41 ± 5.8 [e]	42 ± 2.6 [e]	24 ± 2.8 [e]
Pos.		263 ± 9.8	863 ± 16.9	615 ± 45.1	1 214 ± 20.5	409 ± 11.1	1 236 ± 8.5	541 ± 22.3	854 ± 17.8	899 ± 23.8	358 ± 9.2

[a] μg/plate.

[b] NA, not activated; HLI, hamster-liver S9; RLI, rat-liver S9.

[c] Mean ± S.E.

[d] Positive control.

[e] Partial clearing of background lawn.

Results and discussion

The carcinogens benzene, di(2-ethylhexyl) phthalate, diethylstilbestrol, hexamethylphosphoramide and safrole, and the noncarcinogen caprolactam, were not mutagenic under the conditions of the assay (Tables 3–8). The noncarcinogen benzoin (Table 9), and the carcinogens acrylonitrile (Table 10), phenobarbital (Table 11), and *o*-toluidine (Table 12) were all mutagenic in at least one strain-activation combination. Where multiple experiments run on specific strain-activation combinations produced the same results, only the last set of data are given in Tables 3–12. Where repeat experiments produced equivocal or contradictory results, all experiments are presented.

Benzoin

This noncarcinogen produced a weak but reproducible response in TA1535 without activation, and an equivocal response in the one test of TA100 without activation. These responses were seen at dose levels that approached a toxic dose in a preliminary experiment by the method of Waleh et al. (1982). S9 abolished the mutagenic responses (Table 9).

Acrylonitrile

This carcinogen was clearly mutagenic in TA1535 with both rat and hamster liver S9, and in TA100 with hamster liver S9 (Fig. 1). The positive responses required S9 and increased with increasing S9 concentrations. No mutagenicity was obtained without activation, or in TA97 or TA98 (Table 10).

Phenobarbital

Phenobarbital was mutagenic only in TA1535, producting weak, but reproducible responses without metabolic activation; a weak response was seen with 5% HLI (Table 11). These responses were at dose levels that produced no toxicity in a preliminary experiment by the method of Waleh et al. (1982). A low level mutagenic reponse was obtained with TA100 without S9, but this test was not repeated. TA97 and TA98 showed no increases over negative control values.

o-Toluidine

This carcinogen was mutagenic in TA100 (Fig. 2) and weakly mutagenic in TA1535; both strains responded with HLI only. No mutagenicity was seen with RLI or without S9 (Table 12). Both TA100 and TA1535 were mutagenic with 30% HLI. No mutagenicity was seen in TA97 or TA98.

The low-level responses obtained, and the dependence, in some cases, on higher levels of S9 than are generally recommended in the past (Ames et al., 1975; Maron and Ames, 1983) demonstrate the need to test chemicals at more than one concentration of S9, and with S9 from a second species in addition to rat. They also show that some mutagens are more readily detected in TA1535 than in TA100 and, therefore, TA1535 should not be discarded, contrary to the recommendations of Maron and Ames (1983). The weak mutagenic activity of the noncarcinogen benzoin, which was not seen by the other laboratories using Salmonella, (this volume) deserves further study.

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Mutation tests with Salmonella using the plate-incorporation assay

M.A. Rexroat and G.S. Probst

Toxicology Division, Lilly Research Laboratories, Greenfield, IN (U.S.A.)

Summary

Ten suspect genotoxins (hexamethylphosphoramide, safrole, *o*-toluidine, benzene, diethylhexylphthalate, acrylonitrile, diethylstilbestrol, phenobarbital, caprolactam and benzoin were tested for the induction of bacterial mutation using five histidine auxotrophs of *Salmonella typhimurium* according to the method of Ames et al. (1975). The test was conducted with and without metabolic activation using an S9 fraction prepared from the livers of Aroclor-1254-induced rats. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 2-nitrofluorene (2NF) and 9-aminoacridine (9AmAc) served as the positive controls for the nonactivated test, while 2-aminoanthracene (2AA) served as the positive control for the activated test.

Treatment with MNNG, 2NF, 9AmAc and 2AA resulted in a dose-related induction of reverse mutations in the appropriate Salmonella tester strains in the nonactivated and activated tests. Treatment with phenobarbital resulted in the induction of Salmonella revertants in strain TA1535 and metabolic activation was not required. Either with or without metabolic activation no revertants were induced by treatment with hexamethylphosphoramide, safrole, *o*-toluidine, benzene, diethylhexylphthalate, acrylonitrile, diethylstilbestrol, caprolactam or benzoin.

Bacterial test systems designed to identify the mutagenic potential of a chemical substance are employed in toxicology as predictive tests of potential carcinogenicity. Because of their simplicity and minimal time requirements, such tests have widespread acceptance. In particular, the methodology developed by Ames and associates has enjoyed great popularity (Ames et al., 1973a, b, 1975; McCann et al., 1975a). A very large number of compounds has been evaluated in the various applications of the Ames test now in use (Cline and McMahon, 1977; McCann and Ames, 1977; McCann et al., 1975b; McMahon et al., 1979; Probst et al., 1981; Simmon, 1979; Sugimura et al., 1977) and the correlation between in vivo carcinogenicity and bacterial mutagenicity has been very favorable. Consequently, the Ames Salmonella/mammalian microsome test (Ames et al. 1975) has gained wide acceptance as a suitable microbial system for the identification of genotoxic chemicals.

This report describes experiments to detect mutations in *Salmonella typhimurium* tester strains TA1535, TA1537, TA1538, TA98 and TA100, which were treated with each of ten suspect genotoxins. These included: hexamethylphosphoramide, safrole, *o*-toluidine, benzene, diethylhexylphthalate, acrylonitrile, diethylstilbestrol, phenobarbital, caprolactam and benzoin). The test was conducted with and without metabolic activation using an S9 fraction prepared from the livers of Aroclor-1254-treated rats.

Materials and methods

Chemicals

All test chemicals were provided by Lancaster Synthesis, Ltd. (East Gate, England) and were tested as supplied without further purification attempts to identify impurities. Positive control compounds *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 2-nitrofluorene (2NF), 9-aminoacridine

(AmAc), and 2-aminoanthrocene (2AA) were obtained from Aldrich Chemical Company (Milwaukee, WI). All test chemicals were dissolved and diluted in reagent grade DMSO (Fisher Scientific Co., Pittsburgh, PA). The highest concentration of DMSO (0.05 μl/plate) did not affect survival or mutation in the control cultures.

Bacterial tester strains

Bacterial tester strains employed in this test were kindly provided by B.N. Ames of the university of California, and included the following histidine auxotrophs of *Salmonella typhimurium* LT-2: strains TA1535, TA1537, TA1538, TA98 and TA100. These strains have been maintained in our laboratory for the past several years.

Preparation of cultures

Overnight cultures were prepared from frozen (−80 °C) stocks by inoculating 0.2 ml of each of the tester strains into 20 ml of 2.5% oxoid nutrient broth No. 2 (Oxoid Ltd., Basingstoke, Hants, U.K.) contained in 50-ml screw-cap erlenmeyer flasks followed by incubation at 37 °C in a shaking water bath for 16 h. At the termination of incubation, the cultures were maintained at 0 °C until use (approximately 1 to 2 h). Fresh cultures were prepared for both the activated and nonactivated tests.

TABLE 1

EVALUATION OF PHENOBARBITAL FOR INDUCTION OF BACTERIAL MUTATION USING THE AMES TEST

Treatment	μg/plate	Revertant colony counts (mean ± S.D.) [a]				
		TA1535	TA1537	TA1538	TA98	TA100
Test without metabolic activation						
DMSO [b]	0.05 ml	19 ± 1	12 ± 2	13 ± 5	18 ± 1	128 ± 6
DMSO [c]	0.05 ml	17 ± 4	11 ± 3	12 ± 6	19 ± 3	107 ± 3
Phenobarbital	50	24 ± 3	10 ± 4	14 ± 3	22 ± 4	128 ± 20
	100	24 ± 5	6 ± 2	19 ± 7	16 ± 7	123 ± 7
	500	42 ± 6	10 ± 1	13 ± 2	17 ± 4	141 ± 7
	1000	46 ± 5	8 ± 3	13 ± 2	21 ± 6	143 ± 12
	5000	50 ± 4	7 ± 1	13 ± 2	17 ± 3	144 ± 12
MNNG [d]	2.5	1892 ± 152				1379 ± 314
MNNG	5	3304 ± 24				3173 ± 83
9AmAc [d]	50		123 ± 70			
9AmAc	100		1197 ± 104			
2NF [d]	0.5			109 ± 16	65 ± 7	
2NF	5			848 ± 90	533 ± 56	
Test with metabolic activation						
DMSO [b]	0.05 ml	18 ± 2	10 ± 2	29 ± 6	35 ± 2	103 ± 12
DMSO [c]	0.05 ml	19 ± 7	6 ± 1	24 ± 2	46 ± 8	126 ± 24
Phenobarbital	50	20 ± 3	6 ± 1	25 ± 7	35 ± 5	112 ± 0
	100	20 ± 3	10 ± 1	26 ± 5	39 ± 0	125 ± 3
	500	22 ± 3	8 ± 3	28 ± 9	28 ± 20	129 ± 16
	1000	26 ± 1	10 ± 4	23 ± 5	38 ± 4	129 ± 8
	5000	21 ± 3	10 ± 3	31 ± 1	40 ± 4	142 ± 13
2AA [d]	2.5	187 ± 2	245 ± 15	1738 ± 37	2291 ± 22	2313 ± 61
2AA	5	94 ± 27	181 ± 42	1450 ± 294	3047 ± 24	3048 ± 127

[a] Mean ± standard deviation of counts from triplicate plates. Values represent corrected counts for 100% of the plate area.

[b] DMSO control value for the tester strain plated at the initiation of plating.

[c] DMSO control value for the tester strain plated at the termination of plating.

[d] In the nonactivated test MNNG served as the positive control for strains TA1535 and TA100; 9AmAc was the positive control for strain TA1537; and 2NF served as the positive control for strains TA1538 and TA98. In the activated test, 2AA served as the positive control for all tester strains.

Ames base agar medium

Five days prior to the test, the base layer agar plates were prepared as described by Ames et al., (1975). Four 3-liter batches of agar were prepared and 30-ml aliquots were dispensed into 100-mm^2 round petri dishes. The medium consisted of 1.5% Difco agar (Difco Laboratories, Detroit, MI) prepared in 1 × Vogel–Bonner Salts (Vogel and Bonner, 1956) and contained 2% (w/v) glucose. The plates were inverted and maintained at room temperature for at least five days after which they were inspected for contamination.

Ames top agar medium

At the time of the test the top agar medium was prepared essentially according to the method of Ames et al. (1975). This medium consisted of 0.54% Difco agar prepared in 0.45% NaCl and was maintained (1 h) at 45 °C until used. Just prior to use, L-histidine and biotin were added to give a final concentration of 0.05 mM of each of these components.

Preparation of liver microsomes

The microsomal activation enzymes were prepared as described by Cline and McMahon (1977)

TABLE 2

EVALUATION OF SAFROLE FOR INDUCTION OF BACTERIAL MUTATION USING THE AMES TEST

Treatment	μg/plate	Revertant colony counts (mean ± S.D.) [a]				
		TA1535	TA1537	TA1538	TA98	TA100
Test without metabolic activation						
DMSO [b]	0.05 ml	25 ± 25	11 ± 2	11 ± 1	25 ± 3	115 ± 10
DMSO [c]	0.05 ml	21 ± 2	8 ± 1	13 ± 5	26 ± 3	113 ± 18
Safrole	50	22 ± 5	10 ± 3	9 ± 3	20 ± 6	94 ± 5
	100	20 ± 6	8 ± 2	9 ± 4	25 ± 8	115 ± 10
	500	16 ± 4	7 ± 1	9 ± 2	18 ± 0	102 ± 6
	1000	11 ± 3	8 ± 3	11 ± 3	10 ± 2	76 ± 14
	5000	toxic	toxic	toxic	8 ± 1	68 ± 7
MNNG [d]	2.5	3328 ± 129				2875 ± 196
MNNG	5	3296 ± 35				3224 ± 111
9AmAc [d]	50		76 ± 23			
9AmAc	100		1244 ± 80			
2NF [d]	0.5			111 ± 23	85 ± 9	
2NF	5			905 ± 68	510 ± 16	
Test with metabolic activation						
DMSO [b]	0.05 ml	13 ± 1	9 ± 6	23 ± 8	42 ± 12	114 ± 12
DMSO [c]	0.05 ml	17 ± 4	9 ± 3	22 ± 3	38 ± 9	128 ± 10
Safrole	50	14 ± 5	13 ± 5	18 ± 4	32 ± 6	134 ± 27
	100	19 ± 2	7 ± 4	22 ± 3	40 ± 10	147 ± 5
	500	17 ± 6	11 ± 4	23 ± 3 [e]	42 ± 10	184 ± 20
	1000	17 ± 3	9 ± 3	24 ± 2	33 ± 1	148 ± 4
	5000	7 ± 2 [e]	4 ± 1 [e]	5 ± 2 [e]	25 ± 2	32 ± 2 [e]
2AA [d]	2.5	138 ± 13	80 ± 8	863 ± 135	1033 ± 68	1100 ± 56
2AA	5	231 ± 4	253 ± 41	1763 ± 24	2118 ± 31	2036 ± 89

[a] Mean ± standard deviation of counts from triplicate plates. Values represent corrected counts for 100% of the plate area.

[b] DMSO control value for the tester strain plated at the initiation of plating.

[c] DMSO control value for the tester strain plated at the termination of plating.

[d] In the nonactivated test MNNG served as the positive control for strains TA1535 and TA100; 9AmAc was the positive control fc strain TA1537; and 2NF served as the positive control for strains TA1538 and TA98. In the activated test, 2AA served as th positive control for all tester strains.

[e] Value determined by hand counting colonies due to presence of chemical precipitate.

from the livers of 200–220 g male Fischer 344 rats treated five days prior with a single 100-mg/kg dose of Aroclor 1254 (Thompson et al., 1980). A sterile 25% homogenate was prepared in 0.15 M KCl at 0 °C and centrifuged for 15 min at 9000 × *g*. The supernatant fraction (S9) was frozen in liquid nitrogen and then stored in 2-ml portions (equivalent to 500 mg liver wet weight) at −80 °C. Under these conditions, enzymatic activity was retained for at least six months.

Bacterial mutation assay

At the time of testing, 450 ml of Ames top agar was diluted by the addition of 125 ml of "dilution salts," which consisted of 50 mM KCl and 8 mM $MgCl_2$ prepared in 0.5 M sodium phosphate, pH 7.4. This dilution accounted for the volume of S9 activation mix used in the activated assay and insured that the final concentrations of top agar constituents were equivalent in both the non-activated and activated assays.

The test without metabolic activation was conducted by combining 0.05 ml of the appropriate dilution of test compound, 0.1 ml of the appropriate bacterial tester strain (maintained at 0 °C) with 2.5 ml of the prediluted Ames top agar (45 °C). The solution was quickly mixed by gentle vortexing (avoiding bubbles) and quickly poured

TABLE 3

EVALUATION OF ACRYLONITRILE FOR INDUCTION OF BACTERIAL MUTATION USING THE AMES TEST

Treatment	μg/plate	Revertant colony counts (mean ± S.D.) [a]				
		TA1535	TA1537	TA1538	TA98	TA100
Test without metabolic activation						
DMSO [b]	0.05 ml	19 ± 7	9 ± 2	17 ± 2	39 ± 1	109 ± 11
DMSO [c]	0.05 ml	21 ± 7	11 ± 3	16 ± 3	35 ± 8	103 ± 10
Acrylonitrile	50	20 ± 3	12 ± 2	13 ± 5	36 ± 7	115 ± 4
	100	20 ± 6	8 ± 3	15 ± 0	41 ± 9	99 ± 2
	500	22 ± 4	10 ± 3	13 ± 3	44 ± 16	102 ± 9
	1000	16 ± 5	10 ± 3	19 ± 1	39 ± 9	96 ± 9
	5000	12 ± 5	9 ± 2	16 ± 3	33 ± 10	63 ± 18
MNNG [d]	2.5	1744 ± 228				1362 ± 56
MNNG	5	3229 ± 62				2853 ± 149
9AmAc [d]	50		88 ± 1			
9AmAc	100		1141 ± 188			
2NF [d]	0.5			112 ± 11	104 ± 11	
2NF	5			822 ± 40	572 ± 62	
Test with metabolic activation						
DMSO [b]	0.05 ml	16 ± 5	10 ± 3	26 ± 2	33 ± 3	113 ± 5
DMSO [c]	0.05 ml	13 ± 1	11 ± 3	20 ± 6	22 ± 2	111 ± 3
Acrylonitrile	50	20 ± 3	13 ± 1	21 ± 3	33 ± 6	123 ± 10
	100	17 ± 5	13 ± 2	18 ± 4	28 ± 3	115 ± 5
	500	20 ± 4	12 ± 2	22 ± 5	24 ± 9	118 ± 14
	1000	22 ± 4	9 ± 3	19 ± 3	25 ± 2	123 ± 3
	5000	18 ± 1	14 ± 2	15 ± 4	19 ± 2	87 ± 16
2AA [d]	2.5	122 ± 18	141 ± 19	1120 ± 14	1578 ± 51	1942 ± 90
2AA	5	156 ± 18	248 ± 34	1566 ± 51	2719 ± 102	2951 ± 133

[a] Mean ± standard deviation of counts from triplicate plates. Values represent corrected counts for 100% of the plate area.

[b] DMSO control value for the tester strain plated at the initiation of plating.

[c] DMSO control value for the tester strain plated at the termination of plating.

[d] In the nonactivated test MNNG served as the positive control for strains TA1535 and TA100; 9AmAc was the positive control for strain TA1537; and 2NF served as the positive control for strains TA1538 and TA98. In the activated test, 2AA served as the positive control for all tester strains.

onto a plate containing the base agar medium. The plate was gently swirled to distribute the top agar mix evenly and allowed to gel on a flat surface. For each sample, this operation was conducted in less than 30 sec. For each tester strain, five concentrations of the test chemical were tested and triplicate plates were prepared for each control and test chemical treatment. The top agar layer was allowed to gel for at least 30 min at room temperature and then the plates were inverted and incubated for 48 h at 37 °C.

Revertant colonies were counted using a New Brunswick Biotran II Automated Colony Counter (New Brunswick Scientific, New Brunswick, NJ) with the colony size discriminator set at the lowest limit to maximize the identification of all colonies. Each plate was counted three times, with rotation about 90° between counts. The mean value was recorded as the colony count for the plate. Automated counting permitted counting only approximately 80% of the plate, therefore the area counted and the total area of the plate were measured with the instrument and the counts obtained were corrected accordingly.

TABLE 4

EVALUATION OF DIETHYLSTILBESTROL FOR INDUCTION OF BACTERIAL MUTATION USING THE AMES TEST

Treatment	μg/plate	Revertant colony counts (mean ± S.D.) [a]				
		TA1535	TA1537	TA1538	TA98	TA100
Test without metabolic activation						
DMSO [b]	0.05 ml	22 ± 4	8 ± 2	13 ± 5	23 ± 1	109 ± 11
DMSO [c]	0.05 ml	16 ± 2	7 ± 1	12 ± 3	15 ± 4	104 ± 13
Diethylstilbestrol	50	14 ± 3	8 ± 3	9 ± 3	17 ± 3	92 ± 4
	100	13 ± 2	5 ± 1	7 ± 5	14 ± 3	96 ± 3
	500	7 ± 3	4 ± 1	9 ± 6 [f]	14 ± 1	92 ± 9
	1000	5 ± 1	1 ± 0	28 ± 21 [f]	9 ± 1	85 ± 7
	5000	1837 ± 246 [e]	1975 ± 118 [e]	2061 ± 96 [e]	1635 ± 507 [e]	1392 ± 89 [e]
MNNG [d]	2.5	1737 ± 405				1497 ± 75
MNNG	5	3132 ± 54				2935 ± 158
9AmAc [d]	50		87 ± 38			
9AmAc	100		1238 ± 175			
2NF [d]	0.5			110 ± 8	126 ± 86	
2NF	5			900 ± 32	492 ± 32	
Test with metabolic activation						
DMSO [b]	0.05 ml	18 ± 6	14 ± 3	32 ± 11	44 ± 5	207 ± 12
DMSO [c]	0.05 ml	18 ± 6	7 ± 1	32 ± 10	40 ± 3	257 ± 5
Diethylstilbestrol	50	20 ± 6	7 ± 1	26 ± 6	32 ± 6	219 ± 9
	100	15 ± 4	8 ± 3	27 ± 10	27 ± 3	144 ± 8
	500	7 ± 3	8 ± 4	16 ± 5	21 ± 1	101 ± 19
	1000	6 ± 2	4 ± 2	12 ± 2	17 ± 2	79 ± 36
	5000	1694 ± 662 [e]	2224 ± 181 [e]	1673 ± 111 [e]	2057 ± 195 [e]	2210 ± 305 [e]
2AA [d]	2.5	154 ± 17	106 ± 18	1118 ± 78	1564 ± 16	1863 ± 90
2AA	5	189 ± 8	146 ± 14	1743 ± 34	2993 ± 60	2836 ± 130

[a] Mean ± standard deviation of counts from triplicate plates. Values represent corrected counts for 100% of the plate area.

[b] DMSO control value for the tester strain plated at the initiation of plating.

[c] DMSO control value for the tester strain plated at the termination of plating.

[d] In the nonactivated test MNNG served as the positive control for strains TA1535 and TA100; 9AmAc was the positive control for strain TA1537; and 2NF served as the positive control for strains TA1538 and TA98. In the activated test, 2AA served as the positive control for all tester strains.

[e] The count resulted from the presence of a large chemical precipitate.

[f] Value determined by hand counting colonies due to presence of chemical precipitate.

Bacterial mutation assay (activated)

The test with metabolic activation was conducted exactly the same as the nonactivated test except that "dilution salts" were not added to the Ames top agar since the addition of the S9 activation mixture accounted for this volume in the activated portion of the assay.

The S9 activation mixture contained 0.1 ml of S9, 5 μmoles NADP and 4 μmoles of glucose 6-phosphate in a final volume of 1.0 ml of a salt solution consisting of 33 mM KCl, 8 mM $MgCl_2$ in 0.25 M sodium phosphate (pH 7.4). The activation mixture was maintained at 0 °C throughout the test.

The test with metabolic activation was conducted by combining 0.05 ml of the appropriate dilution of test compound, 0.1 ml of the appropriate bacterial tester strain (maintained at 0 °C), 0.5 ml of the S9 activation mixture (0 °C) and 2.0 ml of undiluted Ames top agar (45 °C). All further procedures were as described above for the nonactivated test.

TABLE 5

EVALUATION OF HEXAMETHYLPHOSPHORAMIDE FOR INDUCTION OF BACTERIAL MUTATION USING THE AMES TEST

Treatment	μg/plate	Revertant colony counts (mean ± S.D.) [a]				
		TA1535	TA1537	TA1538	TA98	TA100
Test without metabolic activation						
DMSO [b]	0.05 ml	27 ± 4	13 ± 2	15 ± 3	34 ± 8	375 ± 44
DMSO [c]	0.05 ml	19 ± 6	14 ± 3	14 ± 3	39 ± 8	361 ± 10
Hexamethyl-	50	21 ± 3	11 ± 1	15 ± 6	30 ± 10	370 ± 9
phosphoramide	100	25 ± 2	12 ± 2	15 ± 6	32 ± 2	357 ± 13
	500	21 ± 7	13 ± 1	15 ± 7	33 ± 7	366 ± 30
	1000	23 ± 5	16 ± 2	13 ± 4	26 ± 3	371 ± 30
	5000	22 ± 2	14 ± 7	14 ± 2	31 ± 3	365 ± 44
MNNG [d]	1	3022 ± 13				2884 ± 46
MNNG	2	3035 ± 57				2757 ± 78
9AmAc [d]	50		47 ± 4			
9AmAc	100		933 ± 242			
2NF [d]	0.5			117 ± 9	97 ± 16	
2NF	5			897 ± 88	607 ± 41	
Test with metabolic activation						
DMSO [b]	0.05 ml	18 ± 1	19 ± 5	25 ± 11	55 ± 4	112 ± 12
DMSO [c]	0.05 ml	20 ± 8	25 ± 7	33 ± 6	56 ± 7	128 ± 27
Hexamethyl-	50	15 ± 2	21 ± 11	29 ± 4	48 ± 10	134 ± 12
phosphoramide	100	14 ± 2	21 ± 5	27 ± 2	49 ± 2	107 ± 14
	500	15 ± 2	17 ± 6	28 ± 4	48 ± 2	123 ± 9
	1000	18 ± 4	19 ± 1	24 ± 8	59 ± 3	124 ± 8
	5000	12 ± 5	20 ± 7	30 ± 3	55 ± 2	120 ± 23
2AA [d]	2.5	132 ± 4	141 ± 16	1389 ± 58	1198 ± 48	1318 ± 70
2AA	5	250 ± 16	407 ± 33	2599 ± 61	2503 ± 47	2537 ± 31

[a] Mean ± standard deviation of counts from triplicate plates. Values represent corrected counts for 100% of the plate area.

[b] DMSO control value for the tester strain plated at the initiation of plating.

[c] DMSO control value for the tester strain plated at the termination of plating.

[d] In the nonactivated test MNNG served as the positive control for strains TA1535 and TA100; 9AmAc was the positive control for strain TA1537; and 2NF served as the positive control for strains TA1538 and TA98. In the activated test, 2AA served as the positive control for all tester strains.

TABLE 6

EVALUATION OF *o*-TOLUIDINE FOR INDUCTION OF BACTERIAL MUTATION USING THE AMES TEST

Treatment	μg/plate	Revertant colony counts (mean ± S.D.) [a]				
		TA1535	TA1537	TA1538	TA98	TA100
Test without metabolic activation						
DMSO [b]	0.05 ml	18 ± 5	6 ± 1	15 ± 3	27 ± 6	141 ± 12
DMSO [c]	0.05 ml	18 ± 2	8 ± 4	13 ± 1	23 ± 2	138 ± 13
o-Toluidine	50	18 ± 6	6 ± 1	13 ± 3	25 ± 5	146 ± 7
	100	25 ± 3	10 ± 3	11 ± 4	32 ± 7	141 ± 16
	500	28 ± 3	8 ± 4	14 ± 3	20 ± 1	140 ± 18
	1000	27 ± 5	8 ± 2	12 ± 6	24 ± 3	130 ± 6
	5000	35 ± 11	6 ± 1	16 ± 2	22 ± 7	153 ± 12
MNNG [d]	2.5	1728 ± 131				1514 ± 230
MNNG	5	3319 ± 56				2937 ± 218
9AmAc [d]	50		141 ± 28			
9AmAc	100		1433 ± 47			
2NF [d]	0.5			105 ± 4	64 ± 10	
2NF	5			900 ± 36	603 ± 66	
Test with metabolic activation						
DMSO [b]	0.05 ml	20 ± 1	8 ± 2	23 ± 3	31 ± 11	102 ± 7
DMSO [c]	0.05 ml	17 ± 1	7 ± 1	20 ± 3	32 ± 1	118 ± 3
o-Toluidine	50	15 ± 1	9 ± 4	19 ± 7	29 ± 3	118 ± 13
	100	13 ± 4	8 ± 3	19 ± 4	32 ± 6	107 ± 4
	500	13 ± 4	4 ± 1	19 ± 2	36 ± 1	117 ± 5
	1000	16 ± 2	8 ± 2	21 ± 3	33 ± 1	127 ± 3
	5000	14 ± 3	12 ± 2	22 ± 3	40 ± 9	142 ± 8
2AA [d]	2.5	176 ± 15	259 ± 22	1700 ± 99	1922 ± 20	2370 ± 37
2AA	5	202 ± 30	156 ± 38	1791 ± 88	2905 ± 53	2849 ± 100

[a] Mean ± standard deviation of counts from triplicate plates. Values represent corrected counts for 100% of the plate area.

[b] DMSO control value for the tester strain plated at the initiation of plating.

[c] DMSO control value for the tester strain plated at the termination of plating.

[d] In the nonactivated test MNNG served as the positive control for strains TA1535 and TA100; 9AmAc was the positive control for strain TA1537; and 2NF served as the positive control for strains TA1538 and TA98. In the activated test, 2AA served as the positive control for all tester strains.

TABLE 7

EVALUATION OF BENZENE FOR INDUCTION OF BACTERIAL MUTATION USING THE AMES TEST

Treatment	μg/plate	Revertant colony counts (mean ± S.D.) [a]				
		TA1535	TA1537	TA1538	TA98	TA100
Test without metabolic activation						
DMSO [b]	0.05 ml	32 ± 2	10 ± 5	18 ± 4	24 ± 3	106 ± 6
DMSO [c]	0.05 ml	29 ± 6	9 ± 3	17 ± 1	22 ± 5	108 ± 3
Benzene	50	25 ± 4	10 ± 4	15 ± 2	23 ± 3	107 ± 10
	100	23 ± 3	11 ± 1	17 ± 1	23 ± 4	112 ± 8
	500	32 ± 5	10 ± 4	17 ± 1	21 ± 3	131 ± 24
	1000	27 ± 1	10 ± 2	15 ± 7	23 ± 5	113 ± 2
	5000	27 ± 7	8 ± 2	14 ± 4	22 ± 6	122 ± 3
MNNG [d]	2.5	1711 ± 67				1855 ± 136
MNNG	5	3337 ± 29				3206 ± 63
9AmAc [d]	50		125 ± 43			
9AmAc	100		1324 ± 39			
2NF [d]	0.5			116 ± 17	105 ± 4	
2NF	5			1024 ± 98	699 ± 86	
Test with metabolic activation						
DMSO [b]	0.05 ml	26 ± 8	10 ± 4	30 ± 6	33 ± 4	129 ± 18
DMSO [c]	0.05 ml	25 ± 4	12 ± 1	34 ± 6	47 ± 8	118 ± 9
Benzene	50	21 ± 4	9 ± 2	25 ± 6	37 ± 2	143 ± 8
	100	30 ± 4	9 ± 2	26 ± 5	39 ± 5	137 ± 16
	500	24 ± 6	10 ± 2	33 ± 5	41 ± 11	138 ± 9
	1000	24 ± 9	11 ± 6	30 ± 2	30 ± 3	135 ± 13
	5000	25 ± 5	10 ± 4	30 ± 5	38 ± 5	146 ± 35
2AA [d]	2.5	224 ± 24	163 ± 31	1941 ± 144	2383 ± 106	2435 ± 63
2AA	5	255 ± 24	349 ± 23	2644 ± 298	3269 ± 133	3288 ± 71

[a] Mean ± standard deviation of counts from triplicate plates. Values represent corrected counts for 100% of the plate area.

[b] DMSO control value for the tester strain plated at the initiation of plating.

[c] DMSO control value for the tester strain plated at the termination of plating.

[d] In the nonactivated test MNNG served as the positive control for strains TA1535 and TA100; 9AmAc was the positive control for strain TA1537; and 2NF served as the positive control for strains TA1538 and TA98. In the activated test, 2AA served as the positive control for all tester strains.

TABLE 8

EVALUATION OF DIETHYLHEXYLPHTHALATE FOR INDUCTION OF BACTERIAL MUTATION USING THE AMES TEST

Treatment	μg/plate	Revertant colony counts (mean ± S.D.) [a]				
		TA1535	TA1537	TA1538	TA98	TA100
Test without metabolic activation						
DMSO [b]	0.05 ml	21 ± 5	13 ± 5	11 ± 1	26 ± 5	115 ± 18
DMSO [c]	0.05 ml	18 ± 4	8 ± 3	12 ± 3	27 ± 2	116 ± 10
Diethylhexyl-	50	15 ± 4	10 ± 2	9 ± 2	22 ± 7	99 ± 12
phthalate	100	22 ± 3	10 ± 3	12 ± 5	23 ± 4	107 ± 7
	500	16 ± 2	7 ± 3	12 ± 4	30 ± 10	102 ± 17
	1000	16 ± 3	12 ± 2	11 ± 4	20 ± 2	110 ± 2
	5000	21 ± 3	8 ± 6	14 ± 4	28 ± 4	120 ± 12
MNNG [d]	2.5	2634 ± 118				
MNNG	5	3373 ± 9				
9AmAc [d]	50		72 ± 13			
9AmAc	100		1293 ± 157			
2NF [d]	0.5			159 ± 15	113 ± 7	2035 ± 57
2NF	5			1262 ± 99	851 ± 65	3091 ± 67
Test with metabolic activation						
DMSO [b]	0.05 ml	16 ± 5	6 ± 4	18 ± 5	38 ± 7	125 ± 1
DMSO [c]	0.05 ml	18 ± 2	8 ± 3	22 ± 7	35 ± 9	127 ± 4 [e]
Diethylhexyl-	50	16 ± 3	9 ± 4	20 ± 11	33 ± 3	110 ± 14
phthalate	100	13 ± 1	8 ± 5	18 ± 2	27 ± 5	111 ± 7
	500	14 ± 2	4 ± 2	20 ± 3	34 ± 3	115 ± 16
	1000	13 ± 1	6 ± 1	20 ± 3	37 ± 6	117 ± 15
	5000	19 ± 3	8 ± 2	19 ± 8	36 ± 1	111 ± 12
2AA [d]	2.5	152 ± 43	238 ± 20	69 ± 18	3161 ± 81	197 ± 38
2AA	5	221 ± 25	83 ± 26	2245 ± 108	173 ± 15	3216 ± 49

[a] Mean ± standard deviation of counts from triplicate plates. Values represent corrected counts for 100% of the plate area.

[b] DMSO control value for the tester strain plated at the initiation of plating.

[c] DMSO control value for the tester strain plated at the termination of plating.

[d] In the nonactivated test MNNG served as the positive control for strains TA1535 and TA100; 9AmAc was the positive control for strain TA1537; and 2NF served as the positive control for strains TA1538 and TA98. In the activated test, 2AA served as the positive control for all tester strains.

[e] Mean of two plates, one plate lost to mold contamination.

TABLE 9

EVALUATION OF CAPROLACTAM FOR INDUCTION OF BACTERIAL MUTATION USING THE AMES TEST

Treatment	μg/plate	Revertant colony counts (mean ± S.D.) [a]				
		TA1535	TA1537	TA1538	TA98	TA100
Test without metabolic activation						
DMSO [b]	0.05 ml	27 ± 8	7 ± 3	16 ± 3	18 ± 6	116 ± 2
DMSO [c]	0.05 ml	20 ± 3	9 ± 2	16 ± 4	25 ± 2	98 ± 15
Caprolactam	50	23 ± 6	7 ± 2	16 ± 2	23 ± 4	108 ± 8
	100	19 ± 6	10 ± 2	14 ± 4	20 ± 10	101 ± 9
	500	21 ± 6	6 ± 4	13 ± 2	18 ± 6	102 ± 11
	1000	24 ± 6	8 ± 2	14 ± 9	16 ± 3	116 ± 14
	5000	24 ± 3	11 ± 6	14 ± 4	20 ± 6	104 ± 6
MNNG [d]	2.5	930 ± 70				898 ± 59
MNNG	5	3022 ± 177				2733 ± 78
9AmAc [d]	50		147 ± 53			
9AmAc	100		1495 ± 34			
2NF [d]	0.5			124 ± 9	77 ± 16	
2NF	5			1027 ± 58	509 ± 22	
Test with metabolic activation						
DMSO [b]	0.05 ml	20 ± 5	9 ± 3	21 ± 3	27 ± 2	100 ± 16
DMSO [c]	0.05 ml	19 ± 2	7 ± 1	25 ± 5	29 ± 8	118 ± 2
Caprolactam	50	18 ± 3	8 ± 5	26 ± 3	31 ± 6	134 ± 4
	100	14 ± 5	10 ± 3	23 ± 8	28 ± 7	114 ± 16
	500	21 ± 3	8 ± 4	24 ± 1	25 ± 1	102 ± 13
	1000	19 ± 4	11 ± 2	21 ± 4	26 ± 6	113 ± 13
	5000	19 ± 5	8 ± 2	26 ± 7	30 ± 6	119 ± 10
2AA [d]	2.5	231 ± 15	28 ± 9	2339 ± 33	2785 ± 255	3008 ± 57
2AA	5	195 ± 2	353 ± 39	3053 ± 342	1448 ± 209	1554 ± 377

[a] Mean ± standard deviation of counts from triplicate plates. Values represent corrected counts for 100% of the plate area.

[b] DMSO control value for the tester strain plated at the initiation of plating.

[c] DMSO control value for the tester strain plated at the termination of plating.

[d] In the nonactivated test MNNG served as the positive control for strains TA1535 and TA100; 9AmAc was the positive control for strain TA1537; and 2NF served as the positive control for strains TA1538 and TA98. In the activated test, 2AA served as the positive control for all tester strains.

TABLE 10

EVALUATION OF BENZOIN FOR INDUCTION OF BACTERIAL MUTATION USING THE AMES TEST

Treatment	μg/plate	Revertant colony counts (mean ± S.D.) [a]				
		TA1535	TA1537	TA1538	TA98	TA100
Test without metabolic activation						
DMSO [b]	0.05 ml	25 ± 2	12 ± 5	11 ± 3	18 ± 6	110 ± 15
DMSO [c]	0.05 ml	22 ± 1	8 ± 2	14 ± 3	21 ± 2	114 ± 14
Benzoin	50	26 ± 1	9 ± 3	13 ± 5	22 ± 2	117 ± 6
	100	25 ± 6	6 ± 1	15 ± 4	19 ± 6	108 ± 24
	500	32 ± 4	10 ± 2	8 ± 1	20 ± 5	124 ± 13
	1000	34 ± 7	6 ± 2	10 ± 2	19 ± 7	130 ± 19
	5000	639 ± 142 [e]	659 ± 100 [e]	507 ± 92 [e]	512 ± 283	636 ± 289 [e]
MNNG [d]	2.5	2114 ± 59				2063 ± 302
MNNG	5	3330 ± 62				2878 ± 429
9AmAc [d]	50		113 ± 33			
9AmAc	100		1386 ± 73			
2NF [d]	0.5			100 ± 12	70 ± 10	
2NF	5			863 ± 21	567 ± 25	
Test with metabolic activation						
DMSO [b]	0.05 ml	21 ± 4	14 ± 6	33 ± 3	39 ± 13	274 ± 12
DMSO [c]	0.05 ml	19 ± 6 [f]	15 ± 4	42 ± 6	46 ± 5	252 ± 14
Benzoin	50	21 ± 8	12 ± 5	31 ± 8	38 ± 8	260 ± 10
	100	26 ± 1	15 ± 2	36 ± 2	41 ± 3	276 ± 7
	500	19 ± 0	13 ± 5	34 ± 7	43 ± 11	266 ± 9
	1000	22 ± 4	13 ± 1	22 ± 2	34 ± 10	257 ± 17
	5000	678 ± 61 [e]	592 ± 58 [e]	757 ± 111 [e]	649 ± 83 [e]	874 ± 156 [e]
2AA [d]	2.5	210 ± 10	177 ± 8	1346 ± 57	1650 ± 127	1960 ± 126
2AA	5	113 ± 36	188 ± 48	1486 ± 19	2804 ± 69	2940 ± 53

[a] Mean ± standard deviation of counts from triplicate plates. Values represent corrected counts for 100% of the plate area.

[b] DMSO control value for the tester strain plated at the initiation of plating.

[c] DMSO control value for the tester strain plated at the termination of plating.

[d] In the nonactivated test MNNG served as the positive control for strains TA1535 and TA100; 9AmAc was the positive control for strain TA1537; and 2NF served as the positive control for strains TA1538 and TA98. In the activated test, 2AA served as the positive control for all tester strains.

[e] The count resulted from the presence of a large chemical precipitate.

[f] Mean of two plates, one plate lost to mold contamination.

Criteria for a positive response

A chemical was judged to have induced a positive response when a dose-related increase in revertants was observed in which the number of revertants exceeded control values by at least two-fold in at least two successive concentrations of the test chemical.

Results and discussion

Ten suspect genotoxins were evaluated for the induction of bacterial mutation using five strains of *Salmonella typhimurium* according to the method of Ames et al. (1975). The test was conducted with and without metabolic activation using a liver S9 fraction from Aroclor-1254-induced rats. Treatment levels were arbitrarily established and resulted in test concentrations of 50, 100, 500, 1000 and 5000 μg/plate of each compound for the nonactivated and activated mutation test. The results are summarized in Tables 1 through 10.

A dose-related increase in Salmonella histidine revertants of strain TA1535 was observed following treatment with phenobarbital and metabolic activation was not required (Table 1); however,

this effect was not observed in the test with S9 activation.

Treatment with safrole (Table 2), acrylonitrile (Table 3), or diethylstilbestrol (Table 4) resulted in toxicity; however, there was no evidence of induced mutation. These toxic effects were evidenced by a reduction in the number of spontaneous revertants in both the activated and nonactivated tests. For acrylonitrile this effect was restricted to strain TA100, whereas with safrole and diethylstilbestrol, all strains were affected.

Treatment with hexamethylphosphoramide (Table 5), *o*-toluidine (Table 6), benzene (Table 7), diethylhexylphthalate (Table 8), caprolactam (Table 9), or benzoin (Table 10) resulted in neither toxicity nor the induction of point mutation in Salmonella either with or without metabolic activation.

In each of the tests performed, a dose-dependent increase in induced revertants was observed with the positive control treatments (MNNG, 2NF and 9AmAc for the nonactivated test, and 2AA for the activated test) and thus confirmed the responsiveness of the tester strains to known genotoxins.

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Mutation tests with Salmonella using 8-azaguanine resistance as the genetic marker

Howard L. Liber

Toxicology Group, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, MA (U.S.A.)

Summary

Ten chemicals were examined for their ability to induce 8-azaguanine-resistance mutations in *S. typhimurium* in the presence or absence of a rat-liver-derived postmitochondrial supernatant (PMS). Acrylonitrile was *mutagenic* in the absence of PMS, but it was not active in the presence of PMS. The remaining 9 compounds: benzene, benzoin, caprolactam, di(2'-ethylhexyl)phthalate, diethylstilbesterol, hexamethylphosphoramide, phenobarbital, safrole and *o*-toluidine, did not induce mutation either in the presence or absence of PMS.

The 10 compounds comprising the CSSTT study were tested for mutagenicity in *Salmonella typhimurium*, using forward mutation to 8-azaguanine resistance as the genetic marker (Skopek et al., 1978a, b). We use forward mutation assays rather than reversion tests because one locus in a single strain of bacteria will theoretically respond to a wider variety of mutagens than a set of reversion strains each of which responds to only a very specific change. The forward assay has been shown to be equisensitive to the Ames' Salmonella test for a variety of mutagens (Skopek et al., 1978b).

The strain used in these studies was TM677, a his^+ revertant of TA1535 into which the plasmid pkM101 had been inserted. In all experiments, cells were treated in suspension culture for 2 h with the test compound, with or without an Aroclor-induced rat-liver postmitochondrial supernatant. The bacteria were then plated in the presence and absence of 8-azaguanine to determine mutant fraction — the number of mutants per survivor.

At the concentrations tested, all of the compounds were inactive in the mutation assay. The single exception was that acrylonitrile without metabolic activation yielded a positive response.

Materials and Methods

Media

(1) Minimal E (0.2 mg/ml $MgSO_4 \cdot 7H_2O$, 2.0 mg/ml citric acid $\cdot H_2O$, 10 mg/ml $K_2HPO_4 \cdot 4H_2O$, 20.0 mg/ml glucose, 0.05 mM biotin; pH 7.0.

(2) Phosphate-buffered saline (PBS) (8.0 mg/ml NaCl, 0.2 mg/ml KCl, 1.15 mg/ml Na_2HPO_4, 0.2 mg/ml KH_2PO_4; pH 7.0).

(3) Brain Heart Infusion (BHI) was purchased from Difco and rehydrated as specified.

Bacterial strain

The cells were stored frozen at −80 °C in BHI with 10% DMSO added as a cryoprotective agent. Each 1.0-ml aliquot contained approximately 10^9 cells. Each day, a vial was thawed into 50 ml minimal E and allowed 1.5 h growth at 37 °C before use.

Preparation of test compound

All compounds were dissolved in reagent grade dimethyl sulfoxide (DMSO) immediately before use.

TABLE 1

MUTAGENICITY OF 10 CSSTT COMPOUNDS TO *Salmonella typhimurium* AT THE 8-AZAGUANINE-RESISTANCE LOCUS

	Conc. tested (μg/ml)	Without PMS			With PMS		
		$8AG^R$ MF $\times 10^5$		Avg. SF	$8AG^R$ MF $\times 10^5$		Avg. SF
		1	2		1	2	
ACN	0	16,11	6,4	1.00	13,11	5,5	1.00
	50	13,11	4,5	1.06	7,7	3,4	1.18
	200	13,13	7,6	0.70	8,7	4,4	1.00
	500	41,33	15,12	0.23	16,13	6,6	0.35
	+	39	85	0.97	171	87	0.54
BEN	0	12,10		1.00	7,8		1.00
	50	12,10		1.02	7,7		1.07
	200	9,9		1.09	8,7		1.18
	500	7,9		1.00	6,7		1.16
	+	38		1.20	106		0.74
ZOIN	0	11,11	6,4	1.00	11,11	5,5	1.00
	30	10,11	–	1.18	6,8	–	0.92
	50	–	6,4	1.02	–	3,4	
	100	12,13	–	0.91	9,8	–	0.97
	200	–	6,6	0.64	–	5,4	0.56
	300	15,14	–	0.54	10,9	–	0.58
	500	–	8,6	0.54	–	5,5	0.58
	+	147	85	0.68	96	87	0.68
HMPA	0	11,12	7,5	1.00	9,8	4,X	1.00
	50	–	5,6	1.08	–	4,4	1.06
	67	12,10	–	1.76	9,9	–	1.12
	200	–	5,5	1.11	–	4,4	1.03
	268	10,12	–	1.76	10,9	–	1.09
	500	–	4,4	1.15	–	4,4	1.07
	670	11,9	–	1.57	10,9	–	1.01
	+	45	104	1.08	125	114	0.54
PB	0	16,11	5,7	1.00	13,11	8,12	1.00
	50	12,11	6,7	1.06	11,12	10,8	1.38
	200	18,X	6,8	0.92	15,16	13,16	0.98
	500	20,18	5,7	0.67	21,18	15,14	0.68
	+	39	118	0.93	171	100	0.79
SAF	0	12,10	6,4	1.00	7,8	5,5	1.00
	50	8,8	5,7	1.09	9,5	4,3	1.24
	200	16,4	2,3	0.03	7,5	5,3	0.94
	500	*,*	*,8	< 0.01	*,*	*,6	0.02
	+	38	85	0.63	106	87	0.50
TOL	0	12,10	5,7	1.00	7,8	3,4	1.00
	50	9,9	7,7	1.16	8,8	8,6	1.05
	200	10,9	9,9	1.00	7,7	3,5	1.31
	500	9,8	6,10	0.71	8,6	7,6	0.79
	+	38	118	0.93	106	88	0.57
CAP	0	11,11	7,5	1.00	11,11	4,X	1.00
	30	13,11	–	1.36	8,8	–	1.24
	50	–	5,5	1.02	–	4,4	0.88
	100	13,11	–	1.32	8,5	–	1.02
	200	–	6,5	0.96	–	4,4	1.06
	300	11,11	–	1.25	9,8	–	1.29
	500	–	6,5	1.01	–	5,4	0.99
	+	147	104	0.60	96	–	0.66

TABLE 1 (continued)

	Conc. tested (μg/ml)	Without PMS			With PMS		
		$8AG^R$ MF $\times 10^5$		Avg. SF	$8AG^R$ MF $\times 10^5$		Avg. SF
		1	2		1	2	
DEHP	0	16,11	7,5	1.00	13,11	4,X	1.00
	50	15,11	5,6	1.18	12,10	8,X	1.23
	200	29,19	6,5	1.13	13,11	3,5	1.23
	500	17,14	5,5	1.25	11,9	4,4	1.23
	+	39	104	0.89	171	114	0.52
DES	0	11,11	5,7	1.00	11,11	8,12	1.00
	30	13,12	–	0.76	5,7	–	0.91
	50	–	6,7	1.06	–	8,9	1.73
	100	12,10	–	0.76	7,7	–	0.93
	200	–	7,8	0.61	–	8,7	1.44
	300	13,13	–	0.74	9,5	–	0.92
	500	–	8,8	0.58	–	7,6	1.24
	+	147	118	0.64	96	100	0.93

Assays were performed as described in Materials and Methods. PMS stands for 5% (v/v) Aroclor-induced rat-liver postmitochondrial supernatant. $8AG^R$ MF $\times 10^5$ is the 8-azaguanine-resistant mutant fraction $\times 10^5$; trials 1 and 2 are listed separately. The average surviving fraction (SF) relative to the zero control is also listed. A dash (–) means the particular concentration was not used in that trial. An X means the point was lost. An * means that mutant fraction could not be determined because excessive toxicity resulted in no mutant colonies being observed. + is the positive control, which was 0.1 μg/ml 4-nitroquinoline-*N*-oxide (without PMS) or 20 μg/ml benzo[*a*]pyrene (with PMS).

Rat-liver metabolizing system

An Aroclor-induced rat-liver postmitochondrial supernatant (PMS) was utilized to provide a mammalian metabolizing system. The PMS was purchased from Litton Bionetics and stored at −80°. An NADPH-generating system was also added. The final concentration of the complete metabolizing system in suspension with bacterial cells was: 0.05 ml/ml PMS, 1.0 mg/ml $NADPH^+$ (Sigma), 1.0 mg/ml glucose 6-phosphate (Sigma), 0.67 mg/ml $MgCl_2 \cdot 6H_2O$, 0.4 units/ml glucose 6-phosphate dehydrogenase (Sigma).

Treatment of cells with test compound

After the preincubation period, half the cells were supplemented with the complete metabolizing system, and then both sets aliquoted as 0.99-ml samples. The test compounds, or the negative or positive control, were added as 10-μl aliquots. The tube was mixed and incubated for 2 h at 37°C in a rotating drum.

The negative control was DMSO; the positive controls were 0.1 μg/ml 4-nitroquinoline-*N*-oxide without PMS and 20 μg/ml benzo[*a*]pyrene with PMS.

Plating protocol

After treatment, 4.0 ml of cold PBS were added to each and the following dilutions were performed: (1) 0.145 ml to 10 ml agar (0.6% with 0.9% NaCl) with 8-azaguanine (350 μg/ml), (2) 0.145 ml (1) to 4 ml PBS, (3) 0.145 ml (2) to 10 ml agar.

Three 2.5-ml aliquots from (1) and three 2.5-ml aliquots from (3) were each layered over a 15-ml minimal E agar plate (0.6% pH 6.5). Thus, the high cell density plates, seeded to determine number of surviving mutants, each contained about 5×10^5 cells. The low density plates, seeded to determine the number of survivors, contained exactly 5.3×10^{-4} as many cells as the high density plates. The final concentration of 8-azaguanine on the high density plates was 50 μg/ml.

Plates were incubated at 37°C for 40 h and counted on an Artek automatic colony counter.

Determination of mutant fraction

After counting the numbers of colonies on the high and low density plates, the 8-azaguanine-resistant mutant fraction was determined by the ratio

$$\frac{\text{number of colonies on mutation plates}}{\text{number of colonies on survival plates}} \times 5.3 \times 10^{-4}$$

The relative surviving fraction was determined by the ratio

$$\frac{\text{avg. number of colonies on treated low density plates}}{\text{avg. number of colonies on untreated low density plates}}$$

Determination of significance

The mutant fraction of a treated culture is considered to be significantly different from the control if it meets two conditions:

(1) The induced value must be significantly greater (at the 99% level) than the background value obtained in the particular experiment. Operationally, this is attained when the value for the induced mutant fraction is 15×10^{-5} higher than the control.

(2) The induced value must be greater than the upper 99% confidence limit for the historical background of the particular batch of bacteria in use which, for all the experiments described here, was 25×10^{-5} (calculated with $n > 300$).

Results and discussion

The results of mutagenicity assays at the 8-azaguanine-resistance locus in *S. typhimurium* for the 10 CSSTT compounds are presented in Table 1. Each compound was tested in the presence and absence of the rat-liver postmitochondrial supernatant (PMS) on two separate days. Each concentration was performed in duplicate, generally resulting in 4 independent determinations of mutant fraction for each treatment condition. The one exception was benzene, which was done on only one occasion.

With the exception of acrylonitrile, the results were uniformly negative. None of the other compounds gave any hint of mutagenic activity. Safrole was toxic at high concentration (500 μg/ml) in the presence or absence of PMS.

In the first assay performed, the highest concentration of acrylonitrile tested, which was 500 μg/ml, yielded mutant fractions of 41×10^{-5}, and 33×10^{-5}, for duplicate treatments without PMS.

These values are both above the 99% upper confidence limit of the background, using either criterion discussed in the Materials and Methods section. When the experiment was repeated, acrylonitrile failed to induce a significant response, although, in fact, the mutant fraction observed at 500 μg/ml was indeed higher than the control. Thus, the results obtained with this compound are equivocal, although they appear to indicate that acrylonitrile is a weak mutagen in this assay system.

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Induction of various genetic effects in the yeast *Saccharomyces cerevisiae* strain D7

P. Arni

Ciba-Geigy Ltd., Basle (Switzerland)

Summary

HMPA, SAF, TOL, CAP, ZOIN, DEN, DEHP, ACN, PB and DES were tested for the induction of genetic effects with strain *Saccharomyces cerevisiae* D7. The investigations were performed without and with addition of an exogenous activation system. HMPA induced mitotic gene conversion and back mutation in experiments without activation. DEHP induced mitotic gene conversion in experiments without and with activation. Treatment with ACN led also to an increase in the incidence of gene conversions, but only in experiments without activation. The other 7 compounds did not induce genetic effects in *S. cerevisiae* D7 under the given test conditions.

Yeasts have, in general, proved to be very suitable for use as indicator organisms in testing chemial substances for possible mutagenic properties. Strain *S. cerevisiae* D7 (Zimmermann, 1975; Zimmermann et al., 1984) permits the detection of induced homozygosis or hemizygosis (mitotic crossing-over, mitotic gene conversion, chromosome loss, deletion and point mutation) leading to adenine-requiring cells, mitotic gene conversion and back mutation.

Adenine requirement can be detected visually as pink or red colonies or sectors on a medium with a low adenine content. Mitotic crossing-over can be distinguished from the other genetic events leading to adenine requirement as pink/red sectored colonies. Mitotic gene conversion is monitored by the appearance of tryptophan nonrequiring colonies on selective media. Mutation induction can be followed by the appearance of isoleucine nonrequiring colonies on selective media. To ensure that mutagenic effects of the metabolites of the test substance formed in mammals would also be detected, experiments were performed in which the cultures were additionally treated with an activation mixture (rat liver microsomes and cofactors) (Ames et al., 1973; Barale et al., 1980; Bronzetti et al., 1981).

Material and methods

Materials

Strain. The D7 strain of *S. cerevisiae* was used as test organism (origin: F.K. Zimmermann, Darmstadt, F.R.G.).

Test compounds. The following substances supplied by IPCS were tested: hexamethylphosphoramide (HMPA), safrole (SAF), *o*-toluidine (TOL), caprolactam (CAP), benzoin (ZOIN), benzene (BEN), diethylhexylphthalate (DEHP), acrylonitrile (ACN), phenobarbital (PB) and diethylstilbestrol (DES).

Media. Basal culture medium (liquid): 2% Bacto peptone (Difco No. 0118-01 Difco Laboratories, Detroit, MI), 1% yeast extract (Difco No. 0127-01) and 2% glucose in double distilled water.

Supplemented medium (solid): to 1 l double distilled water: Bacto yeast nitrogen base without

amino acids (Difco No. 0919-15) 6.7 g

L-arginine HCl	10 mg	L-valine	30 mg
L-isoleucine	60 mg	Uracil	10 mg
L-leucine	60 mg	Adenine sulphate	5 mg
L-lysine HCl	10 mg	L-histidine HCl	10 mg
L-tryptophan	100 mg	L-methionine	10 mg
Agar noble (Difco No. 0142-01)	15 g		
Glucose (Fluka, No. 49140)	20 g		

Tryptophan-free medium: same composition as supplemented medium, but without L-tryptophan.

Isoleucine-free medium: same composition as supplemented medium, but without L-isoleucine.

Method

An inoculum of a stock culture was diluted in fresh growth YEPD medium (1 + 9) and incubated for 5 h at 28°C under shaking. The culture was then adjusted with medium to a density of 0.5–1 $\times 10^8$ cells per ml (as determined with the aid of a counting chamber), and immediately used for the experiment.

All test compounds were dissolved in DMSO. DMSO alone was used for the negative controls.

Incubation without microsomal activation: Into test tubes were introduced: 0.5 ml of the freshly prepared culture, 0.5 ml growth medium, 3.95 ml potassium–sodium phosphate buffer and 0.05 ml of the dissolved test compound. The positive control was treated with 4-nitroquinoline-*N*-oxide (Fluka, Buchs, Switzerland), dissolved in DMSO and diluted with buffer, in a concentration of 0.075 μg/ml.

Incubation with microsomal activation: Into test tubes were introduced: 0.5 ml of the freshly prepared culture, 0.5 ml growth medium, 2.7 ml potassium–sodium phoshate buffer, 0.05 ml of the dissolved test compound and 1.25 ml activation mixture. 1 ml activation mixture contained 0.3 ml S9 fraction of liver from rats induced with Aroclor 1254 (Analabs, North Haven, CT) and 0.7 ml of a solution of cofactors (Ames et al., 1973). The positive control was treated with 60 μg/ml cyclophosphamide monohydrate (Koch Light Labs., Colnbrook Buck, England; No. 1392-60) dissolved in buffer or with 25 or 100 μg/ml 2-acetylaminofluorene (Fluka, Buchs, Switzerland), dissolved in DMSO.

All test tubes were incubated for 16 h on an agitator at 37°C.

The treatment was then discontinued, the suspensions were centrifuged twice, washed and resuspended in buffer. The yeast suspensions were plated out as follows, to determine the counts of surviving cells and the number of mutants produced: 0.1 ml of the suspension was streaked out on each of 5 plates of each of the 2 media. After appropriate dilution in buffer (number of cells determined with a counting chamber), 0.1 ml was streaked out on each of 10 plates of supplemented medium to determine the number of colony-forming units, pink/red sectored colonies (mitotic crossing-over) and pink or red colonies or sectors (aberrant colonies, i.e. other genetic events leading to adenine requirement). The supplemented medium and the tryptophan-free agar were then incubated for 3–6 days and the isoleucine-free agar for about 6–8 days at 28°C; the resultant colonies were counted. The average value for each medium was calculated from the individual values obtained for each plate.

Statistics

The numbers of recombinants and mutants respectively per plate are considered to be Poisson-distributed. The evaluation relates to the number of recombinants or mutants per plate. Concentration groups showing a distinct lower number of colony-forming units than the negative control group (less than 50%) were excluded from the tests. A general p-value was calculated, however. The object of the test is to determine whether plates containing cells treated with the test material reveal a larger number of recombinants and mutants respectively than the negative control plates. The evaluation comprises a comparison of the control group with the treated groups and a trend test. In the case of an increasing trend, the analogue to Williams' test is used for the determination of the lowest effective concentration. In the other case, the analogue to Dunnett's test is used to determine the treated groups which differ from the control group. All tests are based on $C(\alpha)$ statistics. The level of significance was set at $p < 0.01$.

Results

The results are summarized in Table 1. The mean values per plate for each experiment are given in Table 2.

HMPA. In a first experiment the substance was tested at concentrations up to 5000 μg/ml. A significant increase in the incidence of convertants was observed in the experiment without activation at the concentrations of 2500 and 5000 μg/ml. The number of back mutants was significantly enhanced at the highest concentration. These effects could not be observed in the experiment with activation. However, in the gene-conversion system the trend test revealed positive results. In a repeat experiment without activation a significant increase in gene conversions and back mutations was observed at the concentrations of 5000 and 10000 μg/ml.

SAF. No induction of genetic effects could be observed after treatment of the yeast cells with SAF. The compound exhibited an inhibitory effect on the growth of the yeast cells at the concentrations of 30 μg/ml and above.

TOL. Treatment of the yeast cells with TOL did not lead to an induction of genetic effects. A growth-inhibiting effect of the compound was observed at the concentrations of 100–2500 μg/ml (without activation) and 2500 μg/ml (with activation) respectively.

CAP and ZOIN. There was no induction of genetic effects after treatment of the yeast cells with these compounds. An inhibitory effect of the substances on the growth of the yeast cells was not observed.

BEN. Treatment of the yeast cells with BEN did not lead to a significant increase in the incidence of recombinants and mutants. The substance exhibited a growth-inhibiting effect at the concentrations of 1000 μg/ml and above.

TABLE 1

MUTAGENICITY TEST ON *Saccharomyces cerevisiae* D7

Test compound		Genetic endpoint			
		Mitotic crossing-over	Aberrant colonies (total)	Mitotic gene conversion	Back mutation
HMPA	−S9	−	−	+	+
	+S9	−	−	− [a]	−
SAF	−S9	−	−	−	−
	+S9	−	−	−	−
TOL	−S9	−	−	−	−
	+S9	−	−	−	−
CAP	−S9	−	−	−	−
	+S9	−	−	−	−
ZOIN	−S9	−	−	−	−
	+S9	−	−	−	−
BEN	−S9	−	−	−	−
	+S9	−	−	−	−
DEHP	−S9	−	−	+	−
	+S9	−	−	+	−
ACN	−S9	−	−	+	−
	+S9	−	−	−	−
PB	−S9	−	−	−	−
	+S9	−	−	−	−
DES	−S9	−	−	−	−
	+S9	−	−	−	−

[a] Trend test positive.

TABLE 2

COLONY-FORMING UNITS, RECOMBINANTS AND MUTANTS PER PLATE (ARITHMETIC MEAN)

Test compound (μg/ml)		Genetic endpoint				
		Colony-forming units [a]	Mitotic crossing-over [a]	Aberrant colonies (total) [a]	Mitotic gene conversion undiluted suspension	Back mutation undiluted suspension
HMPA −S9	0	167	0	0.1	18.8	0.6
	625	145	0	0.3	19.8	0.4
	1250	142	0	0.2	18.0	0.8
	2500	144	0	0	29.0 *	0.8
	5000	139	0	0	26.0 *	5.0 *
4NQO	0.075	137	0	1.4	162.2	7.6
HMPA +S9	0	245	0	0.2	38.0	3.0
	625	221	0	0.3	30.4	3.2
	1250	212	0	0.4	38.2	2.2
	2500	195	0	0.2	39.0	2.8
	5000	188	0	0.5	46.2	2.6
Cycl.	60	164	0.1	2.7	293.0	86.4
HMPA −S9	0	208	0	0.4	23.4	0.2
	5000	1860	0.1	29.2 *	2.0 *	
	10000	164	0	0.4	32.8 *	4.6 *
4NQO	0.075	90	0	1.1	157.6	2.0
SAF −S9	0	142	0	0.3	19.4	0.8
	3.3	132	0	0.2	25.4	0.2
	10	120	0	0.3	23.4	1.0
	30	16	0	0	3.8	0
	90	0	0	0	0	0
4NQO	0.075	120	0	1.5	197.2	–
SAF +S9	0	279	0	0.7	29.8	0.6
	3.3	271	0	0.4	29.8	2.0
	10	250	0	0.2	33.4	1.8
	30	166	0	0.2	26.2	1.6
	90	9	0	0	1.8	0
Cycl.	60	207	0.9	3.7	332.2	52.6
SAF −S9	0	214	0	0.4	26.0	0.2
	1.5	168	0	0.1	30.4	0
	3	178	0	0.3	25.4	0.4
	6	165	0	0.1	25.4	0.4
4NQO	0.075	94	0	1.1	153.8	2.0
SAF +S9	0	235	0	0.3	37.4	1.8
	1.5	226	0	0.3	33.6	1.4
	3	220	0	0.1	37.2	2.2
	6	174	0	0	24.6	0.6
2-AAF	100	132	0	1.1	171.0	1.6
TOL −S9	0	111	0	0.3	23.4	1.4
	20	101	0	0.1	20.8	1.2
	100	83	0	0.2	16.6	1.2
	500	64	0	0	13.4	0.4
	2500	1	0	0	0.6	0
4NQO	0.075	68	0.1	0.7	185.6	5.8

TABLE 2 (continued)

Test compound (μg/ml)		Genetic endpoint				
		Colony-forming units [a]	Mitotic crossing-over [a]	Aberrant colonies (total) [a]	Mitotic gene conversion undiluted suspension	Back mutation undiluted suspension
TOL +S9	0	135	0	0.4	27.0	0.4
	20	108	0	0.4	20.2	0.6
	100	141	0	0.1	25.2	0.4
	500	108	0.1	0.4	17.6	0.4
	2500	3	0	0	0.6	0
Cycl.	60	98	1.3	3.9	292.4	56.2
CAP −S9	0	145	0	0.1	33.8	0.4
	40	103	0	0.1	27.8	1.6
	200	122	0	0	32.6	0.4
	1000	97	0	0.1	20.4	0.4
	5000	107	0	0.1	28.0	0
4NQO	0.075	57	0	0.9	119.0	1.6
CAP +S9	0	119	0	0.2	39.2	0.8
	40	(471)	0	0.3	32.8	0.6
	200	159	0	0.2	31.8	1.0
	1000	102	0	0.1	31.0	0.4
	5000	112	0.2	0.4	33.2	0.2
Cycl.	60	89	0.4	1.4	210.8	22.6
ZOIN −S9	0	126	0	0.1	42.6	0.6
	20	127	0.1	0.2	29.6	0.8
	100	95	0.1	0.1	24.6	0.6
	500	119	0	0.3	25.8	0.4
	2000	143	0	0.2	33.0	1.3
4NQO	0.075	52	0.1	0.5	127.6	2.2
ZOIN +S9	0	129	0	0.4	29.4	0.2
	20	147	0	0.2	41.6	1.2
	100	138	0	0.1	30.0	1.6
	500	120	0	0.1	20.8	0
	2000	129	0	0.4	22.8	0.6
2-AAF	100	20	0	0	18.8	0
BEN −S9	0	152	0	0.1	19.2	0.2
	40	109	0	0.2	22.2	0.4
	200	111	0	0	14.0	0.4
	1000	91	0	0	13.8	0
	5000	0	0	0	0	0
4NQO	0.075	62	0.2	0.9	180.2	1.6
BEN +S9	0	209	0	0	30.8	0
	40	171	0	0	32.8	0
	200	181	0	0	33.8	0.2
	1000	52	0	0	7.0	0
	5000	0	0	0	0	0
2-AAF	25	127	0.1	0.6	105.6	0.2

TABLE 2 (continued)

Test compound (μg/ml)		Genetic endpoint				
		Colony-forming units [a]	Mitotic crossing-over [a]	Aberrant colonies (total) [a]	Mitotic gene conversion undiluted suspension	Back mutation undiluted suspension
BEN −S9	0	180	0	0.2	32.4	0.2
	4	180	0.1	0.2	34.0	1.0
	20	178	0	0.3	23.0	0.4
	100	181	0	0.3	31.2	1.0
	500	147	0.1	0.2	39.8	0.2
4NQO	0.075	132	0.1	1.8	191.0	2.2
BEN +S9	0	162	0	0.4	41.4	0
	4	169	0.1	0.1	33.6	0.6
	20	192	0	0.1	34.3	0.4
	100	188	0	0.2	26.2	0.4
	500	167	0	0.3	35.2	0
Cycl.	60	177	1.6	3.6	288.0	8.4
DEHP −S9	0	204	0	0.3	26.0	2.2
	40	174	0	0.1	18.2	0.6
	200	180	0.1	0.1	27.5	1.5
	1000	170	0	0.2	27.0	1.2
	5000	167	0	0.2	38.4 *	0.5
4NQO	0.075	120	0.1	1.3	201.3	3.5
DEHP +S9	0	170	0	0.1	25.0	1.0
	40	148	0	0	27.2	1.6
	200	191	0	0	32.4	1.8
	1000	163	0	0	31.2	1.0
	5000	217	0	0.2	43.2 *	1.2
Cycl.	60	123	1.2	2.5	241.2	39.3
ACN −S9	0	175	0	0.1	43.8	1.6
	40	129	0	0.1	52.6	1.6
	200	53	0	0.1	17.2	1.2
	1000	10	0	0	11.2	0
	5000	0	0	0	0	0
4NQO	0.075	111	0.1	1.2	204.4	5.8
ACN +S9	0	232	0	0	57.6	4.8
	40	174	0	0.1	57.8	3.2
	200	67	0	0.3	30.0	1.6
	1000	19	0.1	0.1	20.2	0.2
	5000	0	0	0	0	0
Cycl.	60	153	0.9	1.9	313.4	41.0
ACN −S9	0	(120)	0	0	26.8	0.2
	6.25	83	0	0.1	29.4	0.2
	12.5	83	0	0.1	30.2	0
	25	59	0	0	35.2 *	0.4
	50	69	0	0	38.6 *	0.6
4NQO	0.075	68	0	0.5	154.2	2.6

TABLE 2 (continued)

Test compound (μg/ml)		Genetic endpoint				
		Colony-forming units [a]	Mitotic crossing-over [a]	Aberrant colonies (total) [a]	Mitotic gene conversion undiluted suspension	Back mutation undiluted suspension
ACN +S9	0	150	0	0	42.8	1.2
	6.25	111	0	0	46.0	0
	12.5	102	0	0.1	52.6	0.4
	25	90	0	0.1	47.2	0.2
	50	80	0	0.1	48.2	1.4
Cycl.	60	86	0.4	2.0	324.8	33.2
ACN −S9	0	98	0	0	15.2	0.2
	12.5	76	0	0.1	15.6	0.4
	25	71	0	0	15.4	0
	50	57	0	0.2	26.2 *	0
	100	22	0	0.1	8.2	0
4NQO	0.075	44	0	0.3	134.0	1.0
PB −S9	0	195	0	0.2	42.4	1.4
	80	195	0.1	0.1	26.8	0.4
	400	195	0	0.1	29.6	1.6
	2000	177	0	0.4	38.2	0
	10000	111	0	0	19.2	0.2
4NQO	0.075	103	0.3	1.5	185.4	3.0
PB +S9	0	227	0	0.1	41.0	0.4
	80	221	0.2	0.3	43.0	0.4
	400	192	0	0.1	40.2	0.6
	2000	183	0	0.1	37.2	0.6
	10000	213	0	0.3	39.0	0
Cycl.	60	95	0.5	2.2	294.4	23.2
DES −S9	0	145	0	0.2	123.8	1.6
	4	147	0	0	144.0 *	2.4
	20	135	0	0	123.6	1.2
	100	104	0.1	0.2	138.6	2.0
	500	60	0	0.1	83.4	0.2
4NQO	0.075	98	0	0.9	245.0	6.6
DES +S9	0	174	0	0.2	195.0	2.4
	4	205	0	0.4	202.6	3.0
	20	203	0	0.2	172.8	2.4
	100	183	0	0.3	226.2 *	2.6
	500	142	0	0.2	196.2	3.0
Cycl.	60	142	1.1	3.7	320.6	45.8
DES −S9	0	78	0	0.1	9.6	0
	2.5	96	0	0.2	8.0	0
	5	94	0	0.2	10.2	0.2
	10	55	0	0	11.8	0
	20	44	0	0	2.0	0
4NQO	0.075	44	0.1	0.3	134.0	1.0

[a] Dilution, 10^{-4}. 0.1 ml suspension was streaked out per plate.

* Significantly positive at $P < 0.01$. This is not indicated for the positive controls, which were significantly positive in most cases.

DEHP. In the experiments without and with activation, treatment with DEHP led to a significant increase in the incidence of tryptophan-prototrophic colonies (mitotic gene conversion) at the concentration of 5000 μg/ml.

ACN. Treatment of the yeast cultures with ACN in the concentrations of 25 and 50 μg/ml without activation led to a significant increase in the incidence of tryptophan-prototrophic colonies. This effect was not observed in the experiments with activation. At the concentrations of 100 μg/ml and above, the substance exhibited an inhibitory effect on the growth of the yeast cells.

PB. Treatment of the yeast cultures in concentrations up to 10000 μg/ml with PB without and with activation did not lead to an induction of genetic effects. A growth-inhibiting effect of the substance was also not observed.

DES. In the experiments without activation an inhibitory effect on the growth of the yeast cells was observed at the concentrations of 20 μg/ml and above. A slight but statistically significant increase in the incidence of tryptophan-prototrophic colonies was found at the concentrations of 4 μg/ml without activation and 100 μg/ml with activation. However, these effects showed no dose-dependence and could not be confirmed in an additional experiment without activation. They are therefore attributed to fluctuations in the rate of spontaneously occurring tryptophan-prototrophic colonies.

Discussion

3 of the 10 compounds tested induced genetic effects in *S. cerevisiae* D7. HMPA without activation induced mitotic gene conversion and back mutation. Treatment with ACN without activation and DEHP without and with activation led to an increase in the incidence of gene conversions only. The other 7 compounds did not induce any genetic effect in *S. cerevisiae* D7 under the given test conditions.

The effects that occurred after treatment with HMPA and ACN showed dose-dependence and were reproducible. Due to lack of time, the experiments with DEHP could not be repeated. However, the increase in the incidence of gene conversions observed after treatment with DEHP occurred in the experiments without and with activation at the highest concentration only. It is therefore considered an effect induced by the test compound.

It has been shown that the test protocol used is well suited for the detection of chemical mutagens. Treatment of growing cultures of *S. cerevisiae* D7 for 16 h at 37 °C led to clear-cut positive results with various ultimate mutagens and promutagens (Arni and Müller, 1983a, b). The negative results obtained with the 5 carcinogens SAF, TOL, BEN, PB, and DES may be explained by the fact that these compounds do not induce DNA damage in *S. cerevisiae* D7. However, it is also possible, that the test procedure used is not optimal for detecting these particular substances. The applied evaluation criteria (increase in the number of recombinants, convertants and mutants respectively per plate: statistical significance, dose-dependence and reproducibility) proved to be well suited for the assessment of genetic effects in this test system.

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The induction of mitotic gene conversion in the yeast *Saccharomyces cerevisiae* strain JD1

T.M. Brooks, L.P. Gonzalez, R. Calvert [1] and J.M. Parry [1]

Shell Research Ltd., Sittingbourne Research Centre, Kent and [1] University College of Swansea, Singleton Park, Swansea (Great Britain)

Summary

The results indicate that ACN produces significant increases in the frequency of mitotic gene conversion in stationary-phase and log-phase cultures of the yeast, *Saccharomyces cerevisiae* JD1, in the presence of a rat-liver activation system (S9) and in the optimized yeast P-450 assay.

No activity was observed with HMPA, SAF, TOL, CAP, ZOIN, BEN, DEHP, PB, or DES.

10 chemical compounds selected for testing in the IPCS Collaborative Study Part 1 were assayed for their ability to induce mitotic gene conversion in stationary-phase and log-phase cultures of the yeast *Saccharomyces cerevisiae* JD1. Assays were performed in the presence and in the absence of S9 microsomal fraction obtained from a liver homogenate from rats pretreated with Aroclor 1254, and in a yeast assay optimized for cytochrome P-450 levels in the absence of an exogenous activation system.

9 of the chemicals tested did not increase the mitotic gene conversion frequency in any of the assays. These were HMPA, SAF, TOL, ZOIN, CAP, BEN, DEHP, PB and DES.

Exposure of both stationary-phase and log-phase cultures to acrylonitrile resulted in a significant increase in mitotic gene conversion in the presence of S9 fraction. ACN also showed an increase without the presence of S9 fraction in stationary-phase cells at the Swansea University laboratory only. However, the increase was greatly enhanced in the presence of S9. ACN also showed a significant increase in the yeast P-450 assay.

Materials and methods

Strain

The genotype of *Saccharomyces cerevisiae* JD1 is given below:

a	ade 2-1	ser 7	his 8	his 4C	trp 5 - U9
α	+	+	+	his 4ABC	trp 5 - U6

The use of this strain for the detection of chemical and physical mutagens has been described by Davies et al., 1975. For this study an isolate of JD1 that would not undergo sporulation was used. This is important during long exposure times to chemicals; otherwise convertants may arise by meiotic recombination (Zimmermann, 1975).

Chemicals

HMPA, SAF, TOL, CAP, ZOIN, BEN, DEHP, ACN, PB and DES were supplied by Lancaster Synthesis and J. Ashby, ICI Ltd.

DEHP was prepared as a solution in dimethyl sulphoxide (DMSO) + 1% Tween 80; all other compounds were formulated in DMSO alone.

The positive control compounds cyclophosphamide (Sigma), 4-nitroquinoline-*N*-oxide (J. Ashby, ICI Ltd.) or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Koch-Light) were included in each assay.

Culture media

The cells were exposed to the test compounds in Sorenson phosphate buffer pH 7.0 or in complete growth medium. The growth media used were

yeast complete broth (YC) at Swansea University (Cox and Bevan, 1962) and YEPD broth at Shell Research Ltd. (Davies et al., 1975). Mitotic gene conversion was scored on yeast minimal (YM) plates supplemented with either histidine or tryptophan (Davies et al., 1975). Cell viability was scored on YC agar (Swansea University) or YEPD agar (Shell Research Ltd.).

S9 mix

The S9 mix was prepared from Aroclor-induced rats according to the method of Ames et al. (1975).

Treatment of stationary-phase cells

3–4-day-old colonies grown on solid complete growth medium (YC or YEPD) were suspended in pH 7.0 phosphate buffer at a concentration of 5×10^7 cells/ml (Shell) or 1×10^8 cells/ml (Swansea). This suspension was then divided into 1.6-ml amounts in 30-ml universal containers together with 0.1 ml test compound solution and 0.3 ml pH 7.0 buffer (−S9); these cultures were incubated, with shaking, at 28°C for 18 h. For the experiments with metabolic activation (+S9), test compound (0.1 ml) was added to the yeast cell suspension (1.6 ml), together with S9 mix (0.3 ml); these cultures were incubated with shaking, at 37°C for 2 h and for a further 16 h at 28°C. The treatments were terminated by washing the cells in saline and then resuspending in 2 ml saline. Aliquots (0.1 ml) were then spread onto YM plates supplemented with tryptophan to determine the number of histidine prototrophs and 0.1-ml aliquots (Shell) and dilutions of 1 in 10 (Swansea) were spread onto YM plates supplemented with histidine to determine the number of tryptophan prototrophs. Appropriate dilutions were spread onto complete growth medium plates to determine cell viability.

Treatment of log-phase cells

4-day-old colonies grown on solid complete

TABLE 1

TOXICITY [a] OF TEST COMPOUNDS IN ASSAYS USING *Saccharomyces cerevisiae* JD1

Test compound	Stationary-phase (Swansea)		Stationary-phase (Shell)		Log-phase (Shell)	
	−S9	+S9	−S9	+S9	−S9	+S9
Hexamethylphosphoramide	NT	NT	NT	NT	NT	NT
Safrole	42% at 250 11% at 500 7% at 1000	46% at 500 4% at 1000	43% at 2000	56% at 500 22% at 1000 12% at 2000	33% at 500 5% at 2000	51% at 250 5% at 2000
o-Toluidine	NT	NT	NT	NT	70% at 2000	NT
Caprolactam	NT	NT	NT	NT	NT	65% at 2000
Benzoin	NT	NT	NT	58% at 2000	NT	NT
Benzene	85% at 1000 41% at 2000	NT	64% at 2000	71% at 1000 15% at 2000	79% at 1000 3% at 2000	65% at 1000 23% at 2000
Diethylhexylphthalate	NT	NT	NT	NT	NT	NT
Acrylonitrile	68% at 250 42% at 500 0% at 1000	62% at 500 0% at 1000	76% at 1000 19% at 2000	62% at 250 49% at 500 0% at 1000	90% at 1000 2% at 2000	57% at 500 1% at 1000
Phenobarbital	NT	NT	NT	NT	NT	NT
Diethylstilbestrol	67% at 1000 53% at 2000	47% at 1000 65% at 2000	NT	17–26% at 125–500, 51% at 1000, 78% at 2000	NT	NT

[a] Results expressed in % viability per concentration (μg/ml) test compound.

NT, no toxicity observed up to 2000 μg/ml.

growth medium were suspended in complete growth medium at a concentration of 5×10^6 cells/ml and incubated at 28 °C, with shaking, for 6 h. Aliquots were then treated and plated as for stationary-phase cells.

Optimized yeast P-450 assay

3-day-old colonies grown on solid complete growth medium were suspended in low glucose YC broth (0.5% glucose) at a concentration of 1×10^3 cells/ml, and incubated for 48 h at 28 °C with shaking. The cells were then centrifuged and resuspended in 400 ml high glucose YC broth (20% glucose). This suspension was divided into 1.9-ml amounts in 30-ml universal containers and test compound solution (0.1 ml) was added. The cultures were then incubated at 28 °C for 18 h with shaking. Aliquots were then spread onto YM plates supplemented with histidine or tryptophan to determine the number of prototrophs. Appropriate dilutions were spread onto YC plates to determine cell viability.

Results

The genetic activity of the 10 test compounds was investigated using 3 different methods of exposure of the yeast, *Saccharomyces cerevisiae* JD1, in two different laboratories. Thesse methods were the treatment of stationary-phase cells in buffer (Swansea University and Shell Research Ltd.), log-phase cells in growth medium (Shell) and the optimized yeast P-450 assay (Swansea).

A preliminary experiment was conducted at Swansea University to determine the optimal incubation of the yeast cells with the S9 mix at 37 °C to maximize metabolic activation. The treatment procedure selected involved exposure of cells to the test compound and S9 mix for 2 h at 37 °C prior to further incubation at 28 °C.

The addition of HMPA, SAF, TOL, CAP, ZOIN, BEN, DEHP, PB and DES at concentrations up to 2000 μg/ml to stationary-phase or log-phase cultures of *S. cerevisiae* JD1 did not show any consistent increase in the rate of mitotic gene conversion, either with or without the addition of rat-liver S9 fraction, or in the optimized yeast P-450 assay. The effect of all the test compounds on cell viability is summarized in Table 1.

ACN induced significant levels of mitotic gene conversion at the his_4 and trp_5 loci in both stationary-phase cultures of *S. cerevisiae* JD1 in the presence of rat-liver S9 fraction only (Fig. 1). The data from the two laboratories for stationary-phase cells were in good agreement, and comparable to the results obtained in log-phase cells. ACN also increased mitotic gene conversion at both loci in

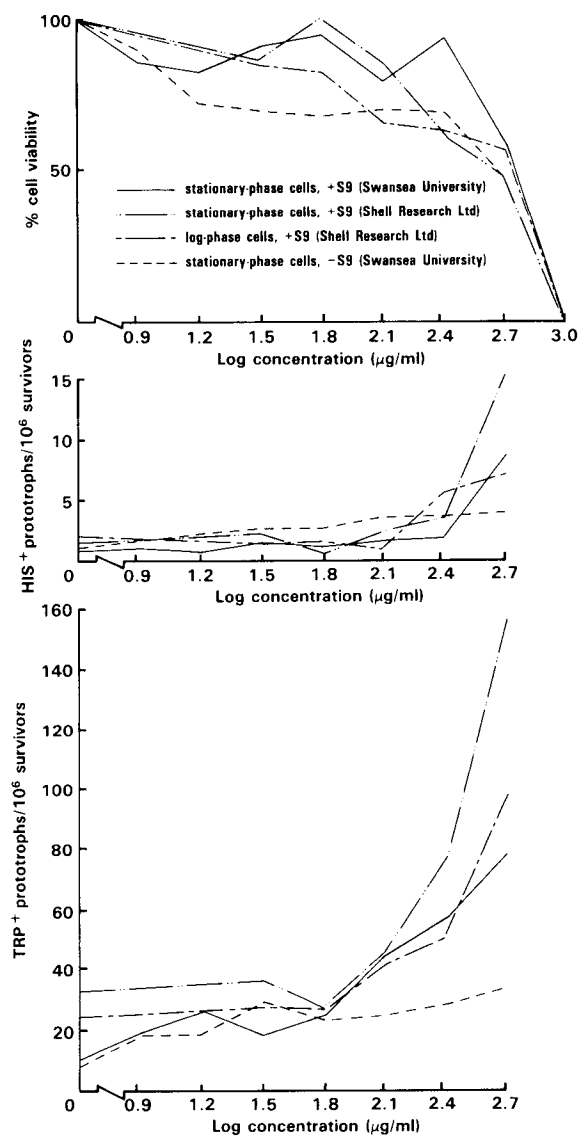

Fig. 1. Survival and induction of mitotic gene conversion in *S. cerevisiae* JD1 after treatment with acrylonitrile in the presence or absence of S9 fraction (+ or − S9).

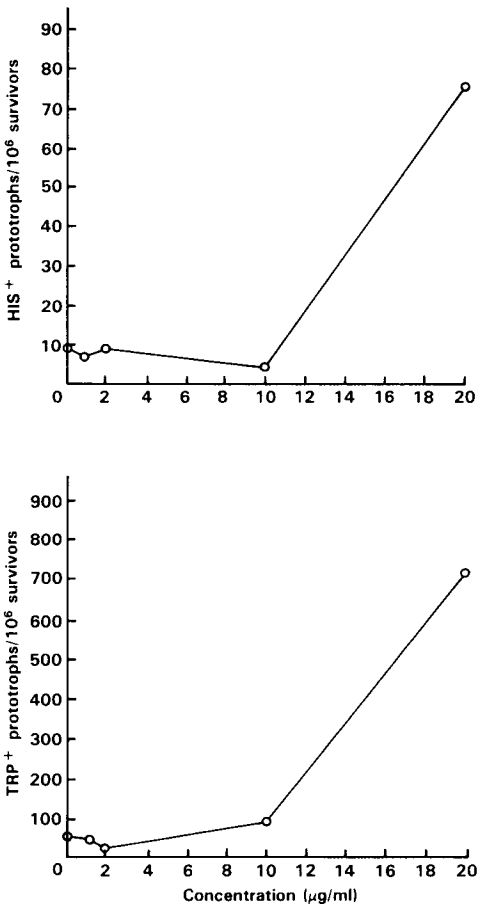

Fig. 2. Mitotic gene econversion in the yeast *S. cerevisiae* JD1 after treatment with acrylonitrile in the optimised P450 assay.

the optimized yeast P-450 assay (Fig. 2). This assay system, utilizing growth in low and, subsequently, high glucose-containing medium to enhance cytochrome P-450/P-448 type metabolism, has previously demonstrated the ability to detect indirect-acting mutagens in the absence of an exogenous activation system (Kelly and Parry, 1983).

Treatment with 4-nitroquinoline-*N*-oxide or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, direct-acting mutagens, was shown to induce mitotic gene conversion in the relevant assays with and without metabolic activation. Treatment with cyclophosphamide, an indirect-acting mutagen, was shown to induce mitotic gene conversion in both log-phase and stationary-phase cells in the presence of S9 fraction.

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Petite mutagenesis in *Saccharomyces cerevisiae* strain D5

Lynnette R. Ferguson

Cancer Research Laboratory, University of Auckland School of Medicine, Auckland (New Zealand)

Summary

All ten compounds in the CSSTT trial were tested as "petite" mutagens, using *S. cerevisiae* strain D5. Each was incubated with the yeast in liquid growth medium for 24 h, using different incubation procedures to optimize toxicity and metabolic activation. DEHP, PB, CAP and ZOIN were not sufficiently toxic to be evaluated in this strain of yeast. Although HMPA and DES failed to induce petite mutants, SAF, TOL, ACN and BEN all induced significant levels under conditions optimizing endogenous metabolic activation.

Many carcinogens such as metal salts, thioacetamide and thiourea which are not active in the Ames' test (Maron and Ames, 1983), cause antimitochondrial effects in yeast assays (Lindegren and Lindegren, 1973; Egilsson et al., 1979). Such antimitochondrial activity is conveniently measured by estimating "petite" or respiratory deficient mutations in the yeast *Saccharomyces cerevisiae*, and a "petite" mutagenesis assay has been proposed (Egilsson et al., 1979; Wilkie and Evans, 1982), as a screen for potential carcinogens.

In the present study, petite mutagenesis has been measured in strain D5 (Zimmermann, 1973) for the 10 compounds in the CSSTT trial.

Materials and methods

Yeast strain

The diploid strain D5 (Zimmermann, 1973) was kindly provided by B.S. Cox (Botany School, Oxford University). In some preliminary experiments, strain 5178B (from D. Wilkie, University College, London) was also used.

Upon receipt, both strains were inoculated into fresh yeast complete medium, grown to stationary phase, DMSO added to 10%, and 1-ml samples stored at −80 °C. For each experiment, a sample was thawed, added to 10 ml fresh medium and grown for exactly 2 h before use. This procedure was important to ensure consistency of results.

Chemicals

8 carcinogens — hexamethylphosphoramide (HMPA), safrole (SAF), *o*-toluidine (TOL), benzene (BEN), di(2-ethylhexyl)phthalate (DEHP), acrylonitrile (ACN), diethylstilbestrol (DES) and phenobarbital (PB) — and 2 noncarcinogens — caprolactam (CAP) and benzoin (ZOIN) — were supplied by J. Ashby, ICI. Ethylmethanesulphonate (EMS), ethidium bromide (EB) (Sigma Chemical Co., U.S.A.) and benzidine (ZID) (ICI Central Toxicology Laboratories) were used as positive controls.

Media

Yeast complete medium (Cox and Bevan, 1962) supplemented with either 2% or 20% glucose, solidified with 1.5% agar where necessary, was used as a growth medium.

Microtitre assay for "petite" mutagenesis

As described in Ferguson and Baguley (1981), a log-phase cell culture (2×10^6 cells/ml) was diluted 100-fold into fresh YC medium. A 96-well (12 column × 8 row) sterile microtitre tray was inoculated with 200 μl in the front row, otherwise

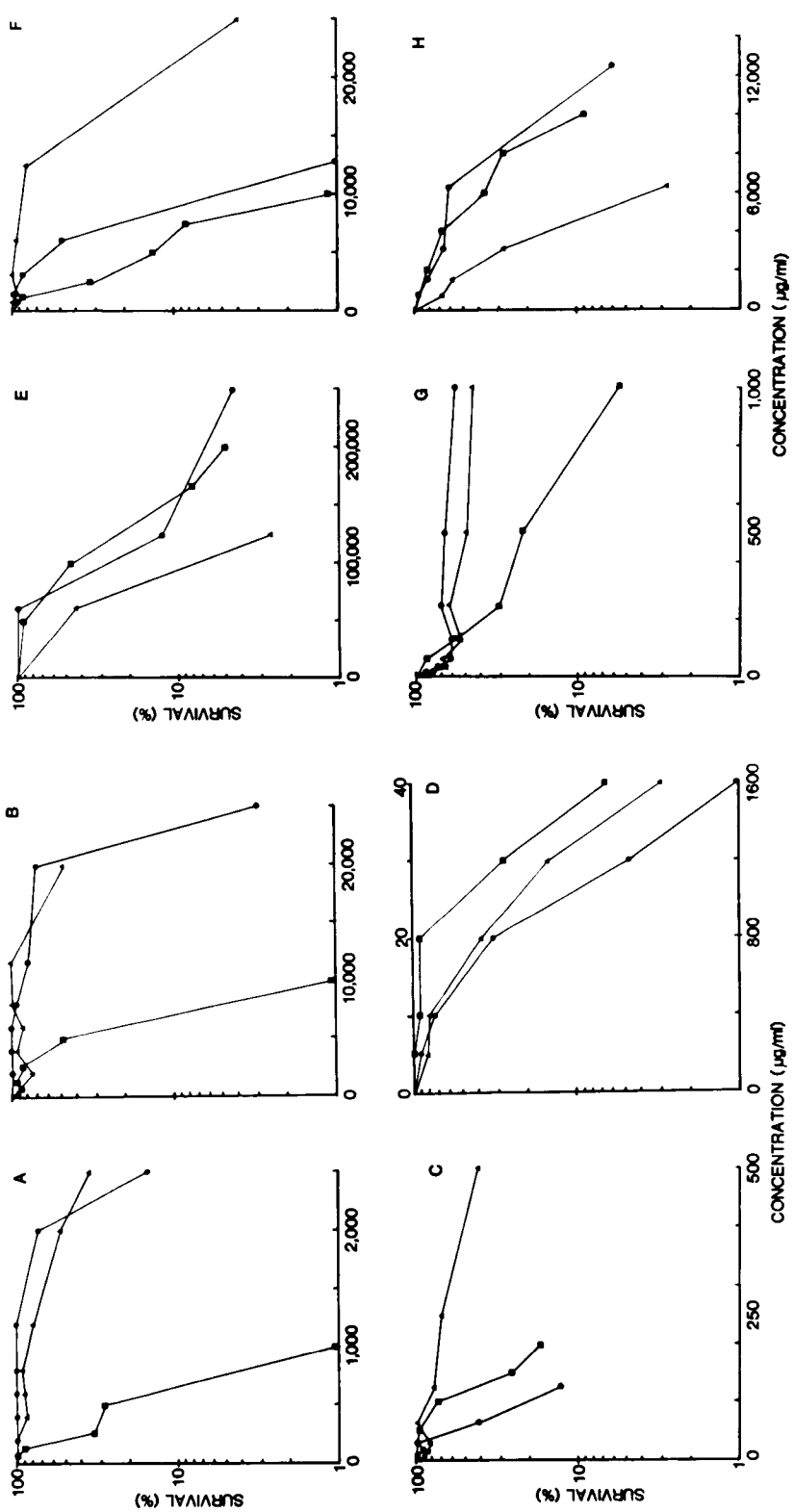

Fig. 1. Survival of *S. cerevisiae* D5 after incubation with a concentration range of various compounds for 24 h, in growth medium. [1A] Compounds incubated in microtitre trays at 30 °C (●, 2% glucose medium; ▲, 20% glucose medium) or in flasks at 37 °C (■). A, SAF; B, TOL; C, DES; D, EB, as positive control for microtitre assays (upper axis), ZID as positive control for flasks (lower axis). [1B] Compounds incubated in McCartney bottles at 30 °C (●, 2% glucose medium; ▲, 20% glucose medium) or in sealed flasks at 37 °C (■). E, HMPA; F, BEN; G, ACN; H, EMS as positive control (for McCartney bottles only).

100-μl aliquots of the diluted yeast culture. For preliminary experiments, 4 μl of a 4 mg/ml solution (in DMSO) was added to the front well in a column (containing 200 μl of yeast suspension). This was mixed using a 100-μl eppendorf pipette, and 100 μl taken into the second well. The process was continued until the seventh row was reached, when the extra 100 μl was discarded. To the eighth row was added 2 μl of DMSO. In this way (Fig. 1), it was possible to expose a given strain of yeast to 12 drugs at 7 different (2-fold) concentrations within a single microtitre tray. In subsequent experiments, the initial drug concentration was adjusted so that the highest dose prevented growth in approximately 90% of the cells. Trays were incubated at 30 °C or 37 °C for 24 h, a 1 in 10^4 dilution was made from each well into saline, and the resulting suspension was plated in triplicate onto YC plates. These were incubated at 30 °C for 3 days and then scored for "petite" and "grande" colonies by tetrazolium overlay (Nagai, 1959).

"Petite" mutagenesis assay using cells grown in McCartney bottles, or shaken in flasks

Chemicals were added to bottles or flasks in no more than 200 μl of DMSO and the yeast suspension (diluted as above) added to give a final volume of 10 ml. Bottles were incubated with (flasks) or without (McCartney bottles) shaking, at 30 °C or 37 °C for 24 h, a 1 in 10^4 dilution into saline was made, and 100 μl of this suspension plated onto each of three YC plates. It was found that washing was unnecessary when chemicals were diluted by this factor. After 3 days incubation at 30 °C, plates were scored for grande and petite colonies by tetrazolium overlay (Nagai, 1959). Where there was a shortage of material, quantities used were reduced but kept in these proportions.

Metabolic activatioan

No exogenous S9 mix was added. Instead, exponentially growing yeast cultures were diluted into 20% glucose medium and grown in 500-ml flasks with medium almost to the top, and incubated at 37 °C for 2 h, with gentle stirring, before use. This has been found to optimise cytochrome P448/P450 activity (Kelly and Parry, 1983). As an alternative method of optimising endogenous metabolic activation, yeast were grown in 2% glucose medium and incubation with the chemicals was at 37 °C rather than the usual 30 °C. These conditions have been found to optimise activation of ZID (used as a positive control), at least in this strain of yeast.

Results

Initial experiments were performed, using the microtitre plate method, for all trial compounds plus the three positive controls, EB EMS and ZID. DEHP, PB, CAP, ZOIN and ZID, did not cause petite mutagenesis at any concentration. However, the last 4 compounds were precipitating out of solution at the highest dose tested (2000 μg/ml) and high levels of toxicity were not reached in any experiment. Testing of DEHP was carried out with or without DMSO, up to levels of 1 : 1 pure compound : yeast suspension.

It was also impossible to reach toxic levels of HMPA, ACN or BEN in the microtitre assay, and this was considered as possibly due to volatility. Also, although EMS (as positive control) gave the expected positive results, positive data were also obtained from wells one or two removed from the inoculum. Again, volatility was considered a problem. For each of these chemicals, subsequent experiments were performed in sealed McCartney bottles, as described in Materials and Methods. Survival and petite induction data are illustrated for each, on figs. 1 and 2. As expected from previously published data, EMS caused petite mutagenesis, and was a suitable positive control for experiments with volatile compounds, using sealed bottles.

HMPA, ACN and BEN all caused toxicity without added DMSO being necessary. From the data, it can be seen that the first of these is not a petite mutagen, while ACN and BEN both increase levels significantly. These increases were unambiguous, dose dependent, and reproducible.

SAF, TOL and DES could all be assayed in the microtitre test, reaching toxic levels (Fig. 1). While DES did not increase "petite" mutagenesis, both SAF and TOL increased "petite" levels in a dose-dependent and reproducible fashion, and could be considered positive in the assay (Fig. 2). Testing the compounds in the haploid strain 5178B did not change the pattern of results seen.

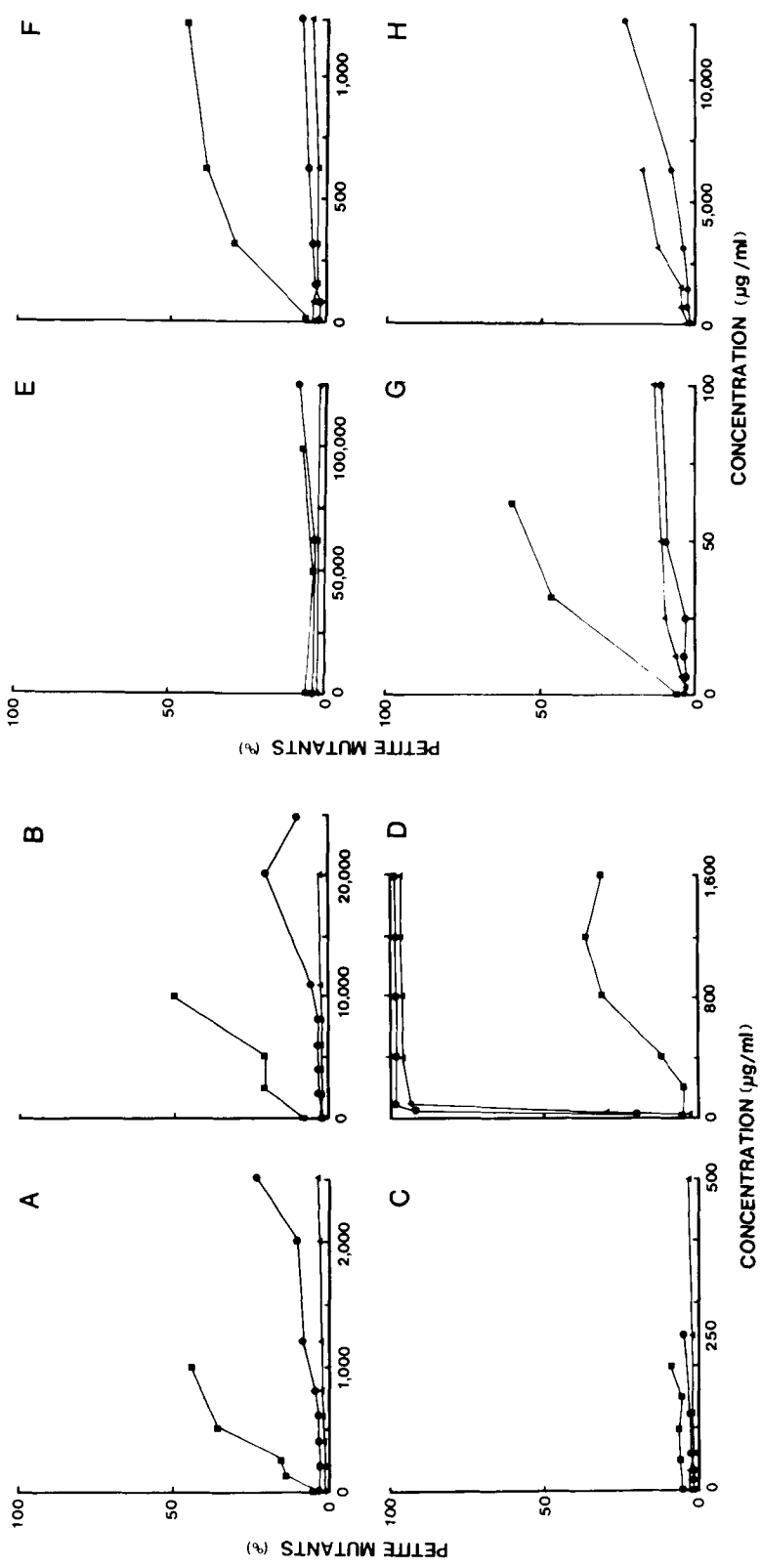

Fig. 2. Petite mutagenesis in *S. cerevisiae* D5 after incubation with a concentratioan range of various compounds for 24 h in growth medium. Numbering and symbols as for Fig. 1.

Growth in 20% glucose medium, which increases metabolic activation of many other compounds (Kelly and Parry, 1983), if anything decreased petite mutagenesis by the present compounds.

In separate experiments (data to be published elsewhere) we have found that ZID can cause toxicity in Strain D5, provided incubation is in flasks, shaken in a water bath, rather than in a microtitre tray. "Petite" induction is optimal for this compound at 37 °C. For this reason, each of the ten compounds under study was retested using this methodology. For HMPA, ACN and BEN, flasks had sealed caps. Although this approach did increase toxicity of most compounds (Fig. 1), DEHP, PB, CAP and ZOIN still did not reach clearly toxic levels. Although HMPA and DES still gave negative results for petite mutagenesis, the 4 compounds causing weak petite induction in previous methodologies caused higher frequencies of such mutants (Fig. 2). Preliminary experiments indicated, however, that these levels of petite induction could be reached using either of the previous methods, providing incubation was at 37 °C. It is only the toxicity of poorly water-soluble compounds that tends to be enhanced by shaking in a flask.

Discussion

Of the 8 carcinogens and 2 noncarcinogens tested in this study, 4 carcinogens gave positive results while neither noncarcinogen was active in the system. Two of the carcinogens (as well as the two noncarcinogens) did not reach toxic levels and it might be worth developing yeast strains with more permeable cell walls for testing compounds of this nature. DES may be more realistically seen as a tumour promotor than an initiator, and might not be expected to give a positive response in this assay (Parry et al., 1982). HMPA was the only carcinogen that gave negative results when it might be expected to show up as positive. It is possible that the compound requires an activation step that cannot be duplicated in this vitro system.

For each of the compounds that do show as positive in this system, the transfer to 20% glucose media slightly decreased, rather than increased, mutagenesis. Incubation at 37 °C, however, significantly enhanced the very weakly positive results seen at 30 °C. Such a result was also found for ZID, as positive control (unpublished) on this strain of yeast. It would seem that 2% glucose medium alone can lead to sufficient P448/P450 activity for metabolic activation in *S. cerevisiae* D5.

Taken together, the results of the present study suggest that petite mutagenesis could be used as a complement to the Ames test for some, but not all, types of compound. More work on metabolic activation with this system would be appropriate. For water-soluble, nonvolatile compounds, use of the microtitre assay gives a simple method, utilizing very small quantities of compound, and yielding results within 3 days. If compounds are only poorly water-soluble, incubation in flasks in a shaking water bath may better permit toxic levels to be reached. Volatile materials require incubation in either sealed McCartney bottles or flasks. Both of these methods are more labor intensive than the microtitre assay.

At present, it must be concluded that the petite mutagenesis assay as a predictor of carcinogenesis is still in developmental stages, but may be worth further investigation and validation.

Acknowledgements

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Tests for the induction of mitotic aneuploidy in the yeast *Saccharomyces cerevisiae* strain D61.M

F.K. Zimmermann, J. Heinisch and I. Scheel

Institute for Microbiology, Technical University of Darmstadt (Federal Republic of Germany)

Summary

A diploid strain of the yeast *Saccharomyces cerevisiae* was used to study the effects of chemicals on the induction of mitotic aneuploidy. The test was designed to detect chromosome loss resulting in a monosomic condition. At the same time, the cumulative effects of induced point mutation, mitotic recombination and deletion of chromosomal fragments could be scored.

Positive control substances were included into the study. Hycanthone strongly induced mitotic segregation and elicited chromosomal loss at a low level. Bavistan was a strong inducer of mitotic chromosome loss but at best caused a weak increase in mitotic segregation. No exogenous source of metabolic activation was used. Hexamethylphosphoramide was totally inactive up to 4.76%. Benzoin, di(2-ethyl hexyl)phthalate and phenobarbital were also completely inactive within solubility limits or up to 1%. Acrylonitrile strongly induced mitotic segregation but no aneuploidy. Benzene induced mitotic segregation and weakly induced mitotic aneuploidy. Caprolactam was a possible weak inducer of aneuploidy. Diethylstilbestrol induced mitotic segregation and aneuploidy at a low level. Safrole was very toxic but did not induce any genetic effects. This was also true for *o*-toluidine.

Mutagenicity test batteries usually include systems to detect point mutations, chromosomal structural aberrations, and various types of repair reactions. The primary targets for the induction of such endpoints are either DNA directly or components of nucleic acid metabolism such as precursor formation, DNA replication and repair. The proper segregation of chromosomes in mitotis and meiosis is mediated by the spindle fiber apparatus and among the most prominent components of this structure are the spindle fibers, which contain tubulin. A number of chemicals induce chromosomal malsegregation on one hand and interfere with microtubule assembly on the other (Kilmartin, 1981). Also, a survey of the literature dealing with mutagenicity testing using fungal systems showed that at least 17 chemicals induce aneuploidy but no other types of genetic effects (Zimmermann, 1983). Therefore, it became urgent to develop test systems for the study of the mechanisms involved in the induction of chromosomal malsegregation. The work reported here was performed without a system of exogenous metabolic activation, the goal being to detect chemicals that directly interfere with chromosomal segregation.

Materials and methods

The Test System. A diploid yeast strain, D61.M, was constructed. It had the following genotype:

$$\frac{cyh2}{CYH2}\ \frac{TRP5}{trp5}\ \frac{leu1}{LEU1}\ centromere\ \frac{ade6}{ADE6}\ \frac{MAL2}{mal0}\ \frac{MAT\alpha}{MATa}$$

$$centromere\ \frac{ade2}{ade2}\ \frac{his}{HIS}\ \frac{SUC}{suc0}\ \frac{ilv1\text{-}92}{ilv1\text{-}92}$$

It formed red colonies because of the homozygous condition of *ade2*, required isoleucine be-

TABLE 1

EFFECTS OF HYCANTHONE ON YEAST STRAIN *D61.M*

Concentration	Titer	Total resistants		White resistants	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Control	31.9	1425	14.90	3	0.31
Bavistan					
10 ppm	12.3	747	20.24	51	13.82
20 ppm	1.3	288	55.36	69	124.45
Hycanthone					
50 ppm	27.5	599 *	36.26	2 *	1.21
100 ppm	23.2	1318 *	94.82	4 *	2.88
125 ppm	16.8	1333 *	131.98	3 *	2.97
150 ppm	3.2	446 *	229.90	2 *	10.31
187 ppm	0.8	1080	432.00	4	16.00

Usually, cells were plated from the cultures directly on the selective medium. However, because of the strong induction of mitotic segregation induced by hycanthone platings were done from a 1:5 dilution of the treatment cultures. This is indicated by *. There were red resistants which reflect the occurrence of mitotic recombination between the centromere and *cyh2* or even a chromosome break resulting in a terminal deletion or point mutation from *CYH2* to *cyh2*, this is cumulatively called mitotic segregation. White colonies were tested for a leucine requirement. Only the ones which expressed *leu1* are listed.

TABLE 2

GENETIC ANALYSIS OF WHITE, LEUCINE-REQUIRING AND CYCLOHEXIMIDE-RESISTANT DERIVATIVES OF YEAST STRAIN *D61.M*

Origin of derivatives or inducing agents	Total	Maximum viable spores per ascus		Other aneuploidies
		4	2	
Control	5	4	1	0
Methylethyl ketone	14	12	2	2: $2n+1$ III
Ethylacetate	20	19	1	1: $2n+1$ III
Bavistan	9	7	2	1: $2n+1$ III
Total	48	42	6	4: $2n+1$ III

All 48 derivatives segregated only *ade6, leu1, TRP5*, but were still heterozygous for *HIS/his*. The trisomic condition for chromosome III could be established by the segregation of mating and nonmating spores. Some of the nonmating spores were able to sporulate.

TABLE 3

EFFECTS OF ACRYLONITRILE ON YEAST STRAIN *D61.M*

Concentration	Titer $\times 10^6$	Total resistants		White resistants	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Control	43.3	1300	12.35	4	0.31
Bavistan					
20 ppm	2.3	236	34.71	76	111.76
Acrylonitrile					
0.249 µl/ml	18.1	1060	19.56	5	0.92
0.498 µl/ml	7.5	625	27.65	1	0.44
0.744 µl/ml	3.2	624	65.00	0	–
0.990 µl/ml	1.7	530	101.92	1	1.92

cause of the homozygous condition of *ilv1-92*. There was no mating reaction because of the presence of both mating-type alleles, no resistance to cycloheximide and no requirements for tryptophan, leucine or histidine. However, this strain could utilize maltose and also raffinoase because the active alleles MAL2 and SUC were dominant. Chromosome loss is indicated when the recessive markers on both sides of the centromere of chromosome VII, *cyh2, leu1* and *ade6*, are expressed. Expression of *cyh2* allows growth on a medium with 1.5 ppm cycloheximide; and the colonies are white instead of red when *ade6* is simultaneously expressed. Such colonies were then tested for expression of *leu1* on a medium without leucine. A genetic analysis of such colonies was performed by sporulation and subsequent tetrad analysis. A monosomic diploid should yield only two viable spores per ascus. Tetrad analysis also revealed other aneuploidies. A trisomic condition for chromosome III should result in two possible conformations: MATa MATa MATα or MATa MATα MATα. Such trisomics will segregate spores carrying MATa and MATα, and these cannot mate. Also, trisomic conditions of the type SUC SUC suc0 of HIS HIS his can also be detected as yielding 3 and 4 HIS spores per ascus.

Media. The full medium contained 1% yeast extract, 2% bactopeptone and 2% glucose. It was solidified if needed with 1% Oxoid agar. Resistance for cycloheximide was selected on this medium supplemented with 1.5 ppm cycloheximide. Survival was determined on plates with a synthetic medium with 1.6 g/l Difco yeast nitrogen base without amino acids and ammonium sulfate. This was supplemented with adenine (4 mg/l, reduced amount for better pigmentation), histidine (10 mg/l), isoleucine and leucine (60 mg/ml) and tryptophan (10 mg/ml). For the determination of the segregation and expression of nutritional markers we omitted the respective supplements. Utilization of maltose and raffinose (indication for the presence of gene *SUC*) was tested on full medium containing either 2% maltose or 2% raffinose and 2 ppm antimycin A to block respiration. The normal carbon source in all other media was 2% glucose and ammonium sulfate at 0.5% the standard nitrogen source.

Growth conditions. The standard incubation temperature was 28°C. Cells for experiments were grown in the full medium up to a titer of 5×10^7 cells/ml in 5-ml volumes. Samples of 0.1 ml were plated on the cycloheximide medium to detect cultures with an unusually high background of resistants. The cultures were stored in a refrigerator for up to two weeks before use.

Treatment. Cells from the refrigerator were resuspended at about $1-2 \times 10^{-6}$ cells per ml in a synthetic medium with the full adenine supplement (10 mg/ml) and incubated for at least 2 h before the agents were added. Incubation was then continued for 16–17 h. The cultures were then placed into an ice bath and immediately plated without washing.

Detection of genetic effects. After 6 days, colonies were counted on the selective and nonselective plates. White colonies were picked and streaked on a synthetic medium without leucine to detect expression of *leu1*. The incidences of all the resistant colonies was expressed as the number per 10^5 colony-forming units. The much rarer white and leucine-requiring colonies were related to 10^6 colony-forming units. It is important to check for the leucine requirement among the white colonies.

Evaluation of genetic effects. A positive control agent, bavistan, was included to make sure that the system was working properly. An agent was classified as definitely positive when there was a dose-related increase in the frequency of white and leucine-requiring resistants.

Results

Hycanthone was used as a positive control agent with respect to the induction of mitotic segregation and aneuploidy. Even though there was a dose-dependent increase in the frequency of white leucine-requiring resistants, the effect has to be considered as weak (Table 1). Genetic analysis of white and leucine-requiring resistants revealed that most of those colonies had become diploid again. Only few gave the expected lethal segregation. However, out of 48 presumptive monosomics, four had become trisomic for an additional chromosome (Table 2). This suggested that there was indeed an induction of aneuploidy, but that the original monosomics returned to the normal disomic state very frequently.

Acrylonitrile induced a clearcut dose-dependent increase in total cycloheximide-resistant colonies and has to be classified as genetically active in strain *D61.M*. However, there was no indication for the induction of mitotic aneuploidy (Table 3).

Benzene turned out to be difficult to evaluate. There was a very sharp toxicity threshold between no effect and all cells killed. There was a weak increase in total resistants in two experiments. Formally speaking, the data could also be interpreted to show an induction of mitotic aneuploidy. But due to the small numbers of colonies actually counted, this cannot be taken as a clear demonstration of induction of chromosomal malsegregation. The results have to be classified as ambiguous. This highlights, on the other hand, the problems with chemicals that exert this sudden toxicity (Table 4).

Caprolactam did not induce mitotic segregation, but there was an increase in white, leucine-requiring resistant colonies indicative of an induction of mitotic aneuploidy. The experiment could have shown a better dose-related increase, but the numbers scored were very small. It has to be classified as a weak inducer of mitotic aneuploidy and inactive in respect to other types of genetic events (Table 5).

Diethylstilbestrol caused the same difficulties as benzene. It gave a very sharp toxicity above a certain concentration. The data show an erratic response and a weak induction of mitotic segregation and mitotic aneuploidy. It is probably geneti-

TABLE 4

EFFECTS OF BENZENE ON YEAST STRAIN *D61.M*

Concentration	Titer $\times 10^6$	Total resistants		White resistants	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Control	38.3	1271	10.06	3	0.26
Bavistan 20 ppm	1.8	56 *	30.00	13 *	69.64
Benzene					
3.5 µl/ml	39.2	1294	11.00	0	–
4.0 µl/ml	34.3	1380	13.40	12	1.17
4.5 µl/ml	no viable cells				
Control	32.9	1305	13.24	0	–
Bavistan 10 ppm	5.6	448	26.67	76	45.24
Benzene					
4.0 µl/ml	19.9	1608	26.98	2	0.34
4.5 µl/ml	16.9	1381	26.76	0	–
5.0 µl/ml	30.1	2204	24.43	11	1.22
6.0 µl/ml	4.3	598	46.72	3	2.34
Control	31.3	1695	18.07	1	0.11
Bavistan 10 ppm	6.3	387	20.48	43	22.75
Benzene					
4.0 µl/ml	33.8	1887	18.63	5	0.49
4.5 µl/ml	21.2	1890	29.67	3	0.47
5.0 µl/ml	10.9	1467	44.86	4	1.22
6.0 µl/ml	no viable cells.				

Colony counts from 3 plates; * only one plate scored. Treatment in synthetic medium for 17 h at 28 °C.

TABLE 5

EFFECTS OF CAPROLACTAM ON YEAST STRAIN *D61.M*

Concentration	Titer $\times 10^6$	Total resistants		White resistants	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Control	40.9	1419	11.56	6	0.49
Bavistan					
20 ppm	1.3	206	51.50	84	210.00
Caprolactam					
5 mg/ml	27.5	1174	14.25	10	1.21
7.5 mg/ml	19.8	822	13.82	15	2.52
10.0 mg/ml	9.7	436	15.03	4	1.38
15.0 mg/ml	3.8	170	15.04	3	2.65

Caprolactam was weighed directly into the culutre tubes before addition of the cell suspension in synthetic medium.

cally active in strain *D61.M* (Table 6).

Safrole falls also in the category of very sharply toxic agents. It may induce mitotic segregation but induction of mitotic aneuploidy was less likely and the numbers of leucine-requiring, white resistant colonies were very small (Table 7).

o-Toluidine did not induce mitotic segregation and there was no indication of induction of mitotic aneuploidy (Table 8).

Hexamethylphosphoramide is active in yeast after

TABLE 6

THE EFFECTS OF DIETHYLSTILBESTROL ON STRAIN *D61.M*

Concentration	Titer $\times 10^6$	Total resistants		White resistants	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Expt. at 37 °C					
Control	34.5	2157	20.84	5	0.48
Bavistan					
20 ppm	6.3	531	27.95	9	4.73
Diethylstilbestrol					
12.35 ppm	25.3	1884	24.79	3	0.39
13.56 ppm	20.5	1906	31.04	6	0.98
14.78 ppm	5.5	775	46.41	8	4.79
15.99 ppm	6.6	826	41.72	7	3.54
17.20 ppm	0.9	236	84.29	0	–
Expt. at 28 °C					
Control	32.9	1305	13.24	0	–
Bavistan					
10 ppm	5.6	448	26.67	76	45.24
Diethylstilbestrol					
10.88 ppm	34.8	1552	14.85	2	0.19
11.86 ppm	23.1	1393	20.06	0	–
12.83 ppm	39.8	1760	14.75	9	0.75
13.81 ppm	5.1	390	25.49	0	–

Diethylstilbestrol was dissolved in ethanol at 1 mg/ml.

TABLE 7

EXPERIMENT WITH SAFROLE AND STRAIN *D61.M*

COncentration	Titer $\times 10^6$	Total resistants		White resistants	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Control	43.3	1605	12.35	4	0.31
Bavistan					
20 ppm	2.3	236	34.71	76	111.76
Safrole					
0.025 μl/ml	41.8	1528	12.15	1	0.08
0.050 μl/ml	3.7	231	20.81	1	0.90
0.062 μl/ml	1.7	171	33.53	1	1.96
0.074 μl/ml	1.1	78	22.94	0	–

Safrole was dissolved in acetone at a concentration of 10 μl/ml.

metabolic activation, but it was negative in the present investigation. It had a mild growth-retarding effect and did not reveal any genetic activity at all. Also totally negative were *benzoin, di-(2-ethyl hexyl)phthalamide* and *phenobarbital* (Table 9).

It must be emphasized that the strain used did respond to agents known to induce mitotic aneuploidy. Bavistan (Wood, 1982) is a reliable inducer of strong effects and it was used as a positive control agent. The negative responses with other agents cannot be ascribed to a lack of response of the strain itself but to a lack of response of yeast in general to the effects of the genetically inactive agents.

TABLE 8

EXPERIMENT WITH *o*-TOLUIDINE AND STRAIN *D61.M*

Concentration	Titer $\times 10^6$	Total resistants		White resistants	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Control	43.3	1605	12.35	4	0.31
Bavistan					
20 ppm	2.3	236	34.71	76	111.76
o-Toluidine					
0.50 μl/ml	35.2	1087	10.29	2	0.19
0.74 μl/ml	19.5	802	13.71	1	0.17
0.99 μl/ml	15.9	532	11.15	3	0.63
1.23 μl/ml	5.7	250	14.53	0	–
1.48 μl/ml	3.6	130	12.15	1	0.93
1.72 μl/ml	2.2	123	18.36	0	–
1.96 μl/ml	1.1	60 *	26.87	0	–
Control	33.8	1767	17.43	2	0.20
Bavistan					
20 ppm	1.2	219	59.19	33	89.19
1.48 μl/ml	1.3	158	22.90	3	4.26
1.96 μl/ml	1.5	119	27.05	0	–
2.44 μl/ml	1.6	98	20.00	0	–

o-Toluidine was predissolved in DMSO at 10%. * only 2 instead of 4 plates scored.

TABLE 9

CHEMICALS COMPLETELY INACTIVE IN TESTS WITH STRAIN *D61.M*

Concentration	Titer $\times 10^6$	Total resistants		White resistants	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Control	38.1	1610	14.09	0	-
Bavistan 20 ppm	1.2	149	41.39	53	147.22
Di(2-ethyl hexyl)phthalate saturated	30.7	1557	16.88	1	0.11
Hexamethylphosphoramide 2.44%	22.3	1528	17.16 [a]	4 *	0.45
Control	40.9	1419	11.56	6	0.49
Bavistan 20 ppm	1.3	206	51.50	84	210.00
Hexamethylphosphoramide 4.76%	29.3	1057	16.03	15	2.28
Control	38.3	1271	10.06	3	0.26
Bavistan 20 ppm	1.8	56 [b]	30.00	13	69.64
Benzoin 9.95 mg/ml	38.7	870	11.25 [c]	0	-.
Phenobarbital 9.95 mg/ml	36.3	1012	9.29	1	0.09

[a] 4 instead of 3 plates scored; [b] only 1 plate scored; [c] only 2 plates scored. Hexamethyl phosphoramide and di(2-ethyl hexyl)phthalate were pipetted into the cell cultures. Phenobarbital and benzoin were weighed directly into the culture tubes and 0.1 ml DMSO added before the cell cultures were added.

Discussion

The strain used monitors induction of mitotic aneuploidy induced by bavistan (Tables 3–9) and hycanthone (Table 1). A genetic analysis of the presumptive aneuploids (Table 2) revealed that there is indeed induction of mitotic aneuploidy as the cause for the occurrence of the white, leucine-requiring resistant colonies. Moreover, the coincident occurrence of trisomics for chromosome III, four of 48 (Table 2), not only showed that there was induction of aneuploidy but also aneuploidy as a result from nondisjunction. The observation of a normal segregation among the clones selected for monosomy suggests furthermore that a monosomic condition can readily revert — at least in yeast — to the normal diploid state. This has also been reported by Wood (1982) for a number of yeast chromosomes.

The conclusion to be drawn is that a mutagen like acrylonitrile may well induce mitotic recombination and mutation but the induction of mitotic chromosomal malsegregation does not have to be associated with this property. This is not especially surprising, since the molecular targets can be quite different. On the other hand, agents like bavistan, which do not induce "classical" types of genetic effects, may be very strong inducers of mitotic chromosomal malsegregation. We know only few such agents. In addition to bavistan there are the solvents acetone (Zimmermann, 1983), ethylacetate, 2-methoxyethylacetate and methylethyl ketone (unpublished), which are very active in the strain used in the present investigation.

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Tests for genetic activity in the yeast *Saccharomyces cerevisiae*: study of forward and reverse mutation, mitotic recombination and illegitimate mating induction

S.G. Inge-Vechtomov, Y.I. Pavlov, V.N. Noskov, M.V. Repnevskaya, T.S. Karpova, N.N. Khromov-Borisov, J. Chekuolene and D. Chitavichus

Department of Genetics and Breeding, Leningrad State University, Leningrad (U.S.S.R.) and Institute of Botany, Academy of Sciences Lit.S.S.R., Vilnius (U.S.S.R.)

Summary

The genetic activity of each of the 10 test chemicals was evaluated on the yeast *Saccharomyces cerevisiae* with assays for forward mutations with strain PV-1, for reverse mutation and for mitotic recombination with strains PV-2 and PV-3, and illegitimate mating induction with strains PV-4a and PV-4b. These tests have shown that ACN and DES were genetically active and that this activity required metabolic activation. Negative tests were obtained for HMPA, CAP, PB, ZOIN, SAF, TOL, BEN and DEHP.

Genetic endpoints traditionally assayed in genetic toxicology tests with yeasts are reverse and forward mutations, mitotic inter- and intra-genic recombination, chromosome loss, and mitochondrial mutations (Zimmermann, 1975; de Serres and Ashby, 1981). It has been established that for some chemicals yeasts may provide better resolution of the genotoxic potential of carcinogens than prokaryotic test systems (de Serres and Ashby, 1981).

The development of new types of genetic systems for genotoxicity studies with yeasts are in progress. Among these are tests based on the observation of low frequency mating of yeast cells of the same mating type (Hawthorne, 1963). Sofar it has been demonstrated that such mating between "a"-type cells may be induced by X-rays (Schiestl and Wintersberger, 1982, and unpublished). It was argued that such treatments induce "true switching events" i.e. intrachromosomal unidirectional conversion leading to a change of the cassette information at the MAT locus. One can predict that a test for "α" with "α" mating would register a wider spectrum of genetic events than the former one. It is known that "a"-specific genes are expressed constitutively and that the MATα locus negatively controls their expression, that is why physical absence of the MATα locus leads to an "a"-mating behaviour (Herskowitz, 1983). Thus, we expect to score among "α" with "α" mating products not only "true switchers" but also the consequences of chromosome breaks near the MAT locus: deletion of MAT or the right arm or entire third chromosome (McCusker and Haber, 1981).

In the present investigation we have studied genetic activity of two groups of chemicals: (a) 7 well-known mutagens, (b) 8 carcinogens and 2 noncarcinogens poorly mutagenic in a prokaryotic test battery. All chemicals were tested with strains capable of detecting reverse mutations, mitotic inter- and intra-genic recombination — both a commonly used genetic endpoints. It has been established that the test for forward mutation induction in the yeast *Schizosaccharomyces pombe* have the highest accuracy in predicting carcinogens among other yeast assays (de Serres and Ashby, 1981). Therefore, we also investigated the utility of the forward Lys$^-$ mutation assay devel-

oped by Chatoo et al. (1979). Finally, we determined the ability of the test for illegitimate "α" with "α" cells mating (α-test) to detect genetically active chemicals.

Materials and methods

Yeast strains. We used 5 strains, which enabled us to detect 6 different genetic events:

Strain PV-1 (*MATα leu2-2 pho1-100*) was used in forward mutation induction experiments. The selective procedure of Chatoo et al. (1979) with aminoadipate as a sole nitrogen source have been used for Lys$^-$ mutants selection. Among Lys$^-$ clones mutants at *LYS2* gene predominated though few *lys5* have been detected after propiolactone mutagenesis.

Strain PV-2$\left(\frac{MAT\alpha}{MATa}\ \frac{his7\text{-}1}{his7\text{-}1}\ \frac{ade2\text{-}475}{ade2\text{-}475}\ \frac{lys2\text{-}67}{lys\text{-}2\text{-}68}\ \frac{can1}{CAN1}\ \frac{rad1\text{-}5}{RAD1}\right)$

was used to study the reversion of the ochre mutation *his7-1*, reversion of the presumed frameshift mutation *ade2-475* (classification based on diagnostic mutagen revertability, i.e. its high response to ICR-170, but lack of response to base-pair substitution mutagens), mitotic gene conversion at *LYS2* gene, mitotic homozygosity of the *can1* recessive-resistance marker. Strain PV-3 is the ultraviolet-light-sensitive derivative of PV-2, homozygous for the *rad1-5* marker.

Strains PV-4a and PV-4b were used for the α-test. Their genotypes are:

*metA*1 + + *his*7-1 *his*4-*B*26 *leu*2-1 *MATα* + *lys*2-*x* and

+ *ade*1-6 *ade*2-163 + + *leu*2-2 *MATα thr*4-*B*15 *lys*2-*A*12

When a mixture of these strains was plated onto selective medium lacking adenine and methionine only rare illegitimate mating products are able to grow. Further genetic characterization of illegitimate hybrids is possible due to the third chromosome markers present as nonselective ones in this strains. Tests for heteroallelic recombination at *LYS2* were utilized to prove the hybrid nature of the selected diploids. Phenotypic expression of the recessive markers: *his4, leu2-1*, or *leu2-2* and *thr4* permitted us to characterize the consequence of $\alpha \times \alpha$ illegitimate mating by the state of the left and right arms of the third chromosome in such hybrids. Heteroallelic recombination or lack of it at *LEU2* was studied in order to demonstrate hemi- (or homo-)zygosity for this marker. If there was no heteroallelic recombination we could identify the allele *leu2-2* as being suppressible by a dominant ochre suppressor and *leu2-1* as a non-suppressible allele: we crossed the hybrids obtained with a *MATa* tester strain bearing the non-suppressible allele *leu2-1* in combination with an ochre suppressor; on leucineless medium we were able to demonstrate these characteristics. It was possible to do this for the great majority of illegitimate hybrids provided they possessed the α mating type (see Results and Discussion).

Strains PV-1, PV-4a, and PV-4b belong to Peterhoff breeding stocks of *Saccharomyces cerevisiae* (Inge-Vechtomov, 1971) while PV-2 and PV-3 were derived from the hybrids between Peterhoff strains and the strain 197/2d *uvs12* obtained from B.S. Cox.

Media. A yeast nitrogen base without amino acids was used for selective medium preparation. In the case of medium selective for Lys$^-$ mutants, 1,5 g/l of α-aminoadipic acid (Sigma, U.S.A.) instead of ammonium sulphate was used, and 30 mg/l of L-lysine was added. When necessary, adenine (20 mg/l), L-histidine (20 mg/l), L-canavanine (40 mg/l), L-leucine (60 mg/l) and D,L-threonine (150 mg/l) were added. Revertants of the *his7-1* mutation were scored on semi-enriched medium containing 1.5 mg of L-histidine per liter. YAPD was a rich medium for scoring survivors and yeast growing cultures. It differs from standard YEPD medium in that the yeast extract was substituted by yeast autolysate (Inge-Vechtomov, 1971).

Experimental procedures. Yeast strains were grown in liquid YAPD medium and stored one month at 0–4 °C. Before an experiment a 1 : 1000 dilution was made with fresh medium and the culture was grown with shaking (150 rev/min) until the logarithmic phase (approximately 18 h at 30 °C). Cells were harvested and resuspended in 0.1 M phosphate buffer pH 7.4. Two different treatment protocols were then employed.

Protocol 1. Treatment in buffer (no growth). To 0.8 ml of cell suspension ($5 \cdot 10^7$–10^8 cells per ml) 0.2 ml of S9 mix or 0.2 ml of buffer were added

and then 10–50 μl of mutagen soltuion was used to initiate the reaction. The samples were incubated with shaking (200 rev/min). Incubation with S9 mix was carried out at 36 °C for 3 h (Pavlov et al., 1981; Pavlov and Khromov-Borisov, 1983). Incubation for direct-acting mutagens was carried at 30 °C for 2 h in buffer alone. Such treatment protocols have been used for direct-acting positive controls and for all chemicals when utilizing strains PV-1 and PV-4a, b. Some experiments with PV-2 and PV-3 were also conducted under such conditions.

Protocol 2. Treatment of growing cells. To 0.5 ml of cell suspension (cell density, $5 \cdot 10^6$ cells per ml) 0.3 ml of liquid complete synthetic medium and 0.2 ml of S9 mix or buffer were added. Then the reaction was initiated with 10–50 μl of mutagen solution, incubation was carried out with shaking at 30 °C for 20 h. Such treatment conditions were employed with PV-2 and PV3 when the 10 chemicals of the Colloborative Study were investigated and with the control promutagen 2-AF. When we studied the genetic activity of the base analogue mutagen HAP we simply grew the cells in liquid YAPD medium containing HAP (Sorenson et al., 1981).

Reactions were terminated by the addition of cold buffer and then standard dilutions and plating were performed (Pavlov and Kromov-Borisov, 1981) 0.1 ml of the treated PV-4a,b mixture ($5 \cdot 10^7$–10^8 cells per ml) was plated onto YAPD medium and was then replica-plated onto selective medium after 24–48 h. This prolonged growth period was necessary for the detection of illegitimate hybrids.

Spot tests were performed as described previously (Pavlov and Khromov-Borisov, 1981).

S9 Preparations. Liver preparations from Aroclor 1254-induced female Wistar rats were produced as described by Ames et al. (1975). It is general practice to prepare S9 from male rats, so our preparations could not be regarded as standard in view of the finding that males and females posess different cytochrome P-450 forms (Kato and Kamataki, 1982). S9 mix was prepared according to Ames et al. (1975).

Chemicals. L-Canavanine was from Sigma. NADP, glucose 6-phosphate, amino acids were from Reanal, Hungary; adenine from Chemapol (Czechoslovakia). All other media components were from Reachim (U.S.S.R.).

Information concerning positive control mutagens is presented in Table 1.

10 chemicals that were the subject of C.S.S.T.T.

TABLE 1

POSITIVE CONTROL MUTAGENS

Mutagen	Abbreviation	Source	Solvent	Treatment protocol
β-Propiolactone	PRO	Sigma (U.S.A.)	water	1
1,2,7,8-Diepoxyoctane	DEO	gift of F.J. de Serres	none [a]	1
Ethylmethanesulfonate	EMS	Sigma (U.S.A.)	none [a]	1
6-*N*-Hydroxylaminopurine	HAP	ICN-Pharmaceuticals, (U.S.A.)	DMSO	2
Acridine mustard ICR-170	ICR	gift of I.A. Zakharov	DMSO-water	1
2-Aminofluorene	2-AF	Fluka (Switzerland)	DMSO	1, 2
Cyclophosphamide	CPA	Saransk Drug factory (U.S.S.R.)	0.1 M phoshate buffer pH 7.0	1

[a] Employed undiluted, in situ.

TABLE 2

SUMMARY OF RESULTS WITH POSITIVE CONTROL MUTAGENS

Mutagen and concentration (μg/ml)	Treatment conditions	Metabolic activation	Strains and genetic endpoints studied									PV-4a and PV-4b illegitimate mating	Total positives	Percent positives
			PV-1 forward mutation	PV-2				PV-3						
				his7-1 reversion	ade2-475 reversion	Gene conversion	Mitotic recombination	his7-1 reversion	ade2-475 reversion	Gene conversion	Mitotic recombination			
PRO 50	2 h 30 °C	no	4/4	15/15	0/3	19/19	15/15	15/15	0/2	16/16	13/13	3/3	100/105	95
DEO 1000	2 h 30 °C	no	+	+	−	+	NT	+	−	+	+	2/2	2/2	100
ICR 1	2 h 30 °C	no	+	12/14	2/2	16/17	12/14	14/15	2/2	16/16	11/13	2/2	87/95	91
EMS 2500	2 h 30 °C	no	2/2	+	−	+	+	+	−	+	+	+	2/2	100
HAP 100	20 h 30 °C	no	5/5	3/3	−	3/3	NT	3/3	−	3/3	NT	−	17/17	100

2-AF 40	3 h 36 °C	yes	3/4	3/3	0/3	3/3	NT	3/3	0/3	3/3	NT	3/3	18/25	72
	3 h 36 °C	no	0/2	0/3	0/3	0/3	NT	0/3	0/3	0/3	NT	NT	0/20	0
	3 h 30 °C	yes	NT	8/14	NT	14/15	6/14	10/10	NT	11/12	6/13	NT	55/78	71
	20 h 30 °C	no	NT	1/14	NT	0/15	1/11	1/10	NT	0/12	1/13	NT	4/75	5
CPA 1000	3 h 36 °C	yes	2/3	NT	NT	NT	NT	NT	NT	NT	NT	NT	2/3	66
	3 h 36 °C	no	0/3	NT	NT	NT	NT	NT	NT	NT	NT	NT	0/3	0
Total			17/24	42/66	2/11	55/75	34/54	46/59	2/10	49/65	31/52	10/10	288/426	
Percent positives			71	79	18	84	63	78	20	75	59	100		67 ± 6.8
Total corrected [a]			17/19	41/49	2/8	55/57	33/43	45/46	2/7	49/50	30/39	10/10	284/328	
Percent positives corrected [a]			89	84	25	96	77	98	28	98	77	100		86 ± 6.2

The numerator is the number of positive experiments, the denominator is the total number of experiments.
(+) means that the chemical was positive in the spot-test, (−) means that the chemical was negative in the spot-test.
[a] Correction in that experiments with promutagens without S9 was not included.

study were routinely dissolved in DMSO; its final concentrations in the incubation mixture was kept lower than 2%. In some cases liquid carcinogens were added undiluted, in situ (HMPA, for example).

Criteria for discrimination between mutagens and nonmutagens

In the present work we used selective methods for monitoring induced genetic events. The mutant yield (the absolute number of colonies on selective medium) is regarded as decisive for the estimation of the mutagenicity of a chemical rather than mutant frequency which may lead to false positives (Pavlov and Khromov-Borisov, 1981). Thus only when the mutant yield after treatment was higher than in the controls was a mutagenic effect suspected and calculations of statistical significance of visually observed differences performed.

The first step in the statistical procedures was to test whether the distribution of colonies on the plates at one data point was uniform (i.e. Poisson) using the known test of homogeneity:

$$\chi^2 = \sum_{i=1}^{k} \frac{(x_i - \bar{x})^2}{\bar{x}} \qquad \nu = k - 1$$

where x_i may be the number of mutant colonies (m_i) or the number of survivor colonies (n_i) on i-th plate,

$$\bar{x} = \frac{1}{k} \sum_{i=1}^{k} x_i$$

k is the number of plates and ν the degrees of freedom.

The second step was calculation of the total numbers $X = \sum_{i=1}^{k} x_1$ of mutants (M_c or M_t) and survivors (N_c or N_t) in control after treatment.

Then the statistical significance of the difference between mutant frequency was tested by the method of Birnbaum (1954) taking survival values into account (when N's and M's were large enough):

$$\chi^2 = \frac{(M_t \cdot N_c - M_c \cdot N_t)^2}{N_c \cdot N_t (M_t + M_c)} \qquad \nu = 1$$

The published tables of the corresponding exact test are useful when $M_c + M_t \leqslant 100$ (Kastenbaum and Bowman, 1970) and $M_c + M_t \leqslant 200$ (Kastenbaum and Bowman, 1966).

When N_c and N_t were small, a different formula has been used:

$$\chi^2 = \frac{(F_c - F_t)^2}{e_c^2 + e_t^2} \qquad \text{where} \qquad F_c = \frac{M_c}{N_c}, \qquad F_t = \frac{M_t}{N_t}$$

$$\text{and} \qquad e_c = F_c \sqrt{\frac{1}{M_c} + \frac{1}{N_c}} \qquad \text{and} \qquad e_t = F_t \sqrt{\frac{1}{M_t} + \frac{1}{N_t}}$$

are standard errors for F_c and F_t calculated under the assumption that M_c, N_c and M_t, N_t are the observed values of pairs of independent Poisson random variables.

In this way we got information as to whether a chemical in an experiment was genetically active. The substance was considered mutagenic if it was positive in more than half of the separate experiments.

Results and Discussion

The results with positive control mutagens are summarized in Table 2 and the results of typical experiments are given in Tables 3–6. From this data the conclusions can be made regarding the positive control mutagens used and the test strains. Comparing columns in Table 2 we can compare mutagens and test strains.

PRO was a good positive control for our strains giving a clear response in 95% of our experiments. It acts with 100% reproducibility because 5% of the tests were performed with the *ade2-475* frameshift allele reversion, the genetic event that PRO failed to induce in all experiments. Lack of *ade2-475* reversion and good reversion of the *his7-1* ochre allele is an indication of base-pair-substituting activity of this chemical. Generally the dose of PRO used in our experiments enhanced the yield of different genetic events studied by about an order of magnitude and caused no more than 50% cell death (Tables 3–6).

Similar analysis of results obtained with DEO and EMS places them in a group with PRO. The striking difference of DEO from EMS and PRO in the types of illegitimate hybrids which it induced

TABLE 3

STRAIN PV-1 TYPICAL POSITIVE CONTROL DATA

Variant [a]	S9	M_t	N_t	Survival (%)	χ^2	P_{obs}
Control	–	99	291	100		
PRO, 50	–	578	117	40	1064.1	< 0.001
Control	–	65	111	100		
EMS, 2500	–	827	48	43	1654.6	< 0.001
Control	–	407	350	100		
HAP, 50	–	851	287	82	259.4	< 0.001
HAP, 100	–	1970	161	46	2906.7	< 0.001
Control	+	120	225	100		
2-AF, 40	+	179	220	98	13.0	< 0.001
CPA, 1000	+	319	213	95	101.5	< 0.001
Control	–	176	162	100		
2-AF, 40	–	162	179	110	NST [b]	
Control	–	99	291	100		
CPA, 1000	–	89	220	75	NST	

[a] Concentration of mutagen in μg/ml.

[b] No statistical treatment because $M_t < M_c$ (see Materials and Methods).

will be described later. The base-pair substituting base analogue mutagen HAP was the most powerful mutagen in comparison with the former three, but induced intragenic recombination less effectively than PRO (Tables 3–5). This observation is in accordance with published data (Pavlov et al., 1979; Sorenson et al., 1981). HAP was unique among all positive controls due to its complete inability to induce illegitimate mating (Table 2, spot test). The reason for this is unclear, but one may speculate that it may be related in some way to its poor recombinogenic activity.

The reproducibility of tests with the frameshifting ICR was high enough but lower than for the base-pair-substituting mutagens. The reasons for this observation are the only moderate induction of ochre mutation reversion (Tables 2, 4, 5) and the variable response of yeast logarithmic cultures to the lethal action of this chemical. ICR was the only chemical capable of inducing reversions of the *ade2-475* marker. ICR's activity was more pronounced in the excision-defective yeast strain PV-3.

The promutagens 2-AF and CPA, when activated by S9 fraction from rats, produced positive results in approximately 75% of the experiments. This relatively low value was rather due to inability of 2-AF to induce reversions of the frameshift marker and the low sensitivity of the *can1* marker homozygosity test with 2-AF (Tables 4, 5). CPA was more suitable as an indirect positive control for the forward mutagenesis test than 2-AF (Table 3). The responses of all test strains to promutagens were never as high as to directly-acting mutagens (Tables 3–6). Thus we may conclude that the efficiency of metabolic activation in our experiments was substantial but not as high as in our experiments with mouse and chicken S9 liver preparations (Pavlov and Khromov-Borisov, 1983).

From the results in Table 2 it is evident that all our test strains respond to positive control mutagens with high efficiency (80–90% positives). Strain PV-1 detected both base-pair substituting and frameshift mutagens and the number of test plates needed is low compared to other tests.

The high cost of aminoadipate is in our opinion the only limitation of this test. The sensitivity of PV-2 and PV-3 is different when different endpoints are assayed: gene conversion (most sensitive — 98–96% positives), *his7-1* reversion (98–84% positives), mitotic recombination (77%

TABLE 4

PV-2 TYPICAL POSITIVE CONTROL DATA

Variant [a]	Incubation time (h)	S9	Genetic endpoint studied	M_i	N_i	S(%)	χ^2	P_{obs}
Control	2	–	conversion	88	429	100		
			his7-1 reversion	23				
			can1 homozygot.	61				
ICR, 1	2	–	conversion	226	422	103	56.6	< 0.001
			his7-1 reversion	36			NC [b]	> 0.05
			can1 homozygot.	187			60.3	< 0.001
PRO, 50	2	–	conversion	712	411	96	514.1	< 0.001
			his7-1 reversion	490			445.6	< 0.001
			can1 homozygot.	372			237.0	< 0.001
Control	20	–	conversion	60	40	100		
			his7-1 reversion	7				
HAP, 200	20	–	conversion	347		90	37.1	< 0.001
			his7-1 reversion	1441			234.9	< 0.001
Control	20	+	conversion	100	488	100		
			his7-1 reversion	30				
			can1 homozygot.	104				
2-AF, 40	20	+	conversion	165	372	76	39.0	< 0.001
			his7-1 reversion	28			NST [c]	
			can1 homozygot.	60			NST	
Control	20	–	conversion	88	475	100		
			his7-1 reversion	9				
			can1 homozygot.	91				
2-AF, 40	20	–	conversion	74	258	54	NST	
			his7-1 reversion	16			NC	< 0.01
			can1 homozygot.	73			NST	

[a] Concentration of mutagen in μg/ml.
[b] Not calculated, $P_{observed}$ estimated from Kastenbaum and Bowman Tables.
[c] No statistical treatment because $M_t < M_c$.

positives) and *ade2-475* reversion (25–28% positives, lowest sensitivity). It is evident that the latter marker is not suitable for mass screening of mutagens, but it may be used when the specificity of a known mutagen is under investigation. The homozygous *rad1-5* mutation in the strain PV-3 somewhat enhanced its overall sensitivity to the mutagens tested (Table 2) due to its better response to ICR and 2-AF (Tables 4, 5). The disadvantage of these two strains is the large number of plates needed to detect the various genetic events. One can overcome this difficulty for mass screening by using only the two most sensitive and relatively nonspecific tests, mitotic gene conversion and ochre reversion.

The α-test was sensitive in the detection of the genetic activity of various chemical mutagens (100% positives in Table 2). However, one should keep in mind, that the powerful mutagenic base analogue HAP failed to induce illegitimate mating. Thus it is evident that only specific DNA alterations can lead to illegitimate mating. Representative samples of illegitimate hybrids were analyzed for the different variants of the experiments (Table 7). The majority of illegitimate mating products were "α" in respect to mating ability and no chromosome III rearrangements were detected. Thus, the precise mechanisms leading to illegitimate mating remain obscure. DEO was strikingly different from the other mutagens in that it was

TABLE 5

STRAIN PV-3 TYPICAL POSITIVE CONTROL DATA

Variant [a]	Incubation time (h)	S9	Genetic endpoint studied	M_i	N_i	S(%)	χ^2	P_{obs}
Control	2	–	conversion	100	470	100		
			his7-1 reversion	19				
			can1 homozygot.	103				
ICR, 1	2	–	conversion	360	284	60	322.0	< 0.001
			his7-1 reversion	187			247.5	< 0.001
			can1 homozygot.	422			407.1	< 0.001
PRO, 50	2	–	conversion	641	318	67	655.1	< 0.001
			his7-1 reversion	437			583.4	< 0.001
			can1 homozygot.	374			286.9	< 0.001
Control	20	–	conversion	43	36	100		
			his7-1 reversion	13				
HAP, 200	200	–	conversion	139	33	91	13.3	< 0.001
			his7-1 reversion	525			29.7	< 0.001
Control	20	+	conversion	95	453	100		
			his7-1 reversion	26				
			can1 homozygot.	93				
2-AF, 40	20	+	conversion	209	449	99	43.7	< 0.001
			his7-1 reversion	176			112.7	< 0.001
			can1 homozygot.	74			NST [b]	< 0.001
Control	20	–	conversion	93	462	100		
			his7-1 reversion	18				
			can1 homozygot.	77				
2-AF, 40	20	–	conversion	68	300	65	NST	
			his7-1 reversion	15			NST	
			can1 homozygot.	82			NC [c]	< 0.01

[a] Concentration of mutagen in μg/ml.

[b] No statistical treatment because $M_i < M_c$.

[c] Not calculated, $P_{observed}$ estimated from Kastenbaum and Bowman Tables.

TABLE 6

STRAINS PV-4a, PV-4b TYPICAL POSITIVE CONTROL DATA

Variant [a]	S9	M_i	N_i	S(%)	χ^2	P_{obs}
Control	–	57	516	100		
PRO, 50	–	481	344	66	547	< 0.001
DEO, 1000	–	457	290	56	625	< 0.001
ICR, 1	–	157	446	86	62	< 0.001
Control	+	76	496	100		
2-AF, 40	+	131	343	73	43	< 0.001

[a] Concentration of mutagens in μg/ml.

TABLE 7

CLASSIFICATION OF ILLEGITIMATE HYBRIDS ARISING SPONTANEOUSLY AND INDUCED BY DIFFERENT MUTAGENS

Variant	Genetic events occurring in one of the parent strains [a]					No changes [b] (α)	Total hybrids analyzed
	Matα loss (hybrids of α-mating type)			Activation or transposition of silent HMR_a in MATα (nonmating hybrids)			
	Chromosome III loss	Chromosome III right arm deletion	Homozygotization of leu2 or his4	Chromosome III right arm deletion	No changes in chromosome III markers		
Control	27	4	3	0	5	139	178
DEO	114	61	13	6	5	59	258
PRO	9	4	5	4	3	46	71
EMS	3	2	2	0	4	63	74

[a] Criteria for chromosome III aberrations were:
thr4 and leu2 expression: PV-4a chromosome III loss,
thr4 expression: PV-4a chromosome III right arm deletion,
his4 and leu2 expression: PV-4b chromosome loss.

[b] α-Mating type hybrids, no changes of chromosome III markers.

able to induce chromosome III loss and deletions at high frequency (Table 7). It is attractive to speculate that DEO induces deletions in yeast with high frequency as in Neurospora (Ong and de Serres, 1975). Surprisingly, such deletions occurred only in one of the test strains, PV-4a (data not shown). Thus the situation is not trivial and further experiments are necessary.

The main conclusions from this part of our work are as follows:

The test battery PV-1, 2, 3, 4a, 4b detects mutagenic substances with high efficiency (including alkylating agents, base analogue, ICR-170 frameshift mutagen and promutagens). They do not detect nonmutagenic substances, as can be judged from the fact that genetic activity of promutagens were detected only rarely in the absence of exogeneous activation (4 four positives in 75 experiments, Table 2). Thus 5% positives should be regarded as "noise" in our experiments. Mutagenic effects should be suspected only when this "noise" level is exceeded at least 4 times.

The summary of our results obtained with the 10 chemicals is presented in Table 8. The picture is quite different from that reported in Table 2. In general only 14% of the experiments proved to be positive, i.e. reproducibility was low. In our opinion this reflects the low genetic activity of chemicals under our test conditions and with our test strains. We classified the 10 chemicals into 3 groups:

(1) Genetically active (ACN and DES, underlined by a solid line in Table 8). The conclusion is based on the observation that these two chemicals yield approximately 20% positives in general and because ACN was twice positive in the α-test in the two experiments, the same being true for DES tested with strain PV-3 upon mitotic gene conversion induction. Both ACN and DES were active mainly with metabolic activation.

(2) Suspected genetically active chemicals (HMPA, CAP, PB and ZOIN, underlined with broken line in Table 8). This conclusion is based on the observation that HMPA, CAP and PB generate more than 20% positives in general in one of the variants (S9 mix absent or present) and that ZOIN was positive in two of the three experiments with PV-1 without activation.

(3) Inactive chemicals (SAF, TOL, BENZ and DEHP), because of their inability to yield positives more frequently than 20% and their inability to yield more than 50% positives in any particular test.

The results of the experiments with the chemi-

TABLE 8

SUMMARY OF RESULTS WITH 10 CHEMICALS

Chemical	Dose range (μg/ml)	Metabolic activation	Strains and genetic endpoint studied								Total positive	Percent positive
			PV-1	PV-2			PV-3			PV-4a, b		
			Forward mutation	his7-1 reversion	Gene conversion	Mitotic recombination	his7-1 reversion	Gene conversion	Mitotic recombination	Illegitimate mating		
HMPA	2 -2054	+	0/4	0/3	0/4	0/2	0/2	1/2	1/2	0/2	1/21	5
		−	1/3	1/3	1/4	1/2	0/2	1/2	0/2	0/2	5/21	23
SAF	0.1- 110	+	1/3	0/3	1/4	0/2	0/2	0/3	0/2	0/2	1/21	5
		−	0/3	0/3	0/4	1/2	0/2	0/3	0/2	1/2	2/21	10
TOL	1 -1000	+	1/3	0/2	0/3	0/2	0/2	0/3	0/2	1/2	2/19	10
		−	1/3	0/2	0/3	0/2	0/2	0/3	0/2	1/2	2/19	10
CAP	1 -1000	+	1/3	0/2	0/3	0/2	0/2	0/3	0/2	1/2	2/19	10
		−	0/3	0/2	1/3	1/2	0/2	0/3	1/2	1/2	4/19	21
ZOIN	1 -1000	+	0/3	1/4	2/4	0/4	0/4	1/4	0/4	1/2	5/28	18
		−	2/3	0/4	0/4	0/4	0/4	1/4	0/4	0/2	3/29	10
BEN	1 - 880	+	0/3	0/3	0/3	1/3	0/4	1/3	1/3	1/2	4/24	17
		−	0/2	0/3	1/3	0/3	0/4	0/3	0/3	0/2	1/23	4
DEHP	1 -1000	+	0/3	0/3	1/3	0/3	0/3	0/2	0/2	1/2	2/21	9
		−	1/3	0/3	0/3	0/3	0/3	0/3	1/3	0/2	2/23	9
ACN	1 - 800	+	0/2	0/2	0/2	1/2	0/2	0/2	0/2	2/2	3/16	19
		−	0/2	0/2	0/2	0/2	0/2	0/2	0/2	1/2	1/16	6
PB	1 -1000	+	0/2	0/2	0/2	1/2	0/2	1/2	0/2	1/2	3/16	19
		−	1/2	1/2	0/2	1/2	0/2	0/2	1/2	0/2	4/16	25
DES	1 - 100	+	1/3	0/2	0/2	1/2	0/2	2/2	0/2	1/2	5/17	29
		−	1/3	0/2	1/2	0/2	0/2	0/2	1/2	0/2	3/17	18
Total positives			11/56	3/52	8/60	8/48	0/50	8/54	6/47	13/40	56/406	
Percent			20	4	13	17	0	15	11	36		14±6.9

Numerator is the number of experiments being positive, denominator is the total number of experiments.

TABLE 9

STUDY OF GENE CONVERSION INDUCTION BY 2-AF AND DEO WITH YEAST STRAIN PV-3

Expt. [a]	Variant	Concentration (μg/ml)	M_t	N_t	S(%)	χ^2	P_{obs}
1	Control	–	426	298	100		
	2-AF	40	860	396	132	50.5	< 0.001
	DES	1	475	342	114	0.2	> 0.05
		10	484	285	96	6.7	< 0.05
		100	64	211	71	NST [b]	
2	Control	–	84	444	100		
	2-AF	40	238	296	66	153.3	< 0.001
	DES	1	131	517	116	4.3	< 0.05
		10	123	530	119	2.1	> 0.05
		100	95	488	109	NC [c]	> 0.05

[a] Incubation for 20 h with S9 at 30 °C.

[b] No statistical treatment because $M_t < M_c$.

[c] Not calculated, $P_{observed}$ estimated from Kastenbaum and Bowman Tables.

cals regarded as genetically active are presented in Tables 9 and 10. It is evident from these data that the genetic activity of ACN and DES was considerably lower than that of the positive controls. This supports our judgment that was drawn when the whole set of data was considered (Table 8). When comparing the ability of our tests to detect the genetic activity of 10 chemicals we may classify the α-test as the most sensitive (36% positives, Table 8), compared to the classical tests on conversion, reversion and mitotic recombination, which were the least sensitive (17–0% positives) and to the forward mutagenesis test as showing median sensitivity (20% of positives). The test for mitotic homozygosity of the recessive *can1* marker was as sensitive as the test for conversion induction, but one may not rely on the former as much as on the latter test because of its inability to detect positive

TABLE 10

ILLEGITIMATE MATING INDUCTION BY 2-AF AND ACN WITH STRAINS PV-4a AND PV-4b

Expt. [a]	Variant	Concentration (μg/ml)	M_t	N_t	S(%)	χ^2	P_{obs}
1	Control	–	449	468	100		
	2-AF	40	528	398	85	25.7	< 0.001
	ACN	0.8	346	425	91	NST [b]	
		8	337	458	98	NST	
		80	317	443	94	NST	
		800	464	360	76	20.0	< 0.001
2	Control	–	144	1556	100		
	2-AF	40	258	1176	75	73.2	< 0.001
	ACN	8	210	1069	68	50.7	< 0.001
		80	183	817	52	67.0	< 0.001
		800	204	1029	66	51.4	< 0.001

[a] Incubation for 3 h with S9 at 36 °C.

[b] No statistical treatment, because $M_t < M_c$.

control mutagens with high efficiency (Table 2).

Our conclusion in conclusion in general is that introduction of strains PV-1 PV-4a and PV-4b may increase the overall sensitivity of yeast as a detector of the genetic activity of environmental mutagens. From the results we may conclude that at present the usefulness of the tests employed for the detection of weekly active in classical microbial assays chemical carcinogens is doubtful.

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Tests for mutagenic activity in growing cells of the yeast *Saccharomyces cerevisiae* strain XV185-14C

Tina R. Harrington and Earle R. Nestmann

Mutagenesis Section, Environmental and Occupational Toxicology Division, Department of National Health and Welfare, Ottawa, Ont. (Canada)

Summary

HMPA, SAF, CAP and ZOIN showed dose-dependent mutagenicity in *Saccharomyces cerevisiae* strain XV185-14C. The experimental protocol involved treatment of growing cells in the absence and presence of S9 for metabolic activation.

In a previous collaborative study (de Serres and Ashby 1981), yeast assays were shown to be promising predictors of carcinogenic activity associated with selected compounds (de Serres and Hoffmann, 1981). In comparison with the popular Salmonella reversion assay (Maron and Ames, 1983), yeast has the potential to be a much more useful test organism since it is eukaryotic and able to detect a wide variety of genetic endpoints (von Borstel, 1981). We used *S. cerevisiae* strain XV185-14C in this study because it has been shown previously to be a sensitive strain for use in a reversion test for the detection of mutagens and carcinogens (Mehta and von Borstel, 1981; Nestmann and Lee, 1983).

Materials and methods

Saccharomyces cerevisiae strain XV185-14C

Haploid strain XV185-14C has the genotype:

a *ade2-1 arg4-17 lys1-1 trp5-48 his1-7 hom3-10*

This strain has been described previously in detail (Mehta and von Borstel, 1981; von Borstel, 1981). We have used the *trp, his* and *hom* markers for this study. The *trp5-48* mutation is ochre suppressible; the *his1-7* marker is a missense mutation; and *hom3-10* appears to be a frameshift mutation (Mehta and von Borstel, 1981).

Chemicals

The test chemicals provided were dissolved in dimethylsulphoxide (DMSO), spectrophotometric grade (Baker). Aroclor 1254-induced rat-liver S9 was prepared according to the protocol of Ames et al. (1975). Positive control compounds were ethyl methanesulfonate (EMS; Eastman) and dimethylnitrosamine (DMN; Aldrich).

Testing procedure

Cultures of XV185-14C in YEPD were inoculated from frozen stocks (−80°) and grown overnight. Subcultures (1 : 10 in YEPD) were prepared and allowed to grow to exponential phase (4 h). Aliquots of the subcultures were combined with either a control or test chemical and with either S9 or buffer in a total volume of 3 ml in loosely-capped glass tubes and were grown for 22 h. All the culturing and treatment steps involved incubation at 30 °C in a gyratory water bath. The contents of the tubes were washed twice with 0.1 M phosphate buffer and pelleted by centrifugation (3000 rpm for 10 min). The cells were resuspended in 5 ml of phosphate buffer and were plated without dilution on both selection plates (lacking tryptophan, histidine or methionine) for mutant determination and on fully-supplemented plates for survivors. The plates were incubated for 4 days at 30 °C before counting.

TABLE 1

EXPERIMENTAL RESULTS

Compound	Expt.	Dose [a] μg (μl)/ml	Survival (%)		Reversion frequency ($\times 10^{-7}$) [b]							
					Trp^+		Hom^+		His^+			
			−S9	+S9	−S9	+S9	−S9	+S9	−S9		+S9	
HMPA	1	(DMSO)	100	100	16.8 (315)	23.6 (543)	1.3 (25)	1.4 (33)	1.1	(20)	1.0	(24)
		17.4 (0.1)	95.0	100	24.8 (442)	20.5 (473)	1.2 (22)	0.87 (20)	–	(0)	1.4	(32)
		174 (1)	88.0	103	30.6 (505)	20.4 (483)	1.6 (27)	1.4 (32)	6.8	(113)	3.0	(72)
		1745 (10)	119	147	23.0 (513)	16.6 (560)	0.54 (12)	0.89 (30)	7.3	(163)	0.95	(32)
		3490 (20)	112	87.0	25.3 (528)	23.3 (468)	0.62 (13)	1.3 (27)	11.4	(238)	5.8	(117)
		(EMS)	21.0	–	389 (1530)	–	20.4 (80)	–	188	(740)	–	
		(DMN)	69.0	83.0	38.2 (485)	26.7 (508)	2.2 (28)	2.0 (37)	16.5	(210)	0.79	(15)
	2	(DMSO)	100	100	56.6 (403)	60 (497)	2.1 (15)	4.4 (37)	14.5	(103)	2.4	(20)
		17.4	85.0	111	74.3 (455)	68 (630)	2.5 (15)	2.4 (22)	17.2	(105)	3.5	(32)
		174	84.0	96.0	71.2 (427)	93 (742)	3.7 (22)	2.5 (20)	20.5	(123)	2.3	(18)
		1745	98.0	76.0	74.5 (520)	92 (580)	2.4 (17)	3.6 (23)	14.0	(98)	5.1	(32)
		3490	65.0	53.0	88.7 (410)	127 (568)	5.8 (27)	9.6 (43)	20.0	(92)	19.0	(83)
		(EMS)	55.0	–	–	–	–	–	–		–	
		(DMN)	97.0	115	63.0 (437)	77 (747)	1.7 (12)	1.9 (18)	9.3	(65)	1.9	(18)
SAF	3	(DMSO)	100	100	9.4 (167)	52.5 (211)	0.17 (3)	1.7 (7)	0.62	(11)	–	(0)
		3.7 (0.025)	12.0	92.8	66.7 (144)	79.6 (297)	1.9 (4)	0.54 (2)	5.1	(11)	4.3	(15)
		7.4 (0.05)	6.50	81.3	142 (163)	29.0 (103)	0.87 (1)	2.0 (7)	7.8	(9)	2.3	(8)
		14.8 (0.1)	0.650	13.1	– (0)	73.9 (39)	– (0)	5.7 (3)	–	(0)	–	(0)
		29.6 (0.2)	–	1.00	– (0)	– (0)	– (0)	– (0)	–	(0)	–	(0)
		(EMS)	5.30	–	293 (277)	–	7.6 (72)	–	4.2	(4)	–	
		(DMN)	16.6	102	5.6 (167)	60.0 (249)	0.27 (8)	–	–		–	
	4	(DMSO)	100	100	37.5 (145)	27.0 (305)	3.0 (12)	2.8 (32)	64.6	(250)	3.1	(35)
		3.7	100	104	50.4 (197)	17.6 (208)	3.8 (15)	1.6 (18)	81.6	(318)	6.8	(80)
		7.4	97.0	55.0	24.0 (90)	20.5 (128)	3.5 (13)	2.4 (15)	66.7	(250)	22.9	(143)
		14.8	2.20	18.0	1930 (165)	105 (212)	197 (17)	5.0 (10)	2580	(218)	125	(252)
		29.6	–	3.1	– (0)	91 (32)	– (0)	20.0 (7)	–	(0)	143	(50)
		(EMS)	93.0	–	776 (2780)	–	12.1 (43)	–	457	(1640)	–	
		(DMN)	183	156	32.3 (228)	34.1 (600)	3.8 (27)	0.76 (13)	33.9	(240)	7.5	(132)
CAP	8	(DMSO)	100	100	1.9 (25)	1.9 (27)	2.1 (28)	0.71 (10)	8.9	(117)	2.4	(33)
		100	103	80.0	2.4 (32)	2.7 (30)	0.88 (12)	2.7 (30)	5.7	(77)	10.7	(120)
		1000	82.0	101	2.4 (27)	1.8 (25)	1.7 (18)	1.6 (23)	12.2	(132)	5.7	(80)
		2000	83.0	96.0	3.2 (35)	2.4 (33)	2.4 (27)	3.2 (43)	9.3	(102)	4.2	(57)
		(EMS)	57.0	–	8.3 (62)	–	10.9 (82)	–	163	(1220)	–	
		(DMN)	137	98.7	2.6 (47)	2.4 (33)	2.5 (45)	1.3 (18)	4.7	(85)	9.3	(128)

	9	(DMSO)	100	100	17.3	(228)	11.0	(263)	7.7	(102)	4.7	(112)	38.5	(508)	8.3	(198)
		100	80.0	77.0	46.5	(493)	15.6	(287)	13.4	(142)	8.3	(152)	33.7	(357)	7.6	(140)
		1000	104	93.0	30.3	(415)	68.0	(151)	8.9	(122)	10.6	(235)	23.0	(315)	8.4	(187)
		2000	151	79.0	16.9	(337)	87.3	(165)	5.4	(108)	5.9	(113)	20.8	(413)	8.0	(152)
		(EMS)	52.0	–	254	(1730)	–		21.6	(147)	–		380	(2580)	–	
		(DMN)	193	78.0	10.3	(263)	20.6	(385)	5.9	(150)	4.8	(90)	4.8	(122)	18.3	(343)
TOL	5	(DMSO)	–	–	–		–		–		–		–		–	
		2.6 (0.025)	100	100	18.8	(270)	15.8	(332)	3.2	(47)	1.1	(23)	15.1	(217)	2.0	(43)
		5.3 (0.05)	96.0	–	25.9	(358)	–		4.0	(55)	–		14.3	(198)	–	
		10.6 (0.1)	120	44.0	11.4	(197)	39.8	(368)	0.87	(15)	1.4	(13)	18.7	(323)	1.1	(10)
		21.2 (0.2)	107	35.0	21.9	(338)	44.9	(333)	3.1	(48)	18.9	(140)	17.7	(273)	8.1	(60)
		(EMS)	59.0	–	185	(1560)	–		6.2	(52)	–		363	(3060)	–	
		(DMN)	108	120	19.2	(297)	20.2	(511)	2.3	(35)	1.5	(38)	21.9	(340)	2.2	(56)
	6	(DMSO)	100	100	9.1	(87)	2.9	(51)	0.35	(3.3)	0.28	(5)	0.31	(3)	0.13	(2.3)
		5.3	155	88.0	5.3	(79)	4.0	(63)	0.09	(1.3)	0.25	(4)	0.29	(4.3)	0.06	(1.0)
		10.6	157	94.0	5.2	(78)	3.1	(51)	0.47	(7.0)	0.40	(6.7)	0.087	(1.3)	0.04	(0.7)
		21.2	93.0	55.0	6.3	(56)	9.9	(97)	0.30	(2.7)	0.58	(5.7)	0.56	(5)	0.03	(0.3)
		(EMS)	61.0	–	92.1	(540)	–		1.4	(8.3)	–		101	(587)	–	
		(DMN)	–	46.0	–		8.7	(71)	–		0.77	(6.3)	–		–	(0)
	7	(DMSO)	–	100	–		1.9	(27)	–		0.71	(10)	–		2.4	(33)
		5.3	–	102	–		3.0	(43)	–		2.0	(28)	–		4.3	(62)
		10.6	–	99.0	–		2.7	(38)	–		1.4	(20)	–		6.3	(88)
		21.2	–	124	–		1.4	(25)	–		2.1	(37)	–		3.9	(68)
		(EMS)	–	57.0	–		8.3	(62)	–		10.9	(82)	–		163	(1220)
		(DMN)	137	97.0	2.6	(47)	2.4	(33)	2.5	(45)	1.3	(18)	4.7	(85)	9.3	(128)
ZOIN	10	(DMSO)	100	100	3.1	(47)	1.6	(37)	4.4	(66)	1.0	(22)	4.4	(67)	2.0	(47)
		25	107	86.0	1.4	(22)	2.0	(40)	1.2	(18)	2.4	(47)	6.5	(105)	2.4	(47)
		250	146	101	1.7	(37)	0.7	(17)	0.8	(18)	1.3	(30)	8.7	(192)	1.3	(30)
		2500	146	83.0	1.6	(35)	2.3	(43)	2.0	(45)	0.9	(18)	5.4	(120)	11.8	(225)
		5000	111	101	3.7	(62)	2.2	(52)	2.6	(43)	2.1	(50)	12.4	(207)	0.86	(20)
		(EMS)	76.0	–	121	(1390)	–		119	(1370)	–		240	(2760)	–	
		(DMN)	103	100	1.2	(18)	1.8	(42)	1.3	(20)	2.1	(48)	6.3	(98)	2.3	(53)
	11	(DMSO)	100	100	23.0	(380)	14.0	(158)	1.4	(23)	2.7	(30)	14.8	(245)	8.7	(98)
		25	97.0	112	22.2	(355)	25.5	(323)	1.9	(30)	2.8	(35)	36.4	(582)	24.9	(317)
		250	82.0	116	23.1	(312)	18.5	(242)	2.7	(37)	2.4	(32)	59.8	(807)	48.7	(638)
		2500	113	132	25.4	(473)	31.0	(462)	1.9	(35)	3.7	(55)	38.3	(712)	28.4	(423)
		5000	92.0	104	25.7	(388)	29.2	(342)	1.4	(22)	2.3	(27)	38.7	(585)	11.1	(130)
		(EMS)	73.0	–	230	(2780)	–		11.4	(138)	–		112	(1360)	–	
		(DMN)	92.0	95.0	28.2	(428)	28.7	(307)	4.2	(63)	4.2	(45)	31.5	(478)	42.2	(451)

[a] The concentrations of control compounds are 10 μl/ml (DMSO) and 0.75 μl/ml (EMS and DMN).

[b] Numbers in parentheses are averages of revertants/ml counted on 3 replicate plates.

Results and discussion

The results of testing the 5 compounds in strain XV185-14C are shown in Table 1. Criteria for the classification of positives and negatives are described in the summary chapter on fungi in this volume. HMPA, SAF, and ZOIN show a positive response in the *his1-7* marker, and CAP is positive at the *hom3-10* locus. ZOIN does not require S9 for a positive effect, but HMPA, SAF and CAP are dependent upon S9 for mutagenicity.

Frequencies of spontaneous mutants (per 10^7 survivors) varied, sometimes widely, from one experiment to the next in the *trp5-48* allele (1.9–56.6, −S9; 1.6–6.0, +S9), the *hom3-10* marker (0.17–7.7, −S9; 0.28–4.7, +S9) and at the *his1-7* locus (0.31–64.6, −S9; 0.13–8.7, +S9). Variation in mutant frequencies of negative controls also has been observed in strain XV185-14C in a previous collaborative study (Mehta and von Borstel, 1981).

The *his1-7* marker was the most sensitive of the 3 loci chosen for study, reverting in response to treatment with HMPA, SAF and ZOIN, but not CAP. Although the *trp5-48* marker responded most frequently in a study of chlorinated compounds and resin acids found in effluents from pulp and paper mills (Nestmann and Lee, 1983), Mehta and von Borstel (1981) have stated that *his1-7* generally appears to be the most sensitive marker in XV185-14C.

Mutagenic activity of 2 carcinogens (HMPA and SAF) and of 2 noncarcinogens (CAP and ZOIN) was detected using yeast strain XV185-14C. As with the noncarcinogens azide and caffeine, it is possible that CAP and ZOIN are microbial mutagens that are not active in mammals in vivo. Alternatively, the negative results of the cancer bioassays could be incorrect, and these compounds could be carcinogens. Another possibility involves experimental artifact, such as selection or even solvent effects. Falsely positive results have been noted previously when DMSO has been used as a solvent in mutagenicity testing (Nestmann et al., 1980, 1983). However, positive results with CAP and ZOIN were found by other investigators in this collaborative study. CAP was detected as a mutagen in Drosophila and in mammalian chromosome aberration and polyploid tests. ZOIN was positive in Salmonella and mammalian cell mutation assays.

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The detection of mitotic gene conversion, point mutation and mitotic segregation using the yeast *Saccharomyces cerevisiae* strain D7

James M. Parry and Friederike Eckardt [a]

Department of Genetics, University College of Swansea, Swansea, (Great Britain) and [a] Gesellschaft für Strahlen- and Umweltforschung, Abteilung Strahlenbiologie, Neuherberg (Federal Republic of Germany)

Summary

The 10 test chemicals were evaluated for their ability to induce mitotic gene conversion, point mutation and mitotic segregation in yeast strain D7. The results obtained were analysed by the determination of integral yields of the levels of plate increases produced.

SAF, ACN and BEN produced increases in mitotic gene conversion and BEN was also capable of inducing point mutation. The results obtained with mitotic segregation were less conclusive, in that they involved the detection of relatively small numbers of the relevant phenotypes.

The diploid yeast strain D7 described by Zimmermann (1975) has been developed as a multipurpose system for the detection of a variety of biological and genetic endpoints. In this study we utilized D7 to determine the effects of the IPCS chemicals upon the following endpoints.

(a) Cellular toxicity, as measured by the growth of cells on complete medium.

(b) The induction of nuclear point mutations at the *ile1-92* allele, as measured by the production of isoleucine-independent prototrophs detected on isoleucine-deficient medium.

(c) The induction of mitotic gene conversion at the *trp-5* locus, as measured by the production of tryptophan-independent prototrophs detected on tryptophan-deficient medium;

(d) The induction of aberrant mitotic segregations at the *ade-2* locus, as detected by the production of red and pink coloured colonies detected in complete medium.

The 10 IPCS chemicals were tested for their ability to induce the 3 genetic endpoints using a protocol designed to optimise the levels of endogenous cytochrome P-450 in yeast (Kelly and Parry, 1983) and in the presence and absence of an exogenous source of liver microsomes and cofactors.

Materials and methods

Strain

The relevant genotype of strain D7 is:

Chromosome VII	centromere	$\frac{trp\ 5\text{-}12\ cyh_2^r}{trp\ 5\text{-}27\ CYH_2^S}$
Chromosome V	centromere	$\frac{ilv\ 1\text{-}92}{ilv\ 1\text{-}92}$
Chromosome XV	centromere	$\frac{ade\ 2\text{-}40}{ade\ 2\text{-}119}$

trp 5-12 and *trp 5-27* are heteroalleles of the *TRP-5* locus and undergo mitotic gene conversion to produce prototrophic colonies carrying one wild-type allele that allows for growth on selective medium lacking tryptophan. *ade 2-40* is a completely inactive allele of *ADE-2*, which produces deep red colonies, whereas *ade 2-119* is a leaky allele causing accumulation of only a small amount of red pigment and thus produces pink colonies. In heteroallelic diploids the *ade 2-40* and *ade 2-119* alleles complement to give rise to white

adenine-independent colonies. A variety of genetic events including mitotic crossing-over in D7 may give rise to the production of cells homoallelic for the *ade-2* mutations and thus lead to the observation of red and pink colonies. The frequency of mitotic crossing-over between the *ADE-2* gene and the centromere of chromosome XV can be unambiguously confirmed in D7 by the observation of red/pink half sectored colonies.

Base-substitution mutation in D7 can be assayed at the *ILV-1* gene by the production of isoleucine-independent colonies by reversion of the homozygous *ilv 1-92* mutations on chromosome V.

Treatment protocol

The test compounds were assayed for their genetic activity using a treatment procedure developed to optimise the endogenous levels of cytochrome P-450 in yeast (Kelly and Parry, 1983). In this protocol cells were inoculated into liquid yeast complete medium containing 0.5% glycerol and grown on an orbital shaker at 28°C for 2 days. Cells were washed and resuspended in liquid complete medium containing 20% glucose at a cell concentration of 5×10^6 cells/ml. Under these conditions yeast cells produce their maximal levels of cytochrome P-450 in approximately 24 h. Prior growth in medium containing glycerol as a carbon source results in the elimination of pre-existing petite mutants from the experimental culture.

After inoculation into 20% glucose yeast complete medium the cells were placed on an orbital shaker at 28°C for 4 h before the introduction of the test chemicals. At this time suitable concentrations of the test chemical were added to the treatment medium and the cultures were then grown in sealed bottles for 18 h at 28°C. Cells were exposed to the test chemicals both in the presence and absence of an Aroclor-induced rat-liver S9 mix as an exogenous activation mix prepared as described in Parry and Sharp (1981). The bottles treated with S9 mix were incubated for 2 h at 37° (without shaking) prior to their 18-h growth period.

In the case of ACN, CAP and DEHP, the sealed bottles were made of glass and the other 7 chemicals were tested in plastic bottles. After treatment, cells were washed 3 times in saline and after appropriate dilution plated upon complete medium to measure cell viability and aberrant colonies, and upon selective medium to detect point mutation and mitotic gene conversion.

All the cultures were examined after treatment for the presence of spore tetrads, indicative of sporulation. In none of the experiments described here was there any indication of meiosis and cell sporulation.

In the case of aberrant segregation we attempted to screen at least 1000 individual colonies for their phenotypes.

Chemicals

The following chemicals supplied by the IPCS programme via Lancaster Synthesis were tested for their ability to induce mitotic segregation, mitotic gene conversion and nuclear point mutation in strain D7 at concentrations of up to 2000 μg/ml.

Acrylonitrile (ACN), *o*-toluidine (TOL), phenobarbital (PB), safrole (SAF), benzene (BEN), diethylstilbestrol (DES), caprolactam (CAP), benzoin (ZOIN), diethylhexylphthalate (DEHP) and hexamethylphosphoramide (HMPA). 8 of the chemicals were prepared by dissolving in DMSO prior to the preparation of the aqueous concentration ranges. DES was prepared in 95% ethanol and DEHP was prepared in 99% DMSO plus 1% Tween-80.

Cyclophosphamide at 50 μg/ml was used as a positive control chemical. A number of range-finder studies were performed with the chemicals to determine appropriate dose ranges. The results shown here represent those from a single experiment in which all ten chemicals were assayed simultaneously.

Results

Induction of mitotic gene conversion and point mutation

The results of the assay of all 10 chemicals are shown in Table 1.

The results of the study of the 10 test compounds have been evaluated by a number of criteria. We have analysed the data according to the methods of Eckhardt and Haynes (1980). For each biological endpoint and exposure dose, the net induction of viable mutants, the "mutant yield" (defined as induced mutants per treated cell) was calculated. When these values were positive the test agent was classified as showing inducing

TABLE 1

RESULTS OF THE INDUCTION OF MITOTIC GENE CONVERSION (trp^+), POINT MUTATION (iso^+) AND MITOTIC SEGREGATION (COLOURED COLONIES) IN YEAST STRAIN D7 AFTER TREATMENT WITH THE 10 IPCS CHEMICALS

Treatment (μg/ml)	+/− S9 mix	Mean number of viable cells 10^{-4} dilution	Mean number of trp^+ prototrophs 10^0 dilution	Mean number of iso^+ prototrophs 10^0 dilution	Aberrant colonies (%)
Control 2% DMSO	−	317.3	277.2 ±89	30.7 ± 4.3	0.157
Control 2% DMSO	+	307.8	283.2 ±38.2	29.2 ± 5.5	0.108
50 Cyclophosphamide	−	112.2	404.7 ±17.2	65.7 ± 6.0	1.07
50 Cyclophosphamide	+	131.5	415.3 ±19.9	51.7 ± 3.1	1.01
ACN					
20	−	111.2	563.3 ±50.0	42.3 ± 6.1	0.18
50	−	81.1	492.3 ±27.4	44.7 ± 3.2	0.49
100	−	41.6	122.3 ±35.2	41.7 ±129	0
200	−	113 (10^{-3} dilution)	86.7 ±20.8	5.7 ± 1.5	0.76
ACN					
20	+	115.5	299.7 ±25.8	46.7 ± 2.5	0.26
50	+	71.1	358.4 ±23.4	38.3 ± 3.1	0
100	+	48.8	107.3 ±11.7	41.7 ±129	0.21
200	+	55 329 (10^{-3} dilution)	340.0 ±64.8	17.7 ± 3.1	0.87
BEN					
50	−	218.3	192.7 ±20.7	41.00± 2.6	0.23
100	−	216.7	253.0 ± 8.3	61.3 ± 9.61	0.51
200	−	240.00	473.0 ±76.7	58 ± 5.57	0.41
500	−	112.6	285.3 ±36.7	37.3 ± 8.5	0.18
1000	−	74.4	174.7 ±16.3	43.00± 6.0	0.134
2000	−	27.7 150.7 (10^{-3} dilution)	279.0 ±14.7	12.3 ± 2.52	0
BEN					
50	+	243.2	372.0 ±23.6	41.0 ± 2.6	0.23
100	+	287.3	356.3 ±24.3	61.0 ± 4.0	0
200	+	216.7	465.7 ±56.1	117.7 ± 15.1	0
500	+	77.6	260.0 ±15.6	42.3 ± 4.73	0.258
1000	+	111.3	443.7 ±19.4	48.3 ± 2.08	0.36
2000	+	40.3 218 (10^{-3} dilution)	253 ±13.2	20.7 ± 3.2	0.77
CAP					
50	−	213.7	123.3 ± 7.6	22.3 ± 3.2	0
100	−	203.7	117.5 ± 6.8	17.3 ± 4.9	0
200	−	94.1	123.7 ±10.7	10.7 ± 1.2	0.213
500	−	64.1	90.0 ±16.5	5.7 ± 1.5	0
1000	−	31.3	40.0 ± 6.2	5.3 ± 0.6	0.38
2000	−	48.3 (10^{-3} dilution)	43.3 ± 7.6	6.3 ± 0.58	0

TABLE 1 (continued)

Treatment (μg/ml)	+/− S9 mix	Mean number of viable cells 10^{-4} dilution	Mean number of trp$^+$ prototrophs 10^0 dilution	Mean number of iso$^+$ prototrophs 10^0 dilution	Aberrant colonies (%)
CAP					
50	+	198.3	214.7 ± 20.6	20.3 ± 5.9	0
100	+	201.0	184.7 ± 20.2	21.3 ± 4.5	0.33
200	+	106.7	104.0 ± 16.5	7.3 ± 2.3	0.38
500	+	85.5	61.0 ± 20.1	5.3 ± 0.6	0.35
1000	+	47.7	55.0 ± 18.3	6.3 ± 0.6	0.21
2000	+	211.0 (10^{-3} dilution)	58.0 ± 13.2	5.7 ± 0.6	0.98
DEHP					
200	−	261.3	19.07 ± 13.1	27 ± 2.7	0
500	−	68.3	120 ± 17.3	20.3 ± 2.5	0.44
1000	−	137.9	161 ± 26.9	10.3 ± 3.1	0
2000	−	27.6	167 ± 25.7	11 ± 2.6	0.36
3000	−	144.9 (10^{-3} dilution)	204 ± 41.8	11.7 ± 2.1	0.35
5000	−	76.6 (10^{-3} dilution)	150 ± 39.8	9.3 ± 2.5	1.17
DEHP					
200	+	216	208 ± 13.5	29 ± 6.1	0
500	+	137.9	220.3 ± 17.2	19.7 ± 3.1	contaminated
1000	+	77.7	188.3 ± 32.3	15.3 ± 2.5	0.9
2000	+	45.5	205.7 ± 6.7	13.3 ± 1.5	1.32
3000	+	138	197.3 ± 39.8	10 ± 1.73	0.66
5000	+	55.6 (10^{-3} dilution)	87 ± 22.7	2.7 ± 2.5	1.44
DES					
0.05	−	314	335.7 ± 41.3	30.3 ± 3.2	0.74
0.1	−	307.3	221 ± 10.4	25.3 ± 4.0	0.22
0.2	−	225.3	142 ± 24.8	18 ± 2.0	0.18
0.5	−	127.0	195.3 ± 22.2	10.3 ± 1.5	0.079
1	−	51.0	184.5 ± 2.5	5 ± 1	0
2	−	119.2 (10^{-2} dilution)	0	0	0
DES					
0.05	+	453.7	211 ± 55.4	24.7 ± 5.1	0
0.1	+	368.3	279 ± 33.2	20.3 ± 2.5	0
0.2	+	186.2	225.5 ± 32	19.7 ± 3.1	0.054
0.5	+	119.2	246.7 ± 21.2	10.3 ± 2.1	0.168
1	+	44.2	184.5 ± 2.5	5 ± 1	0.45
2	+	399.9 (10^{-3} dilution)	18.7 ± 5.0	1.67 ± 0.6	0.23
HMPA					
10	−	247.3	369.7 ± 26.1	29.3 ± 3.1	0
20	−	114.2	204.7 ± 13.8	28.3 ± 9.7	0.09
50	−	106.0	244.3 ± 4.2	21.7 ± 8.96	0.09
100	−	65.0 347 (10^{-3} dilution)	189.0 ± 20.6	12.0 ± 4.1	0.32
200	−	49.0 274.3 (10^{-3} dilution)	155.0 ± 8.7	14.3 ± 1.53	0.21

TABLE 1 (continued)

Treatment (μg/ml)	+/− S9 mix	Mean number of viable cells 10^{-4} dilution	Mean number of trp^+ prototrophs 10^0 dilution	Mean number of iso^+ prototrophs 10^0 dilution	Aberrant colonies (%)
HMPA (continued)					
300	−	39.0 190 (10^{-3} dilution)	134.3 ± 5.86	5.67 ± 2.1	0.15
500	−	276.0 (10^{-2} dilution)	61.0 ± 9.6	3.3 ± 1.5	0.76
HMPA					
10	+	187.3	204.7 ± 13.8	23.0 ± 2.65	0.18
20	+	149.9	302.3 ± 16.2	28.0 ± 9.5	0.07
50	+	122.6	273.7 ± 25.6	27.0 ± 10.6	0.49
100	+	54.7 192.8 (10^{-3} dilution)	156.0 ± 11.3	15.7 ± 0.58	0.44
200	+	76.0 235 (10^{-3} dilution)	148.0 ± 11.1	16.7 ± 1.53	0.22
300	+	42.6 185.3 (10^{-3} dilution)	98.7 ± 9.3	8.67 ± 1.52	0.29
500	+	325.3	61.0 ± 9.6	3.3 ± 1.5	0.76
PB					
100	−	217.3	310.0 ± 14.0	23.7 ± 2.5	0.153
200	−	193.8	259.7 ± 23.4	18.3 ± 2.5	0
500	−	149.2	217.0 ± 36.1	23.0 ± 1.53	0.27
1000	−	61.6	128.3 ± 29.7	33.3 ± 2.1	0
2000	−	61.7 377.3 (10^{-3} dilution)	118.0 ± 2.1	27.0 ± 4.73	0.76
5000	−	37.3 246.7 (10^{-3} dilution)	89.0 ± 11.3	17.3 ± 2.5	0
PB					
100	+	240.3	310.0 ± 14.0	23.7 ± 2.5	0.14
200	+	195.5	264.3 ± 19.6	20.3 ± 2.5	0.051
500	+	164.5	266.7 ± 5.9	31.0 ± 3.0	0
1000	+	59.7	183.3 ± 3.5	37.0 ± 5.3	0
2000	+	61.3 360 (10^{-3} dilution)	131.3 ± 21.6	26.3 ± 3.5	0.079
5000	+	21.7 1557 (10^{-3} dilution)	100.0 ± 24.8	17.7 ± 3.5	0.19
SAF					
10	−	299.1	586.0 ± 24.6	29.3 ± 3.5	0
20	−	303.7	709.7 ± 29.3	30.7 ± 2.5	0
50	−	352	620.3 ± 74.1	40.7 ± 6.7	0.66
100	−	37.1	115.6 ± 21.1	12.0 ± 3.6	0
200	−	68.3 (10^{-3} dilution)	1.67 ± 0.58	3.0 ± 1.0	0.59

TABLE I (continued)

Treatment (μg/ml)	+/− S9 mix	Mean number of viable cells 10^{-4} dilution	Mean number of trp^+ prototrophs 10^0 dilution	Mean number of iso^+ prototrophs 10^0 dilution	Aberrant colonies (%)
SAF					
10	+	297.3	456.0 ±15.9	31.3 ± 2.5	0.24
20	+	188	485.0 ±18.8	29.7 ± 3.8	0.35
50	+	170	553.3 ±69.0	52.7 ± 8.6	0
100	+	37.1	184.0 ±11.3	23.0 ± 1.0	1.05
200	+	147.2 (10^{-3} dilution)	1.67± 0.58	2.67± 0.58	0.27
TOL					
10	−	229.4	300.0 ±23.3	22.7 ± 3.5	0.13
20	−	98.2	185.7 ±15.8	8.0 ± 1.7	0
50	−	82.5	164.3 ±22.8	10.3 ± 2.3	0
100	−	52.5	144.0 ±2.64	11.0 ± 1	0.19
200	−	47.8	110.0 ±13.5	13.6 ± 3.2	0
300	−	30.3 109.7 (10^{-3} dilution)	73.7 ± 9.2	13.0 ± 2.7	0.48
500	−	22.7	108.3 ± 9.5	7.67± 2.5	0.25
TOL					
10	+	190	315.0 ± 7.5	27.0 ± 7.9	0.15
20	+	140.1	195.3 ±14.5	12.3 ± 1.2	0.071
50	+	89.2	173.3 ±37.0	11.0 ± 1.73	0.112
100	+	70.8	171.7 ±17.5	8.67± 0.6	0.28
200	+	76.6	142.0 ± 9.64	11.33± 1.5	0
300	+	25.2 111.3 (10^{-3} dilution)	85.0 ±12.1	10.0 ± 0.6	0.5
500	+	30.7 119.3 (10^{-3} dilution)	125.0 ±11.1	9.0 ± 1.7	0.22
ZOIN					
100	−	251.3	129 ±15.6	22.7 ± 1.5	0.114
200	−	230.7	201 ± 9.5	23.7 ± 3.1	0.145
500	−	178.7	223.3 ±23.5	23.7 ± 2.5	0
1000	−	40.9	228.7 ± 8.7	16.7 ± 3.1	0.73
2000	−	24 126.7 (10^{-3} dilution)	168.3 ±15.6	20.3 ± 2.1	1.08
3000		64 (10^{-3} dilution)	51.2 ± 8.2	6.7 ± 0.6	1.56
5000	−	96.3 (10^{-3} dilution)	7.33± 2.3	1.33± 0.58	0.28
ZOIN					
100	+	292	228 ±32.9	30.3 ± 4.5	0
200	+	191.3	176.7 ±13.2	32.3 ± 2.5	0
500	+	235.4	246 ±33.1	27 ± 4.0	0.13
1000	+	94.3	184.3 ±20.3	18.7 ± 1.53	0.53
2000	+	18.7 95.6 (10^{-3} dilution)	98 ±11	7.3 ± 2.1	0.38
3000	+	contaminated			
5000	+	250.3 (10^{-3} dilution)	21 ± 6.1	1.33± 0.58	0.46

capacity whereas negative values indicated that the test agent was genetically inactive under the experimental conditions. In addition, the integral under the yield curves plotted against lethal hits (negative logarithm of the surviving fraction) for each exposure dose was calculated and is shown in Table 2. As has been shown by Eckardt and Haynes (1980), the yield integral, which reflects the total number of viable mutants produced over the entire dose range of an agent, can be used for a quantitative comparison of the mutagenic efficiency of genotoxic agents if the appropriate conditions are met.

Positive numbers for the integral yield values indicate that at least some doses of the agent induce the genetic endpoint under consideration. Significant yields as large as the background yield are comparable to the doubling of plate mutants since the background yield is subtracted from the induced yield in our calculations. For comparative purposes we have classified the results into 3 groups on the following basis.

(1) +, significant induction at 3 or more doses that produce yields as large as the background yield,

(2) ±, 2 doses produce yields at least as large as the background yield,

(3) −, no induction at all, or yields are smaller than background, or only a single dose that produces yields above background.

When analysed in this manner it can be seen that only SAF, BEN and ACN produce results that were positive for mitotic gene conversion; BEN and ACN were also positive for the induction of point mutation. None of the other test chemicals gave any indication of a positive re-

TABLE 2

INTEGRAL YIELDS OBTAINED IN ASSAYS FOR MITOTIC GENE CONVERSION AND POINT MUTATION WITH THE TEST CHEMICALS IN YEAST STRAIN D7

Chemical	± S9	Maximum lethal hits	Mitotic gene conversion $\times 10^{-5}$	Point mutation $\times 10^{-6}$	Conclusion
DES	−	3.28	3.5 (−)	0 (−)	2−
	+	2.04	6.4 (−)	0 (−)	2−
ZOIN	−	2.9	8.4 (−)	0.94 (−)	2−
	+	2.0	3.4 (−)	0.78 (−)	2−
CAP	−	4.2	0 (−)	0 (−)	2−
	+	2.7	0 (−)	0 (−)	2−
DEHP	−	3.72	15.6 (−)	1.0 (−)	2−
	+	4.0	14.8 (−)	1.0 (−)	2−
HMPA	−	4.6	14.2 (−)	0.66 (−)	2−
	+	3.86	11.0 (−)	0.95 (−)	2−
BEN	−	2.7	12.4 (−)	2.1 (±)	1± 1−
	+	2.3	17.2 (±)	3.1 (+)	1+ 1±
PB	−	2.33	4.47 (−)	0.79 (−)	2−
	+	2.81	7.09 (−)	0.97 (−)	2−
SAF	−	2.15	11.7 (+)	0.49 (−)	1+ 1−
	+	2.15	18.5 (±)	1.6 (−)	1± 1−
ACN	−	3.3	12.5 (+)	2.1 (+)	2+
	+	1.95	19.4 (+)	1.07 (+)	2+
TOL	−	2.94	7.0 (−)	0.96 (−)	2−
	+	2.67	7.46 (−)	0.31 (−)	2−

TABLE 3

COMPARISON OF THE INCREASE IN PLATE COUNTS ABOVE CONTROL VALUE FOR 10 TEST CHEMICALS FOR MITOTIC GENE CONVERSION AND POINT MUTATION

Chemical	±S9	Gene conversion	Point mutation
DES	−	−	−
	+	−	−
ZOIN	−	−	−
	+	−	−
CAP	−	−	−
	+	−	−
DEHP	−	−	−
	+	−	−
HMPA	−	−	−
	+	−	−
BEN	−	−	−
	+	+	+
PB	−	−	−
	+	−	−
SAF	−	+ +	−
	+	+ +	−
ACN	−	+	−
	+	−	−
TOL	−	−	−
	+	−	−

sponse in terms of integral yields in D7.

The results were also assayed using the following criteria:

(1) + +, significant increase in plate counts above the control (2 × increase in counts) for at least 3 doses.

(2) +, significant increase in plate counts above control (2 × increase in counts) for 2 doses,

(3) −, no increase in plate counts above control or only single point produces yields above background.

The results of these comparisons are shown in Table 3. They demonstrate that SAF and ACN (in the absence of exogenous S9) produce significant increases in the induction of mitotic gene conversion. BEN (in the presence of exogenous S9) gave a significant increase in mitotic gene conversion and point mutation. None of the other 7 chemicals gave any evidence of a significant increase in the induction of gene conversion or point mutation in yeast strain D7.

Induction of mitotic segregation

Table 1 shows the summary data for the production of aberrant colonies. This data represents the numbers of red, pink, red/pink, red/white, and pink/white colonies obtained on complete medium. In view of the relatively small numbers of aberrant colonies observed, it was necessary to use "robust" criteria for the classification of a positive result. In our case the following criteria were used:

(1) +, increase of at least 5 × at 3 doses,

(2) ±, increase of at least 5 × at 2 doses,

(3) −, no increases of 5 × or increase at only 1 dose.

The summary data in Table 3 indicate that significant results were obtained with DEHP (in presence of exogenous S9), SAF (in the presence of S9), and ZOIN (in the absence of S9). None of the remaining 7 chemicals showed significant increases that met our criteria. Unfortunately the actual plate numbers of aberrant colonies obtained were low for all the treatment and the experiment must be considered less than optimal for this endpoint.

Conclusions

The results presented here demonstrate that using the criteria of integral yield (Eckardt and Haynes, 1980) and a 2 × increase in plate counts both safrole and acrylonitrile were capable of inducing mitotic gene conversion at the *trp-5* locus in yeast strain D7. In addition, benzene was capable of inducing base substitution point mutation at the *ile1* locus and mitotic gene conversion in the presence of S9 mix. None of the remaining 7 chemicals showed any significant induction of either mitotic gene conversion or point mutation within the dose ranges tested in an experimental protocol that had previously been used to assay the activity of the test chemicals using yeast strains D61-M and D6.

The results of the study of aberrant segregation were less conclusive in that they involved the detection of relatively small numbers of the relevent phenotypes. In this study 3 chemicals, i.e. DEHP, SAF and ZOIN, produced significant

TABLE 4

SUMMARY OF RESULTS OF THE INDUCTION OF MITOTIC SEGREGATION IN D7 INDUCED BY THE 10 TEST CHEMICALS

	Chemicals									
	DES	ZOIN	CAP	DEHP	HMPA	BEN	PB	SAF	ACN	TOL
− S9	−	+	−	−	−	−	−	+/−	−	−
+ S9	−	−	−	+	−	−	−	−	−	−

numbers of aberrant colonies. However, it should be pointed out that none of the chemicals produced significant increases in pink/red colonies indicative of the induction of reciprocal mitotic crossing-over. In the case of DEHP only 1 pink/red colony was observed over all the dose points and it is most likely that aberrant segregation in this case arises from the induction of aneuploidy colonies as observed with D6 and D61-M.

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Tests for genetic activity in the yeast *Saccharomyces cerevisiae* using strains D7-144, XV185-14C and RM52

R.D. Mehta and R.C. von Borstel

Department of Genetics, The University of Alberta, Edmonton, Alberta (Canada)

Summary

All of the 7 carcinogens and the 2 noncarcinogens tested on log phase cells of 3 strains of *Saccharomyces cerevisiae* (D7-144, XV185-14C and RM52) induced mutagenic activity in at least 2 of the 3 strains for each compound assayed. When treated with the compounds in growth medium, the log-phase cells of strain D7-144 (a nonsporulating derivative of strain D7) responded to all but hexamethylphosphoramide, strain XV185-14C responded to all of the compounds, and strain RM52 responded only to diethylhexylphthalate, hexamethylphosphoramide, phenobarbitone and benzoin.

The 3 strains of yeast developed in our laboratory and used in this study were the haploid yeast XV185-14C and the diploid strains D7-144 and RM52. The chemicals were selected by John Ashby because they had all been negative or were very weakly positive in studies carried out with the Salmonella tester set. 8 of the chemicals are carcinogens in animals, and 2 are putative noncarcinogens. Of the chemicals available, we tested all but diethylstilbestrol in this study. Diethylstilbestrol has already been tested extensively in our laboratory, and shown to be negative in stationary-phase cells (Mehta and von Borstel, 1981), weakly positive in log-phase cells, and strongly positive after being oxidized (Mehta and von Borstel, 1982). Therefore, for this international program, we devoted our attention to the other 9 compounds.

In the previous international program (de Serres and Ashby, 1981) we found that the carcinogens selected by Ashby, also selected mostly on the basis of being negative in the Salmonella assays devised by Ames and his collaborators (Ames et al., 1975), were usually negative in stationary-phase assays in yeast (Mehta and von Borstel, 1981). This was also found by the other yeast investigators in that study. On the other hand, most of the carcinogens were positive in log-phase cells of yeast. Consequently, for this study, we did not use stationary-phase cells in any of the assays. In this study, we found that log-phase cells, recently harvested and exposed to the chemicals in buffer, did not respond to chemicals nearly as well as log-phase cells exposed to the chemicals while the cells were still dividing in the growth medium itself.

Materials and methods

Strains

The strains used were: XV185-14C, a *trp5-48 arg4-17 lys1-1 ade2-1 his1-7 hom3-10*; D7-144,

$$\frac{\alpha}{a}\ \frac{ade2\text{-}40}{ade2\text{-}119}\ \frac{trp5\text{-}12}{trp5\text{-}27}\ \frac{ilv1\text{-}92}{ilv1\text{-}92}$$

and RM52,

$$\frac{a}{\alpha}\ \frac{trp1}{trp1}\ \frac{ade2\text{-}40}{ade2\text{-}119}\ \frac{his1\text{-}7}{his1\text{-}7}$$

The haploid strain XV185-14C has been described

previously (cf. Mehta and von Borstel, 1981). The diploid strains D7-144 and RM52 have been developed in our laboratory; D7-144 is a nonsporulating derivative of strain D7, and RM52 was derived from the cross (D5 × Y0600-25C). The strains D5 and D7 were developed by Zimmermann (1973) and Zimmermann et al. (1975).

Media

The assay protocol, including media and growth of cultures, has been described by Mehta and von Borstel (1982). The following media were used:

YEPD, yeast extract peptone dextrose medium: yeast extract 1%, peptone 2%, dextrose 2%, agar 2%.

SC, synthetic complete medium: Difco yeast nitrogen base 0.67%, dextrose 2%, agar 2%, plus the following amino acids and bases, per liter: 20 mg each of L-tryptophan, L-histidine–HCl, L-arginine–HCl, L-methionine, L-isoleucine, L-tyrosine, L-lysine–HCl, adenine sulfate, uracil, and 30 mg L-leucine, 350 mg L-threonine, and 75 mg L-valine.

SCA, synthetic complete medium with limited adenine: the same as SC except the concentration of adenine is reduced to 5 mg/l.

OM, omission media: synthetic complete medium lacking one of the nutritional supplements, e.g., –his (SC minus histidine), –arg (SC minus arginine).

Growth of cultures

All strains were maintained on YEPD-agar under refrigeration. A cell suspension (1×10^7 cells/ml) of each strain was prepared either in liquid YEPD or in liquid SC medium containing the following additional nutrient supplements: tryptophan, arginine, lysine, histidine, methionine at 50 μg/ml, threonine at 750 μg/ml, and adenine at 12.5 μg/ml. The medium was dispensed (at 1.45 ml/tube) in glass test tubes (18 mm × 150 mm). The tubes were incubated at 30 °C in a waterbath shaker. Cultures were allowed to grow for 4–6 h before an appropriate incubation mix was prepared by adding an aliquot of either a solution of the test compound or that of the solvent. However, where it is indicated, the cell suspension in the incubation mix was replaced with the cell suspension in phosphate buffer (pH 7.0) prepared from cultures in logarithmic growth. (Mehta et al., 1982).

Incubation mix

Each of the treatment and control tubes contained 1.45 ml of cell suspension plus 0.5 ml S9 mix or buffer. The positive control tubes were dispensed either with 50 μl of cyclophosphamide stock solution or with 10 μl of ethyl methanesulfonate. An aliquot of 50 μl of dimethyl sulfoxide was added to the negative control tubes. Depending upon the various concentrations used, 5–200 μl aliquots of each test agent were added to the treatment tubes. A final volume of the incubation mix was brought up to 2.0 ml per tube by adding appropriate amounts of dimethyl sulfoxide, such that the concentration of dimethyl sulfoxide was maintained at 2.5% v/v in each tube. The concentration of the S9 microsomal fraction was kept at 25–50 μl/ml of the incubation mix.

Incubating and plating

All of the control and treatment tubes were sealed with parafilm and incubated at 30 °C in a waterbath shaker for the duration of the treatment. At the end of the treatment period, samples (1.0 ml) were taken from each tube and added to plastic tubes (12 mm × 75 mm) containing 1.0 ml of prechilled 0.1 M phosphate buffer (pH 7.0). The cells were either washed once and resuspended in 2.0 ml of buffer or left unwashed, depending upon the toxicity of the test substance. All of the tubes were kept on ice during this operation. Where required, serial dilutions were prepared in phosphate buffer. The cells from appropriate dilutions were plated (0.2 ml/plate) on various media plates.

To score for different prototroph colonies originating as a result of reverse mutation or gene conversion, cells are spread onto appropriate omission media. The plating for strains D7-144 and RM52 was done on SCA medium to score for survival and for aberrant (pigmented) colonies. Survival count for strain XV185-14C was determined by plating the cells on SC medium. A minimum of 4 plates were used for each determination. The plates were incubated at 30 °C for 3–5 days to score for prototroph colonies, or for 6–10 days to score for aberrant colonies.

TABLE 1

MUTAGENIC RESPONSE OF STRAINS D7-144 AND XV185-14C [a]

Chemical	Incubation medium	Gene conversion		Point mutations			
		D7-144		XV185-14C			
		TRP^+		HIS^+		TRP^+	
		−S9	+S9	−S9	+S9	−S9	+S9
ACN [b]	SC [c] (pH 4.1)	++	−	−	−	+	−
BEN	Buffer (pH 7.0)	−	−	−	+	−	−
CAP	SC (pH 4.1)	−	−	+	−	+	−
DEHP	Buffer (pH 7.0)	−	−	−	+	−	−
HMPA	SC (pH 4.1)	−	++	+	−	−	+
PB	SC (pH 4.1)	−	+	+	−	−	++
SAF	SC (pH 4.1)	−	+	−	−	−	+
TOL	SC (pH 4.1)	−	+	−	−	−	−
ZOIN	Buffer (pH 7.0)	−	−	−	−	−	−

[a] Summary refers to the data in Tables 3, 4 and 5.

[b] *Abbreviations*: ACN, acrylonitrile; BEN, benzene; CAP, caprolactam; DEHP, diethylhexylphthalate; HMPA, hexamethylphosphoramide; PB, phenobarbitone; SAF, safrole; TOL, *o*-toluidine; ZOIN, benzoin.

[c] SC is synthetic complete medium (see Materials and Methods).

Metabolic activation system

For metabolic activation with an exogenous preparation, the S9 liver microsomal fraction was derived from rat livers induced with Aroclor 1254. The preparation was stored at −70 °C. We usually obtain this preparation from the Carcinogen Testing Laboratory, British Columbia Cancer Foundation, Vancouver, BC (Canada). The standard procedure for preparation and use of the S9 mix is described by Ames et al. (1975).

TABLE 2

MUTAGENIC RESPONSE OF STRAINS XV185-14C, D7-144 AND RM52 WITH TREATMENT IN YEPD MEDIUM (pH 6.3) [a]

Chemical	Gene conversion		Point mutations							
	D7-144		RM52		XV185-14C					
	TRP^+		HIS^+		ARG^+		HIS^+		TRP^+	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
ACN	++	++	−	−	+	−	++	−	+	+
BEN	+	+	−	−	++	−	+	+	+	−
CAP	+	+	−	−	++	++	+	−	++	−
DEHP	+	+	+	−	+	+	+	+	−	+
HMPA	−	−	++	+	++	+	+	−	++	++
PB	++	+	−	−	+	−	+	−	+	++
SAF	+	+	+	++	+	−	−	−	+	+
TOL	−	+	+	−	+	−	+	−	+	+
ZOIN	−	++	−	−	−	−	+	−	−	−

[a] Summary refers to the data in Tables 6, 7 and 8.

TABLE 3

INDUCTION OF GENETIC ACTIVITY BY ACRYLONITRILE, *o*-TOLUIDINE, CAPROLACTAM, HEXAMETHYLPHOSPHORAMIDE, SAFROLE AND PHENOBARBITONE IN STRAIN D7-144 OF *Saccharomyces cerevisiae* [a]

Chemical	Concentration (μg/ml)	Survival %		TRP^+ convertants $\times 10^{-5}$		Mutagenic response	
		−S9	+S9	−S9	+S9	−S9	+S9
DMSO [b]	Control	100	100	0.8 ± 0.09 (75) [c]	0.5 ± 0.03 (155)		
ACN	0.8	164	159	0.7 ± 0.05 (106)	0.3 ± 0.01 (135)	+ +	−
	8.1	94	151	1.7 ± 0.11 (152) [e]	0.3 ± 0.02 (155)		
	80.6	59	80	1.1 ± 0.07 (62)	0.8 ± 0.03 (219)		
	806.0	10	2	17.7 ± 0.68 (170)	17.4 ± 3.10 (94)		
TOL	378	95	84	1.2 ± 0.06 (110)	0.5 ± 0.07 (130)	−	+
	756	139	20	0.9 ± 0.03 (123)	0.6 ± 0.11 (41)		
	1512	42	5	1.3 ± 0.07 (51)	1.3 ± 0.37 (19)		
	3024	8	2	1.6 ± 0.25 (13)	1.4 ± 0.59 (10)		
CAP	10	122	156	0.9 ± 0.05 (103)	0.3 ± 0.02 (157)	−	−
	25	113	188	1.0 ± 0.12 (111)	0.3 ± 0.02 (212)		
	50	108	92	0.9 ± 0.12 (94)	0.5 ± 0.02 (148)		
	100	118	93	0.9 ± 0.10 (105)	0.4 ± 0.04 (131)		
EMS	Positive control	0.1	−	120 ± 29.1 (7)	−		
CP	Positive control	167	51	3.1 ± 0.05 (1884)	12.3 ± 0.05 (1652)		
DMSO	Control	100	100	1.1 ± 0.23 (129)	0.5 ± 0.06 (117)		
DMSO [d]	Control	100	100	0.1 ± 0.01 (195)	0.2 ± 0.01 (291)		
HMPA	5140	393	113	0.5 ± 0.02 (247)	0.8 ± 0.03 (229)	−	+ +
	10270	306	128	0.8 ± 0.08 (298)	1.0 ± 0.06 (315)		
	20540	330	100	1.1 ± 0.06 (426)	1.5 ± 0.09 (369)		
	41080	213	43	1.4 ± 0.07 (354)	1.4 ± 0.15 (154)		
SAF	10.96	67	36	1.1 ± 0.17 (87)	1.4 ± 0.13 (130)	−	+
	21.92	99	28	0.9 ± 0.08 (107)	2.3 ± 0.10 (165)		
	43.84	45	18	1.4 ± 0.20 (75)	1.7 ± 0.09 (76)		
	87.68	30	6	1.1 ± 0.19 (38)	1.9 ± 0.14 (28)		
PB	250	103	80	0.1 ± 0.005 (263)	0.3 ± 0.01 (306)	−	+
	500	97	49	0.1 ± 0.005 (194)	0.4 ± 0.02 (260)		
	1000	62	36	0.1 ± 0.005 (260)	0.5 ± 0.04 (238)		
	2000	26	21	0.3 ± 0.01 (242)	0.7 ± 0.04 (206)		
EMS	Positive control	0.3	−	424 ± 8.18 (475)	−		
CP	Positive control	175	66	0.6 ± 0.03 (240)	2.0 ± 0.12 (327)		

[a] Cells growing in synthetic complete medium (pH 4.1) were treated for 17 h at 30 °C.
[b] *Abbreviations*: DMSO, dimethyl sulfoxide; EMS, ethyl methanesulfonate; CP, cyclophosphamide.
[c] Figures in parentheses represent total number of colonies scored.
[d] Control applies to PB treatment data.
[e] Underlined figures indicate a positive response.

A 30-ml sample of S9 mix contained the following ingredients:

S9 fraction	3.00 ml
NADP (0.1 M)	0.60 ml
glucose 6-phosphate (1.0 M)	0.08 ml
$MgCl_2 \cdot 6H_2O$ (0.4 M) + KCl (1.65 M)	0.60 ml
phosphate buffer (0.1 M) pH 7.4	25.10 ml
distilled water	0.68 ml

For a mix without the S9 microsomal fraction, the S9 fraction was replaced with an equal volume of phosphate buffer.

Chemical solutions and doses

The solvent of choice for water-insoluble compounds is dimethyl sulfoxide. Alternatively, acetone, alcohol or dimethyl formamide can be used. An appropriate dose range and exposure time are selected on the basis of the cell toxicity and solubility of a given test chemical. The test compound is usually assayed at a minimum of 4 concentrations covering a cell survival range of 10–100%.

Positive controls

Cyclophosphamide (4 mg/ml) was used as a positive control to test the activity of the S9 fraction. For tests carried out without the addition of the S9 fraction, ethyl methanesulfonate (10 μl/ml) or 4-nitroquinoline *N*-oxide (2.5 μg/ml) was used as the positive control. For each experiment, the positive and the negative controls (the solvent) were run in parallel with the test agent(s) to monitor the validity of the assay procedure.

Source of chemicals

Cyclophosphamide, NADP, D-glucose 6-phosphate, and 4-nitroquinoline *N*-oxide were supplied by Sigma Chemical Company, St. Louis, MO. Dimethyl sulfoxide and ethyl methanesulfonate were obtained from Terochem Laboratories, Edmonton, Alberta (Canada).

Criteria for evaluation of mutagenicity

(1) A minimum of a 2-fold increase in mutation frequency with an increase in mutation yield as well as exhibiting a dose-related response was regarded as strong evidence for mutagenicity of a compound (++).

(2) A minimum of a 2-fold increase in mutation frequency without a corresponding increase in mutation yield was regarded as good evidence for mutagenicity of a compound, particularly if the compound was toxic (+). In instances where a dose-dependent response was not obtained, but the test compound exhibited at least a 2-fold increase in mutation frequency as well as an increase in mutation yield, its response was also considered as positive (+).

(3) No increase in mutation frequency or yield, or any response that was inconsistent, e.g., a sporadic increase in frequency without any relation to dose, was regarded as negative (−).

Results and discussion

Log-phase cells of two strains of yeast, D7-144 and XV185-14C, were tested with the chemicals in buffer at pH 7.0 or in synthetic complete medium at pH 4.1. A summary of the results is shown in Table 1; the detailed data are presented in Tables 3, 4 and 5. It can be seen that all compounds except benzoin were positive in one test or another.

When the log-phase cells of these strains, as well as strain RM52 and an additional genetic endpoint in strain XV185-14C, were tested in the presence of the chemicals in the standard growth medium (yeast extract and peptone at pH 6.3), the chemicals reacted much more strongly with the yeast (Table 2). All of the chemicals, including benzoin, responded in one test or another. Table 2 is a summary of the detailed data presentation in Tables 6, 7 and 8.

There are two points worth noting: First, the noncarcinogens, caprolactam and benzoin were mutagenic in strains D7-144 and XV185-14C but neither was mutagenic in strain RM52. It is important to note that benzoin may indeed be a weak carcinogen that needs further testing. Moreover, both benzoin and caprolactam have been positive in other short-term assays besides the yeast assays. Thus, the efficacy of yeast as an efficient and simple short-term assay should not be denigrated because it yielded a false positive in this study. Indeed, most noncarcinogenic compounds tested with the yeast assays are negative, thus a positive found with the yeast system should not be regarded as an invalidation of the system.

TABLE 4

GENETIC ACTIVITY OF BENZENE, DIETHYLHEXYLPHTHALATE, AND BENZOIN IN STRAINS XV185-14C AND D7-144 OF *Saccharomyces cerevisiae* [a]

Chemical		S9	Survival (%)	Strain XV185-14C			Strain D7-144		
				Revertant $\times 10^{-7}$			Survival (%)	Convertant $\times 10^{-5}$	
				His^+				Trp^+	
DMSO [b]	Control	–	100	6.5 ± 1.0	(17) [c]	40.0 ± 2.1 (105)	100	0.79 ± 0.08	(458)
DMSO + 1% Tween 80	Control [d]	–	100	8.3 ± 3.8	(15)	51.8 ± 4.1 (94)	100	0.62 ± 0.03	(433)
BEN	274.7	–	96	5.2 ± 1.2	(13)	39.0 ± 2.5 (98)	88	0.80 ± 0.03	(496)
	549.4	–	56	11.0 ± 1.9	(16)	28.9 ± 8.7 (42)	67	0.78 ± 0.03	(368)
	1098.8	–	6	33.3 ± 8.3	(4) [e]	33.3 ± 16.7 (4)	20	0.94 ± 0.15	(130)
	2197.5	–	0.03	0	(0)	0 (0)	0.01	13.30 ± 13.30	(1)
	Mutagenic response			–		–		–	
DEHP	1541	–	149	7.4 ± 0.9	(20)	64.7 ± 6.5 (175)	85	1.13 ± 0.07	(552)
	3081	–	132	9.6 ± 1.4	(23)	66.7 ± 7.8 (160)	83	1.08 ± 0.05	(518)
	6163	–	136	4.1 ± 1.0	(10)	51.0 ± 12.4 (126)	73	1.11 ± 0.03	(470)
	12325	–	132	6.7 ± 2.6	(16)	49.2 ± 5.4 (118)	73	0.98 ± 0.10	(415)
	Mutagenic response			–		–		–	
ZOIN	125	–	105	4.7 ± 0.4	(13)	57.2 ± 2.6 (157)	99	0.79 ± 0.03	(548)
	250	–	102	3.0 ± 1.1	(8)	27.7 ± 2.3 (74)	88	0.78 ± 0.04	(480)
	625	–	92	7.1 ± 2.8	(17)	27.1 ± 8.7 (65)	80	0.84 ± 0.03	(473)
	1250	–	76	7.0 ± 1.9	(14)	49.2 ± 4.5 (98)	77	0.94 ± 0.10	(505)
	Mutagenic response			–		–		–	
DMSO	Control	+	100	3.7 ± 0.9	(13)	65.4 ± 12.1 (228)	100	0.70 ± 0.04	(423)

DMSO + 1% Tween 80	Control	+	100	4.9 ± 0.7 (18)	50.2 ± 5.5 (86)	100	0.67 ± 0.03 (379)
BEN	274.7	+	99	3.2 ± 1.2 (11)	49.6 ± 10.3 (171)	158	0.57 ± 0.07 (516)
	549.4	+	50	11.6 ± 3.8 (20	47.5 ± 7.8 (82)	105	0.84 ± 0.01 (502)
	1098.8	+	0.2	366.3 ± 183.2 (2)	549.5 ± 317.2 (3)	3	1.18 ± 0.03 (18)
	2197.5	+	0.1	0 (0)	0 (0)	0	0 (0)
	Mutagenic response			+	−		−
DEHP	1541	+	102	35.3 ± 1.5 (133)	72.2 ± 8.5 (272)	112	0.81 ± 0.08 (548)
	3081	+	72	6.0 ± 1.6 (16)	76.6 ± 7.2 (204)	94	0.88 ± 0.09 (503)
	6163	+	71	7.3 ± 2.2 (19)	60.0 ± 11.0 (157)	89	0.80 ± 0.02 (430)
	12325	+	67	13.0 ± 1.3 (32)	55.6 ± 3.5 (137)	70	1.17 ± 0.07 (493)
	Mutagenic response			−	+		−
ZOIN	125	+	81	6.8 ± 1.1 (19)	39.9 ± 8.1 (112)	122	0.67 ± 0.05 (462)
	250	+	70	10.3 ± 1.4 (25)	75.8 ± 9.6 (185)	119	0.75 ± 0.05 (507)
	625	+	69	5.4 ± 1.3 (13)	88.5 ± 9.1 (213)	116	0.62 ± 0.04 (409)
	1250	+	63	4.5 ± 0.9 (10)	84.2 ± 11.5 (186)	118	0.69 ± 0.03 (461)
	Mutagenic response			−	−		−
EMS	Control	−	24	11551.3 ± 406.7 (7208)	769.2 ± 18.3 (480)	6	118.39 ± 2.35 (5020)
CP	Control	−	82	54.6 ± 4.7 (118)	36.6 ± 2.9 (79)	94	7.07 ± 0.35 (4664)
CP	Control	+	78	202.4 ± 13.7 (552)	101.2 ± 6.2 (276)	79	34.28 ± 2.00 (16346)

[a] Cells harvested at logarithmic phase of growth were treated with the test compound in phosphate buffer pH 7.0 for 17 h.
[b] *Abbreviations*: DMSO, dimethyl sulfoxide; EMS, ethyl methanesulfonate; CP, cyclophosphamide.
[c] Figures in parentheses are total number of colonies counted.
[d] This control applies to DEHP treatment.
[e] Underlined figures indicate a positive response.

TABLE 5

INDUCTION OF GENETIC ACTIVITY BY ACRYLONITRILE, SAFROLE, *o*-TOLUIDINE, CAPROLACTAM, PHENOBARBITONE AND HEXAMETHYLPHOSPHORAMIDE IN STRAIN XV185-14C OF *Saccharomyces cerevisiae* [a]

Chemical	Concentration (μg/ml)	Survival %		Revertants per 10^7 survival			
				HIS$^+$		TRP$^+$	
		−S9	+S9	−S9	+S9	−S9	+S9
DMSO [b]	Control	100	100	2.8 ± 1.54 (4) [c]	11.7 ± 1.96 (9)	15.7 ± 3.95 (17)	80.7 ± 9.25 (62)
ACN	0.8	106	109	8.5 ± 1.55 (13)	11.9 ± 3.59 (10)	25.5 ± 4.32 (39) [d]	10.7 ± 2.81 (9)
	8.1	97	121	2.1 ± 0.56 (3)	12.9 ± 2.40 (12)	2.9 ± 1.30 (4)	97.0 ± 11.13 (90)
	80.6	81	102	5.1 ± 1.74 (6)	3.8 ± 1.00 (3)	104.5 ± 7.09 (122)	25.5 ± 1.64 (20)
	806.0	72	93	0 ± 0.0 (0)	11.2 ± 0.00 (8)	56.7 ± 7.55 (59)	16.9 ± 2.55 (12)
	Mutagenic response			−	−	+	−
SAF	10.93	18	81	0 ± 0.0 (0)	3.2 ± 2.52 (2)	31.3 ± 14.20 (8)	51.3 ± 13.19 (32)
	21.92	10	57	6.9 ± 5.47 (1)	6.8 ± 3.43 (3)	0 ± 0.0 (0)	102.3 ± 7.94 (45)
	43.84	6	26	11.4 ± 8.95 (1)	5.0 ± 3.94 (1)	0 ± 0.0 (0)	160.0 ± 45.15 (24)
	87.68	1	7	0 ± 0.0 (0)	0 ± 0.0 (00)	62.5 ± 9.20 (1)	0 ± 0.0 (0)
	Mutagenic response			−	−	−	−
TOL	378	11	60	0 ± 0.0 (0)	10.8 ± 6.42 (5)	31.3 ± 9.42 (5)	12.9 ± 5.88 (6)
	756	9	20	15.6 ± 12.30 (2)	0 ± 0.0 (0)	23.4 ± 11.78 (3)	13.2 ± 10.36 (2)
	1512	9	17	0 ± 0.0 (0)	25.0 ± 6.56 (3)	15.6 ± 12.30 (2)	8.3 ± 6.56 (1)
	3024	6	9	0 ± 0.0 (0)	13.9 ± 10.94 (1)	11.4 ± 8.95 (1)	0 ± 0.0 (0)
	Mutagenic response			−	−	−	−
CAP	100	55	89	11.3 ± 0.99 (9)	14.7 ± 4.01 (10)	6.3 ± 2.48 (5)	148.5 ± 6.38 (101)
	200	47	78	11.8 ± 2.67 (8)	1.7 ± 1.31 (1)	83.8 ± 11.56 (57)	51.7 ± 1.60 (31)
	400	20	54	3.4 ± 2.66 (1)	0 ± 0.0 (0)	10.1 ± 2.66 (3)	2.4 ± 1.89 (1)
	800	8	28	25.0 ± 12.56 (5)	0 ± 0.0 (0)	41.7 ± 12.64 (5)	37.0 ± 12.36 (8)
	Mutagenic response			+	−	+	−
PB	250	51	120	5.4 ± 1.75 (4)	9.8 ± 1.64 (9)	12.2 ± 3.21 (9)	69.6 ± 9.78 (64)
	500	45	112	22.9 ± 5.67 (15)	22.0 ± 4.55 (19)	130.1 ± 9.42 (64)	268.5 ± 18.65 (232)
	1000	36	109	9.5 ± 3.75 (5)	3.6 ± 1.80 (3)	20.2 ± 5.85 (8)	209.5 ± 23.16 (176)
	2000	48	112	26.2 ± 2.38 (18)	2.4 ± 0.91 (1)	16.0 ± 2.70 (11)	291.6 ± 12.50 (126)
	Mutagenic response			+	−	+	+

EMS	Positive control	0.55	–	31625 ±2029 (253)	–	1750 ±113.64 (7)	–
CP	Positive control	134	135	19.4± 2.18 (11)	50.8± 3.50 (39)	19.4± 1.41 (11)	52.3 ± 5.74 (59)
DMSO	Control	100	100	20.7± 2.56 (22)	25.0± 2.51 (45)	9.4± 3.91 (10)	5.6± 1.13 (10)
HMPA	5140	186	83	22.3± 3.11 (44)	43.7± 4.84 (65)	6.1± 1.13 (12)	6.7 ± 2.53 (10)
	10270	121	76	54.5± 7.64 (70)	31.6± 3.05 (43)	9.3± 3.15 (12)	11.0 ± 1.46 (15)
	20540	121	86	38.8± 4.74 (50)	45.3± 2.70 (70)	8.5± 1.17 (11)	17.5± 0.98 (27)
	41080	120	83	52.7± 3.10 (67)	41.7± 3.51 (62)	8.6± 2.55 (11)	6.7 ± 1.37 (10)
	Mutagenic response			+	–	–	+
SAF	10.93	119	94	25.3± 2.69 (32)	29.6± 1.20 (50)	7.9± 2.39 (10)	4.2 ± 0.89 (7)
	21.92	70	76	48.4± 7.53 (36)	30.7± 2.90 (42)	12.1± 4.36 (9)	11.7 ± 0.94 (16)
	43.84	73	52	32.2± 3.46 (25)	38.8± 1.39 (36)	12.9± 2.62 (10)	14.0 ± 2.55 (13)
	87.68	12	52	15.6± 12.30 (2)	33.1± 5.21 (31)	7.8± 6.15 (1)	9.6 ± 1.62 (9)
	Mutagenic response			–	–	–	+
PB	250	97	79	27.1± 4.82 (28)	44.9± 3.25 (64)	11.6± 4.82 (12)	10.5 ± 1.39 (15)
	500	92	74	29.7± 4.44 (29)	25.5± 2.04 (34)	16.4± 2.95 (16)	9.7 ± 2.43 (13)
	1000	93	75	34.3± 4.94 (34)	30.5± 4.10 (41)	6.1± 0.92 (6)	11.9 ± 1.66 (16)
	2000	91	63	52.7± 9.24 (51)	38.7± 3.92 (44)	8.3± 2.30 (8)	24.7 ± 1.96 (28)
	Mutagenic response			+	–	–	+ +
EMS	Positive control	1	–	42063 ±1955 (673)	–	2250 ±385 (36)	–
CP	Positive control	117	77	44.1± 4.42 (55)	49.7± 5.04 (70)	2.4± 1.21 (3)	9.21± 1.95 (13)

[a] Cells growing in synthetic complete (pH 4.1) were treated for 17 h at 30 °C.

[b] *Abbreviations*: DMSO, dimethyl sulfoxide; EMS, ethyl methanesulfate; CP, cyclophosphamide.

[c] Figures in parentheses represent total number of colonies scored.

[d] Underlined figures indicate a positive response.

TABLE 6

INDUCTION OF GENETIC ACTIVITY IN STRAIN D7-144 BY TREATMENT OF CELLS GROWING IN YEPD MEDIUM (pH 6.3)

Chemical	Concentration (μg/ml)	Survival %		Trp$^+$ convertants $\times 10^{-5}$		Mutagenic response	
		− S9	+ S9	− S9	+ S9	− S9	+ S9
Solvent (DMSO) [a]	Control	100	100	0.20 ± 0.06 (484) [b]	0.20 ± 0.05 (511)		
DMSO + 1% Tween 80 [c]	Control	100	100	0.42 ± 0.04 (502)	0.21 ± 0.03 (485)		
ACN	0.8	55	35	0.38 ± 0.07 (596) [d]	0.51 ± 0.07 (462)	+ +	+ +
	8.1	24	33	0.86 ± 0.06 (573)	0.62 ± 0.05 (530)		
	80.6	22	31	0.91 ± 0.02 (558)	0.81 ± 0.07 (661)		
	806.0	6	5	6.76 ± 0.08 (1225)	6.48 ± 0.05 (832)		
BEN	274.7	70	50	0.32 ± 0.06 (627)	0.62 ± 0.06 (816)	+	+
	549.4	72	51	0.30 ± 0.05 (604)	0.56 ± 0.12 (744)		
	1098.8	49	38	0.47 ± 0.04 (653)	0.76 ± 0.48 (760)		
	2197.5	45	32	0.47 ± 0.04 (609)	0.57 ± 0.02 (475)		
ZOIN	125	129	57	0.10 ± 0.02 (360)	0.20 ± 0.05 (511)	−	+ +
	250	107	43	0.12 ± 0.02 (368)	0.41 ± 0.05 (616)		
	625	113	36	0.10 ± 0.03 (324)	0.63 ± 0.06 (721)		
	1250	75	31	0.28 ± 0.05 (591)	0.54 ± 0.08 (510)		
CAP	100	78	76	0.39 ± 0.04 (867)	0.32 ± 0.04 (646)	+	+
	200	69	74	0.32 ± 0.07 (630)	0.31 ± 0.04 (610)		
	400	48	76	0.41 ± 0.06 (559)	0.23 ± 0.05 (454)		
	800	33	65	0.60 ± 0.04 (561)	0.40 ± 0.08 (685)		
DEHP	1541	75	53	0.54 ± 0.02 (491)	0.58 ± 0.04 (717)	+	+
	3081	72	50	0.80 ± 0.03 (690)	0.38 ± 0.05 (899)		
	6163	71	48	1.08 ± 0.05 (910)	0.69 ± 0.06 (768)		
	12325	63	48	0.86 ± 0.03 (644)	0.70 ± 0.05 (717)		
HMPA	5140	152	165	0.20 ± 0.07 (484)	0.15 ± 0.04 (635)	−	−
	10270	137	162	0.15 ± 0.06 (627)	0.17 ± 0.06 (721)		
	20540	119	142	0.26 ± 0.06 (997)	0.23 ± 0.04 (856)		
	41080	119	147	0.30 ± 0.10 (1005)	0.25 ± 0.03 (949)		
PB	250	57	70	0.33 ± 0.03 (538)	0.38 ± 0.03 (703)	+ +	+
	500	52	67	0.37 ± 0.03 (539)	0.32 ± 0.04 (567)		
	1000	54	56	0.52 ± 0.09 (800)	0.52 ± 0.06 (769)		
	2000	49	54	0.58 ± 0.07 (805)	0.47 ± 0.05 (665)		
SAF	10.96	127	35	0.14 ± 0.04 (496)	0.57 ± 0.04 (523)	+	+
	21.92	98	36	0.23 ± 0.04 (641)	0.59 ± 0.05 (562)		
	43.84	34	31	0.56 ± 0.06 (541)	0.54 ± 0.04 (445)		
	87.68	17	18	0.57 ± 0.05 (266)	0.72 ± 0.03 (337)		
TOL	378	90	33	0.19 ± 0.01 (483)	0.71 ± 0.05 (605)	−	+
	756	82	22	0.24 ± 0.02 (572)	0.92 ± 0.04 (540)		
	1512	53	14	0.34 ± 0.02 (515)	1.05 ± 0.03 (395)		
	3024	1	1	0.68 ± 0.09 (26)	1.11 ± 0.01 (36)		
CP	4000	71	81	0.41 ± 0.01 (820)	0.42 ± 0.05 (895)		
EMS	5840	0.06	−	474 ± 0.11 (569)	−		

[a] *Abbreviations*: DMSO, dimethyl sulfoxide; EMS, ethyl methanesulfonate; CP, cyclophosphamide.

[b] Figures in parentheses represent total number of colonies scored.

[c] This control applies to diethylhexylphthalate treatment.

[d] Underlined figures indicate at least a 2-fold increase in the mutation frequency over the control.

TABLE 7

INDUCTION OF GENETIC ACTIVITY IN STRAIN RM52 BY TREATMENT OF CELLS GROWING IN YEPD MEDIUM (pH 6.3)

Chemical	Concentration (μg/ml)	Survival %		His^+ revertants $\times 10^{-7}$		Mutagenic response	
		−S9	+S9	−S9	+S9	−S9	+S9
Solvent (DMSO) [a]	Control	100	100	1.62 ± 1.01 (65) [b]	1.94 ± 0.81 (71)		
DMSO + 1% Tween 80 [c]	Control	100	100	2.14 ± 0.91 (76)	7.00 ± 1.19 (185)		
ACN	0.8	80	75	3.93 ± 1.05 (122) [d]	1.35 ± 0.46 (37)	−	−
	8.1	67	64	2.14 ± 0.65 (56)	1.96 ± 0.73 (46)		
	80.6	61	62	3.00 ± 1.71 (71)	1.28 ± 0.80 (29)		
	806.0	53	49	2.64 ± 0.60 (54)	4.22 ± 1.12 (76)		
BEN	274.7	76	96	1.05 ± 0.88 (31)	3.65 ± 2.05 (128)	−	−
	549.4	74	81	1.36 ± 0.64 (39)	3.18 ± 1.19 (94)		
	1098.8	72	61	1.54 ± 0.86 (43)	0.95 ± 0.50 (21)		
	2197.5	73	48	1.61 ± 0.62 (46)	3.23 ± 0.55 (57)		
ZOIN	125	61	75	4.33 ± 1.03 (103)	0.91 ± 0.74 (25)	−	−
	250	59	75	2.04 ± 0.77 (47)	3.23 ± 0.89 (88)		
	500	53	70	3.32 ± 1.50 (69)	1.91 ± 0.69 (49)		
	1000	51	68	2.18 ± 0.86 (43)	2.10 ± 1.04 (52)		
CAP	100	99	74	0.83 ± 0.73 (32)	2.00 ± 0.59 (54)	−	−
	200	86	71	2.74 ± 1.13 (92)	1.32 ± 0.56 (34)		
	400	85	62	2.82 ± 1.35 (94)	3.30 ± 1.46 (75)		
	800	82	48	2.52 ± 0.69 (81)	1.66 ± 0.63 (29)		
DEHP	1541	68	99	2.91 ± 1.28 (70)	1.50 ± 1.01 (39)	+	−
	3081	60	87	2.19 ± 1.26 (47)	6.79 ± 1.43 (156)		
	6163	60	86	2.60 ± 0.72 (55)	5.41 ± 1.31 (123)		
	12325	61	82	4.65 ± 0.93 (100)	2.12 ± 0.80 (46)		
HMPA	5140	89	80	4.34 ± 1.79 (151)	3.64 ± 2.06 (106)	+ +	+
	10270	84	74	4.49 ± 1.52 (148)	5.93 ± 1.63 (160)		
	20540	70	61	8.42 ± 2.13 (231)	4.69 ± 0.82 (104)		
	41080	69	49	7.50 ± 1.66 (202)	3.49 ± 0.81 (63)		
PB	250	71	86	1.58 ± 0.78 (44)	2.36 ± 0.75 (74)	−	−
	500	71	64	1.38 ± 0.86 (38)	2.98 ± 1.01 (70)		
	1000	71	60	2.53 ± 0.53 (70)	2.59 ± 1.14 (57)		
	2000	58	49	2.17 ± 1.26 (49)	1.18 ± 0.53 (21)		
SAF	10.96	84	65	2.26 ± 0.88 (74)	2.28 ± 0.90 (54)	+	+ +
	21.92	73	59	3.38 ± 0.96 (96)	3.60 ± 0.94 (78)		
	43.84	59	47	3.34 ± 0.92 (77)	4.93 ± 1.22 (85)		
	87.68	24	34	2.35 ± 0.45 (22)	11.94 ± 1.29 (149)		
TOL	378	82	67	5.81 ± 1.19 (185)	5.28 ± 0.88 (130)	+	−
	756	58	67	6.32 ± 1.38 (144)	1.11 ± 0.78 (27)		
	1512	56	44	1.97 ± 0.68 (43)	3.00 ± 0.80 (48)		
	3024	7	4	1.55 ± 0.19 (4)	1.30 ± 0.75 (2)		
CP	4000	83	64	2.38 ± 2.00 (77)	41.10 ± 1.73 (958)		
EMS	5840	1	−	2.90×10^4 (8544)	−		

[a] *Abbreviations*: DMSO; dimethyl sulfoxide; EMS, ethyl methanesulfonate; CP, cyclophosphamide.

[b] Figures in parentheses represent total number of colonies scored.

[c] This control applies to diethylhexylphthalate treatment.

[d] Underlined igures show at least a 2-fold increase in the mutation frequency over the control.

TABLE 8

INDUCTION OF REVERSE MUTATIONS IN STRAIN XV185-14C WITH TREATMENT IN YEPD MEDIUM (pH 6.3)

Chemical	Conc. (μg/ml)	Survival %		Revertants per 10^7 survival									
				HIS$^+$		TRP$^+$		ARG$^+$					
		−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9				
Control A [a]		100	100	4.1 ± 0.63 (11) [b]	14.2 ± 1.32 (58)	21.2 ± 2.14 (57)	40.5 ± 5.14 (165)	3.7 ± 0.50 (10)	11.3 ± 1.26 (46)				
Control B [a]		100	100	6.5 ± 1.80 (19)	8.2 ± 1.31 (49)	17.5 ± 2.95 (51)	45.0 ± 2.27 (268)	3.1 ± 1.03 (9)	5.5 ± 0.25 (33)				
ACN	0.8	71	97	13.7 ± 1.85 (26) [d]	9.1 ± 2.08 (36)	63.0 ± 7.21 (120)	48.3 ± 3.71 (143)	18.9 ± 1.08 (36)	9.4 ± 1.11 (37)				
	8.1	57	67	20.7 ± 1.08 (70)	5.5 ± 1.03 (15)	38.0 ± 2.60 (44)	71.3 ± 5.84 (145)	14.9 ± 0.25 (23)	11.8 ± 0.41 (32)				
	80.6	4	34	(1)	12.2 ± 0.95 (17)	107.0 ± 0.58 (9)	118.8 ± 5.90 (124)	71.4 ± 1.22 (8)	3.6 ± 0.25 (5)				
	806.0	0.3	0.1	0 ± 0 (0)	0 ± 0 (0)	0 ± 0 (0)	(1)	(1)	(1)				
Mutagenic response				+ +	−	+	+	+	−				
BEN	274.7	144	51	14.0 ± 4.17 (54)	25.4 ± 1.80 (53)	61.1 ± 4.51 (177)	71.8 ± 2.22 (150)	17.9 ± 2.17 (69)	26.8 ± 2.08 (42)				
	549.2	95	49	10.2 ± 0.87 (26)	14.6 ± 2.29 (29)	64.1 ± 5.51 (123)	52.4 ± 2.65 (78)	18.4 ± 2.39 (47)	9.6 ± 1.11 (19)				
	1098.8	39	45	7.7 ± 0.71 (8)	47.6 ± 4.06 (88)	41.0 ± 0.33 (32)	46.9 ± 0.67 (65)	24.0 ± 2.17 (25)	16.8 ± 1.44 (31)				
	2197.5	3	15	(1)	8.1 ± 0.48 (5)	87.5 ± 0.63 (7)	32.5 ± 0.91 (20)	37.5 ± 0.48 (3)	9.7 ± 0.50 (6)				
Mutagenic response				+	+	+	−	+ +	−				
ZOIN	125	72	154	6.7 ± 1.31 (13)	5.3 ± 1.11 (33)	20.7 ± 1.53 (30)	65.5 ± 10.21 (309)	6.2 ± 0.41 (12)	3.2 ± 1.78 (20)				
	250	70	151	15.4 ± 0.75 (29)	3.4 ± 1.65 (21)	50.9 ± 2.65 (72)	63.4 ± 9.35 (293)	9.5 ± 0.96 (18)	6.8 ± 0.65 (42)				
	625	69	88	8.7 ± 2.12 (16)	16.7 ± 1.73 (60)	13.7 ± 0.67 (19)	60.1 ± 7.37 (162)	(1)	4.2 ± 0.25 (15)				
	1250	61	81	9.2 ± 0.48 (15)	15.7 ± 3.19 (52)	32.9 ± 2.50 (54)	40.5 ± 0.96 (134)	6.7 ± 0.48 (11)	10.0 ± 2.78 (33)				
Mutagenic response				+	−	−	−	−	−				
CAP	100	155	191	8.2 ± 1.32 (34)	4.4 ± 1.44 (34)	50.1 ± 4.73 (156)	54.6 ± 1.86 (319)	6.3 ± 0.87 (26)	11.4 ± 3.52 (89)				
	200	151	184	15.8 ± 2.12 (64)	3.8 ± 0.82 (28)	49.4 ± 1.53 (150)	42.8 ± 8.50 (240)	15.3 ± 2.63 (62)	13.6 ± 1.50 (102)				
	400	141	166	4.2 ± 1.22 (16)	3.7 ± 1.97 (25)	62.7 ± 0.88 (178)	54.9 ± 7.13 (278)	13.2 ± 2.72 (50)	23.7 ± 3.92 (160)				
	800	92	136	8.9 ± 1.26 (22)	11.8 ± 1.38 (65)	72.4 ± 1.80 (179)	58.7 ± 5.85 (324)	16.6 ± 0.75 (41)	25.2 ± 1.97 (139)				
Mutagenic response				+	−	+ +	−	+ +	+ +				
DEHP	1541	77	56	6.2 ± 0.50 (14)	17.6 ± 4.73 (59)	26.1 ± 1.76 (44)	81.4 ± 3.38 (205)	4.5 ± 0.65 (10)	11.3 ± 1.71 (38)				
	3081	60	54	8.0 ± 0.96 (14)	20.6 ± 1.19 (66)	33.3 ± 1.20 (44)	88.1 ± 8.19 (212)	10.2 ± 1.71 (18)	16.5 ± 1.65 (53)				
	6163	62	52	7.8 ± 1.19 (14)	7.5 ± 1.11 (23)	22.2 ± 0.58 (30)	92.4 ± 11.98 (214)	8.3 ± 0.63 (15)	16.8 ± 1.78 (52)				
	12325	52	53	13.7 ± 1.49 (21)	8.8 ± 0.91 (28)	20.9 ± 2.35 (32)	106.8 ± 3.03 (340)	(1)	22.0 ± 4.52 (70)				
Mutagenic response				+	+	−	+	+	+				

HMPA	5140	149	147	21.8 ± 1.18	(87)	4.3 ± 1.19	(26)	91.2 ±	7.55	(273)	73.2 ± 15.81	(329)	18.0 ± 1.78	(72)	4.8 ± 0.48	(29)
	10270	144	148	12.2 ± 0.48	(47)	9.8 ± 1.75	(59)	94.9 ±	7.86	(275)	115.2 ± 11.02	(519)	30.0 ± 1.00	(116)	12.8 ± 2.59	(77)
	20540	123	142	33.8 ± 2.80	(112)	26.2 ± 3.87	(152)	166.3 ±	13.45	(411)	105.7 ± 15.72	(459)	28.8 ± 0.75	(95)	17.4 ± 3.09	(101)
	41080	107	143	19.6 ± 3.12	(61)	5.3 ± 1.03	(31)	40.4 ±	4.21	(126)	127.9 ± 14.61	(748)	18.6 ± 1.04	(58)	27.1 ± 3.52	(158)
Mutagenic response				+		−		+ +			+ +		+ +		+	
PB	250	192	158	6.2 ± 2.04	(32)	3.1 ± 1.00	(20)	80.5 ±	8.69	(311)	61.3 ±	6.23 (295)	4.9 ± 0.63	(25)	20.9 ± 3.77	(134)
	500	184	141	3.2 ± 1.41	(16)	9.2 ± 0.63	(53)	25.4 ±	5.78	(94)	87.9 ±	8.21 (379)	20.0 ± 2.50	(99)	11.0 ± 1.03	(63)
	1000	185	140	9.0 ± 1.44	(45)	14.9 ± 2.14	(85)	23.3 ±	5.57	(87)	91.4 ±	12.66 (390)	5.0 ± 1.60	(25)	14.2 ± 1.76	(81)
	2000	181	139	9.9 ± 2.27	(48)	14.8 ± 1.91	(84)	84.7 ±	3.49	(412)	117.9 ±	4.18 (668)	16.5 ± 3.56	(80)	17.0 ± 1.68	(96)
Mutagenic response				+		−		+			+ +		+		−	
SAF	10.93	199	118	6.7 ± 1.22	(36)	10.8 ± 2.74	(52)	45.8 ±	1.45	(184)	90.0 ±	2.08 (324)	15.0 ± 0.91	(80)	11.3 ± 0.96	(54)
	21.92	165	92	5.4 ± 1.68	(24)	10.7 ± 2.12	(40)	44.3 ±	8.08	(147)	95.6 ±	1.76 (269)	11.1 ± 0.85	(49)	12.0 ± 2.87	(45)
	43.84	35	94	4.4 ± 0.63	(5)	8.1 ± 2.06	(31)	19.6 ±	0.67	(14)	94.8 ±	8.19 (273)	5.3 ± 0.63	(5)	9.1 ± 1.11	(35)
	87.68	3	10	0 ± 0	(0)	12.3 ± 0.25	(5)	27.8 ±	0.50	(2)	49.0 ±	1.78 (20)		(1)	7.4 ± 0.48	(3)
Mutagenic response				−		−		+			+		+		−	
TOL	378	47	88	15.7 ± 1.35	(20)	3.6 ± 0.95	(13)	75.5 ±	0.58	(72)	86.3 ±	9.84 (233)	23.8 ± 0.75	(19)	11.4 ± 2.06	(41)
	756	0.9	28	0 ± 0	(0)	16.8 ± 0.25	(19)			(1)	16.8 ±	2.33 (66)		(1)	3.6 ± 0.41	(4)
	1512	0.8	3	0 ± 0	(0)		(1)	0 ±	0	(0)	135.4 ±	1.33 (13)		(1)	15.6 ± 0.29	(2)
	3024	0.6	0.2	0 ± 0	(0)	0 ± 0	(0)	0 ±	0	(0)	0 ±	0 (0)		(1)	0 ± 0	(0)
Mutagenic response				+		−		+			+		+		−	
EMS [c]	5840	0.1	−	15833 ± 1.76	(19)			0 ±	0	(0)				(1)		
CP [c]	4000	90	112	10.0 ± 0.41	(24)	38.4 ± 6.87	(175)	53.2 ±	5.28	(128)	52.0 ±	5.20 (241)	8.7 ± 0.63	(21)	7.3 ± 1.31	(33)

[a] Control A is dimethyl sulfoxide (DMSO) and Control B is DMSO + 1% Tween 80; Control B applies to DEHP only.

[b] Figures in parentheses represent total number of colonies scored; a figure of less than 5 for the total number of colonies scored was not considered significant for evaluating mutagenic response.

[c] *Abbreviations*: EMS, ethyl methanesulfonate; CP, cyclophosphamide.

[d] Underlined figures indicate an increase in the induced mutation frequencies over the control values by a factor of 2.

Quite the contrary, it should be regarded as a warning that even though the compound under investigation may not be a carcinogen, it may be contaminated with a mutagen that is detectable by yeast.

Second, generally speaking, though the *his1-7* reversion assay was employed with both strains RM52 and XV185-14C, the results were different with the carcinogens and noncarcinogens tested in this study. For example, phenobarbital was not a mutagen with strain RM52, whereas it was a mutagen with strain XV185-14C when *his1-7* reversion was the criterion. Other instances can be found among the data. At this time we cannot tell whether the diploid repair is involved with strain RM52 for phenobarbital-induced lesions, whether the high level of cytochrome P-450 present in strain RM52 is responsible for the inactivation of the phenobarbital as a mutagen, or whether the lack of liquid-holding recovery in strain XV185-14C is responsible for the appearance of the *his1-7* reversions. These alternative explanations are testable.

Conclusion

The 3 yeast strains (D7-144, XV185-14C and RM52) used in this study of 7 carcinogens and 2 noncarcinogens exhibited their general usefulness as short-term assays, as well as demonstrating the special characteristics that make them useful as a series of yeast tests when particularly troublesome compounds require a thorough investigation. Strain XV185-14C is the most sensitive, and RM52 is the least sensitive of the assays.

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The induction of mitotic aneuploidy, point mutation and mitotic crossing-over in the yeast *Saccharomyces cerevisiae* strains D61-M and D6

James M. Parry and Friederike Eckardt [1]

Department of Genetics, University College of Swansea, Swansea (Great Britain) and [1] Gesellschaft fur Strahlen- und Umweltforschung, Abteilung Strahlenbiologie, Neuherberg (Federal Republic of Germany)

Summary

The 10 test chemicals were evaluated for their ability to induce mitotic chromosome aneuploidy, mitotic recombination and point mutation in two strains of yeast using a variety of protocols. The results obtained were evaluated for their ability to induce the 3 endpoints by determination of integral yields and the levels of plate increases produced.

HMPA, TOL, PB, SAF, ACN, BEN, DES and DEHP all produced increases in mitotic aneuploidy, HMPA and ACN were capable of inducing point mutation and ACN gave positive results for the induction of mitotic recombination.

The diploid yeast cultures D6 and D61-M have been developed as screening systems for the detection of induced mitotic chromosome aneuploidy (Parry and Zimmermann, 1976; Zimmermann, personal communication). Monosomic cells ($2n - 1$) produced by chromosome aneuploidy are detected in these strains by the expression of recessive markers that result from the loss of one of a pair of homologous chromosomes carrying a series of linked genetic markers in a heterozygous condition. Monosomic colonies are detectable by their white colony colour and their resistance to cycloheximide, in contrast to the red colony colour and sensitivity to cycloheximide of the original diploid D6 and D61-M cultures.

In addition to their ability to detect the induction of mitotic aneuploidy the two strains may also be used to assay the induction of both mitotic crossing-over and point mutation. In both strains mitotic crossing-over may be assayed by the production of homozygous cychoheximide-resistant colonies produced by crossing-over in the right arm of chromosome VII. In strain D6 point mutation may be assayed by the production of reversions at the homozygous adenine-2 locus and in strain D61-M by the production of reversions at the homozygous isoleucine-1 locus.

A number of testing protocols were utilized to evaluate the profile of genotoxicity produced by the 10 test compounds. These involved the use of stationary-phase and log-phase cells, the presence and absence of an exogenous source of liver microsomes and cofactors and a protocol optimised for the presence of maximal levels of endogenous yeast cytochrome P-450 (Kelly and Parry, 1983).

Materials and methods

Strains

The relevant genetic markers of strains D6 and D61-M are shown below:
D6

$$\text{Chromosome XV}\ \frac{\text{ade 2-40}}{\text{ade 2-40}}$$

$$\text{Chromosome VII}\ \frac{\text{ade 3}}{+}\ \text{centromere}\ \frac{\text{leu}_1\ \ \text{trp}_2\ \ \text{cyh}^r_2\ \ \text{met13}}{+\ \ +\ \ \text{CYH}^S_2\ \ +}$$

D61-M

Chromosome XV $\frac{ade\ 2\text{-}40}{ade\ 2\text{-}40}$

Chromosome VII $\frac{ade\ 6}{+}$ centromere $\frac{leu_1\ +\ cyh^r_2}{+\ trp_2\ CYH^S_2}$

Chromosome V $\frac{ilv\ 1\text{-}92}{ilv\ 1\text{-}92}$

Both strains carry a series of recessive markers on chromosome VII. They produce red colonies on high glucose complete medium due to the presence of defective alleles *ade 2-40* of the gene adenine-2 in a homozygous condition on chromosome XV, require adenine for growth, and are sensitive to the presence of 2 μg/ml of cycloheximide in the growth medium. The loss of a copy of chromosome VII carrying the wild-type markers results in the production of cells carrying only the recessive and mutant alleles shown on chromosome VII. The resulting monosomic cells ($2n - 1$) are white in colour due to the presence of the defective allele of the gene adenine-3 in the case of D6 and adenine-6 in the case of D61-M in the left arm of chromosome VII, which result in a mutant block in the adenine biosynthetic pathway prior to the adenine-2 gene, thus preventing the formation of the red pigment characteristic of colonies carrying adenine-2 mutations.

The expression in monosomic colonies of the recessive form of cycloheximide-2 carried by the right arm of chromosome VII results in the production of colonies resistant to the presence of 2 μg/ml cycloheximide in the growth medium.

Representative samples of presumptive monosomic colonies were tested for the expression of the mutant alleles carried on chromosome VII by plating upon appropriate selective medium.

The frequencies of mitotic crossing-over in both D6 and D61-M were measured by the detection of red cycloheximide-resistant colonies on growth medium containing 2 μg/ml of cycloheximide. Such colonies are produced by crossing-over in the right arm of chromosome VII between its centromere and the cycloheximide-2 locus.

Point mutation in strain D6 was assayed by the detection of adenine independent prototrophs produced by reversion of the *adenine 2-40* mutation carried on chromosome XV. Such colonies were detected by plating on adenine-deficient minimal media. Point mutation in strain D61-M was assayed by the detection of isoleucine-independent prototrophs produced by reversion of the *isoleucine 1-92* mutation carried on chromosome V. Such colonies were detected by plating on isoleucine-deficient minimal media.

Treatment protocol

In Expts. 2, 3 and 4–5 the test compounds were assayed using a treatment developed to optimise the endogenous levels of cytochrome P-450 in yeast (Kelly and Parry, 1983). In this protocol, cells were inoculated into liquid yeast complete medium (0.5% glucose) and grown on an orbital shaker at 28°C for 2 days. Cells were washed and resuspended in liquid yeast complete medium (20% glucose) at a cell concentration of 5×10^6 cells/ml. Under these conditions yeast cells produce their maximal levels of cytochrome P-450 in approximately 6 h and maintain these levels for a period of approximately 24 h.

In Expts. 3 and 4–3, the chemicals were added to the cells at the time of inoculation into high glucose medium, whereas in Expts. 2 and 4–5 the cells were allowed to grow for 4 h before the introduction of the test chemicals. In Expt. 4–3 the culture in high glucose was initiated from cells derived from a stationary phase culture from 3-day-old cultures from solid medium. In Expts. 4–3 and 4–5 the cells were exposed to the test chemical both in the presence and the absence of Aroclor-induced rat-liver S9 mix as an exogenous activation mix. These samples were prepared as described by Parry and Sharp (1981).

After the introduction of the test chemicals all cultures were grown for 18 h on an orbital shaker at 28°C in sealed bottles. In the case of ACN, CAP and DEHP the sealed bottles were made of glass, while the other 7 chemicals were tested in plastic bottles. After treatment, cells were washed 3 times in saline and after appropriate dilution plated upon selective medium to detect cell viability, mitotic aneuploidy, point mutation and mitotic crossing-over.

All cultures were examined after treatment for the presence of spore tetrads indicative of sporulation. In none of the experiments was there any indication of meiosis and cell sporulation.

Chemicals

The following chemicals supplied by the IPCS programme via Lancaster Synthesis were tested for their ability to induce mitotic aneuploidy, point mutation and mitotic segregation in strains D6 and D61-M:

Acrylonitrile (ACN), *o*-toluidine (TOL), phenobarbital (PB), safrole (SAF), benzene (BEN), diethylstilboestrol (DES), caprolactam (CAP), benzoin (ZOIN), diethylhexylphthalate (DEHP), and hexamethylphosphoramide (HMPA). 8 of the chemicals were prepared by dissolving them in dimethyl sulphoxide (DMSO) prior to the preparation of aqueous concentration ranges of up to 5000 μg/ml. DES was prepared in 95% ethanol and DEHP was prepared in 99% DMSO plus 1% Tween 80.

Results

The range of experiments performed with the various test chemicals are illustrated in Table 1. Representative samples of presumptive monosomic colonies (i.e. white and cycloheximide-resistant) were tested for the expression of the recessive auxotrophic markers on chromosome VII.

Tables 2 and 3 illustrate the results obtained in experiment 2 for strain D6 and in Expts. 3, 4–3, and 4–5 for strain D61-M. The results demonstrate that 77.8% of the presumptive monosomies in D6 and 86% in D61-M expressed such markers, thus indicating that the selective procedure utilized here did in fact result mainly in the detection of monosomic ($2n - 1$) colonies.

The data derived from the individual experiments indicate some variation in the response of the chemicals in their ability to induce the various

TABLE 1

SUMMARY OF EXPERIMENTS WITH 10 TEST COMPOUNDS

Expt. No.	Exogenous S9 source	Strain	Initial culture	Chemical addition	Post chemical	Positive control chemical
2–1	–	D6	2 days low glucose (0.5%)	4 h in high glucose	High glucose medium (20%)	Cyclophosphamide 50 μg/ml
3	–	D61-M	2 days low glucose (0.5%)	0 time in high glucose	High glucose medium (20%)	Chenodeoxycholic acid 250 and 500 μg/ml
4–3	–	D61-M	3 days stationary cells from solid medium	0 time in 4% glucose medium	4% Glucose medium	
	+	D61-M	3 days stationary cells from medium	0 time in 4% glucose medium	4% Glucose medium	Cyclophosphamide 50 μg/ml
4–5	–	D61-M	2 days low glucose (0.5%)	4 h in high glucose medium	High glucose medium (20%)	
	+	D61-M	2 days low glucose (0.5%)	4 h in high glucose medium	High glucose medium (20%)	Cyclophosphamide 50 μg/ml

TABLE 2

Expt. 2-1 D6 HIGH GLUCOSE

Test for phenotype of white cycloheximide-resistant colonies [a]

	Number of white Cyc^r colonies tested	Number of colonies requiring leucine and tryptophan
Overall	544	423
Control (pooled colonies from 3 Expts.)	53	41
Cyclophosphamide	32	22
ACN	54	43
BEN	41	31
CAP	16	13
DEHP	61	45
DES	68	50
HMPA	37	21
PB	65	47
SAF	40	29
TOL	75	58
ZOIN	29	23

[a] For test chemicals, white Cyc^r colonies were pooled from a range of treatment concentrations. Only colonies remaining white on YC medium after selection were tested.

Overall frequency of $2n-1$ colonies out of total white Cyc^r = 77.8%.

TABLE 3

Expts. 3, 4-3 AND 4-5 HIGH GLUCOSE D61-M

Test for phenotype of white cycloheximide-resistant colonies [a]

	Number of white Cyc^r colonies	Number of colonies requiring leucine
Overall	664	571
Control pooled for 3 Expts.	49	42
Cyclophosphamide	64	58
Chenodeoxycholic acid	55	47
ACN	41	36
BEN	68	61
CAP	32	20
DEHP	60	51
DES	47	42
HMPA	72	63
PB	51	39
SAF	56	51
TOL	59	48
ZOIN	64	39

[a] For test chemicals white Cyc^r colonies were pooled from a range of treatment concentrations. Only colonies remaining white on YC medium after selection were tested for leucine requirement.

Overall frequency of $2n-1$ colonies out of total white Cyc^r = 86%.

TABLE 4

INTEGRAL YIELDS OBTAINED IN 6 Expts. [a]

Expt. No.	Strain	±S9	Cell stage	Maximum lethal hits	Aneuploidy $\times 10^{-6}$	Mitotic segregation cyh^r $\times 10^{-4}$	Point mutation adenine$^+$ $\times 10^{-6}$	Point mutation isoleucine$^+$ $\times 10^{-6}$
(a) SAF								
2-1	D6	−	High glucose	1.33	4.4 (+)	1.27 (−)	0.36 (−)	
3	D61-M	−	High glucose	2.0	0.82 (−)	7.9 (−)		0.11 (−)
4-3	D61-M	−	Stationary	2.0	1.52 (+)	7.4 (−)		1.27 (−)
4-3	D61-M	+	Stationary	0.65	4.38 (+)	16.6 (−)		1.06 (±)
4-5	D61-M	−	High glucose	1.0	1.34 (−)	23.3 (±)		1.05 (+)
4-5	D61-M	+	High glucose	0.7	0.52 (+)	14.0 (±)		0.61 (+)
(b) ACN								
2-1	D6	−	High glucose	3.7	9.6 (+)	4.52 (+)	0.34 (+)	
3	D61-M	−	High glucose	2.0	2.76 (+)	7.3 (−)		0.29 (−)

TABLE 4 (continued)

Expt. No.	Strain	± S9	Cell stage	Maximum lethal hits	Aneuploidy $\times 10^{-6}$	Mitotic segregation $cyh^r \times 10^{-4}$	Point mutation adenine$^+$ $\times 10^{-6}$	Point mutation isoleucine$^+$ $\times 10^{-6}$
4–3	D61-M	–	Stationary	1.17	4.15 (+)	12.9 (±)		0.97 (+)
4–3	D61-M	+	Stationary	0.62	5.09 (+)	17.6 (+)		1.35 (+)
4–5	D61-M	–	High glucose	1.0	0.56 (+)	23.5 (+)		0.53 (+)
4–5	D61-M	+	High glucose	1.0	1.96 (+)	40.6 (+)		0.83 (±)
(c) BEN								
2–1	D6	–	High glucose	1.76	4.6 (+)	9.0 (+)	4.0 (–)	
3	D61-M	–	High glucose	1.2	2.8 (+)	3.5 (–)		0.36 (–)
4–3	D61-M	–	Stationary	0.74	3.0 (±)	15.8 (–)		0.76 (–)
4–3	D61-M	+	Stationary	1.0	11.8 (+)	11.0 (–)		0.93 (±)
4–5	D61-M	–	High glucose	1.0	0 (–)	8.3 (–)		0.99 (+)
4–5	D61-M	+	High glucose	1.0	0 (–)	28.8 (±)		1.19 (±)
(d) ZOIN								
2–1	D6	–	High glucose	1.23	0.17 (–)	2.0 (–)	0.06 (–)	
3	D61-M	–	High glucose	0.9	3.4 (+)	9.1 (+)		0.44 (+)
4–3	D61-M	–	Stationary	1.93	– (–)	2.6 (–)		– (–)
4–3	D61-M	+	Stationary	2.50	8.7 (+)	0.3 (–)		1.6 (–)
4–5	D61-M	–	High glucose	0.88	– (–)	– (–)		– (–)
4–5	D61-M	+	High glucose	0.49	– (–)	– (–)		0.3 (–)
(e) DES								
2–1	D6	–	High glucose	1.86	3.26 (+)	0.44 (–)	– (–)	
3	D61-M	–	High glucose	1.12	1.2 (±)	3.3 (–)		0.57 (±)
4–3	D61-M	–	Stationary	1.69	15.4 (+)	– (–)		– (–)
4–3	D61-M	+	Stationary	0.09	– (–)	– (–)		– (–)
4–5	D61-M	–	High glucose	2.69	8.25 (+)	3.3 (–)		– (–)
4–5	D61-M	+	High glucose	0.68	1.9 (+)	– (–)		– (–)
(f) TOL								
2–1	D6	–	High glucose	0.94	6.18 (+)	1.64 (–)	1.0 (–)	
3	D61-M	–	High glucose	1.0	1.6 (+)	8.4 (+)		0.28 (+)
4–3	D61-M	–	Stationary	2.2	10.2 (+)	10.1 (–)		1.83 (+)
4–3	D61-M	+	Stationary	1.8	12.3 (+)	23.8 (–)		1.61 (–)
4–5	D61-M	–	High glucose	2.3	5.1 (±)	10.1 (–)		1.1 (–)
4–5	D61-M	+	High glucose	0.75	0.68 (±)	11.3 (–)		0.09 (–)

TABLE 4 (continued)

Expt. No.	Strain	± S9	Cell stage	Maximum lethal hits	Aneuploidy $\times 10^{-6}$	Mitotic segregation $cyh^r \times 10^{-4}$	Point mutation $adenine^+ \times 10^{-6}$	Point mutation $isoleucine^+ \times 10^{-6}$
(g) PB								
2–1	D6	–	High glucose	1.12	3.42 (+)	3.6 (–)	1.25 (–)	
3	D61-M	–	High glucose	1.44	3.35 (+)	10.1 (±)		0.75 (+)
4–3	D61-M	–	Stationary	1.43	1.15 (–)	9.3 (–)		0.59 (–)
4–3	D61-M	+	Stationary	2.23	5.68 (+)	6.2 (–)		0.92 (–)
4–5	D61-M	–	High glucose	1.13	0.8 (–)	43.5 (+)		1.07 (+)
4–5	D61-M	+	High glucose	0.88	0.38 (–)	15.3 (–)		0.91 (–)
(h) DEHP								
2–1	D6	–	High glucose	1.15	7.9 (+)	5.9 (–)	3.84 (–)	
3	D61-M	–	High glucose	0.76	1.26 (+)	1.8 (–)		0.18 (–)
4–3	D61-M	–	Stationary	1.85	2.9 (+)	4.5 (–)		0.84 (–)
4–3	D61-M	+	Stationary	1.25	3.0 (+)	3.9 (–)		0.43 (–)
4–5	D61-M	–	High glucose	0.76	0.32 (–)	10.3 (±)		0.42 (+)
4–5	D61-M	+	High glucose	2.14	5.49 (+)	36.3 (–)		3.25 (+)
(j) CAP								
2–1	D6	–	High glucose	0.62	– (–)	– (–)	– (–)	
3	D61-M	–	High glucose	0.69	0.16 (–)	– (–)		0.06 (–)
4–3	D61-M	–	Stationary	0	– (–)	– (–)		– (–)
4–3	D61-M	+	Stationary	0.3	0.1 (–)	– (–)		– (–)
4–5	D61-M	–	High glucose	0.1	– (–)	– (–)		– (–)
4–5	D61-M	+	High glucose	0.71	0.24 (–)	1.3 (–)		0.19 (–)
(k) HMPA								
2–1	D6	–	High glucose	0.59	2.2 (–)	0 (–)	1.64 (–)	
3	D61-M	–	High glucose	1.0	2.96 (–)	8.3 (+)		0.27 (–)
4–3	D61-M	–	Stationary	0.5	0.37 (+)	4.4 (–)		0.77 (+)
4–3	D61-M	+	Stationary	0.8	3.68 (+)	15.0 (–)		1.95 (+)
4–5	D61-M	–	High glucose	0.5	0.90 (–)	0.65 (+)		0.65 (+)
4–5	D61-M	+	High glucose	0.32	0.93 (+)	0.45 (±)		0.45 (±)

[a] Classification of data: +, significant (3 doses produce yields > background yield). ±, 2 doses produce > background yields. –, no induction, yields are smaller than background or only single dose produces yield > background.

endpoints. To avoid the problems of the use of potentially subjective criteria for the evaluation of the results we have analysed the data using the criteria of Eckardt and Haynes (1980), in which the level of induction of each endpoint has been compared on the basis of the lethal events induced at each test concentration.

For each genetic endpoint and dose the net induction of viable mutants (in this context used as a general term to cover all endpoints) the "mutant yield" defined as the induced mutants per treated cell was calculated. If these values are positive the test agent has some inducing capacity for the endpoint under consideration, whereas if the values are negative the agent is defined as being inactive for the endpoint under the experimental conditions being used. The integral under the yield curves plotted against lethal hits (negative logarithm of the surviving fraction) was determined for each test concentration and are shown in Table 4a–j.

Haynes and Eckardt (1979) have shown that the integral yield (which reflects the total number of viable mutants produced over the dose range of an agent) can be used for a quantitative comparison of the genetic activity of the agent under test provided the appropriate conditions are met. Positive numbers for the integral yields indicate that at least some doses of the test agent induce the particular genetic endpoint and a yield of zero

TABLE 5

COMPARISON OF ABILITY OF 10 TEST COMPOUNDS TO INDUCE MITOTIC ANEUPLOIDY, POINT MUTATION AND MITOTIC CROSSING-OVER IN 6 Expts.

Chemical	Aneuploidy	Mitotic crossing-over (cyh^r)	Point mutation
HMPA	3+, 3− } 3	1+, 1±, 4− } 1.5	3+, 1±, 2− } 3.5
TOL	4+, 2± } 5	1+, 5− } 1	2+, 4− } 2
PB	3+, 3− } 3	1+, 1±, 4− } 1.5	2+, 4− } 2
SAF	4+, 2− } 4	2±, 4− } 1	2+, 1±, 3− } 2.5
ACN	5+, 1− } 5	4+, 1±, 1− } 4.5	4+, 1±, 1− } 4.5
BEN	3+, 1±, 2− } 3.5	1+, 1±, 4− } 1.5	1+, 2±, 3− } 2
ZOIN	2+, 4− } 2	1+, 5− } 1	1+, 5− } 1
CAP	6− 0	6− 0	6− 0
DES	4+, 1±, 2− } 4.5	6− 0	1±, 5− } 0.5
DEHP	5+, 1− } 5	1±, 5− } 0.5	2+, 4− } 2

TABLE 6

COMPARISON OF ABILITY OF 10 TEST COMPOUNDS TO INDUCE 3 GENETIC ENDPOINTS BASED ON ABILITY TO INDUCE GREATER THAN 2× INCREASE IN PLATE COUNTS

Expt. No.	Strain	± S9	Cell stage	Aneuploidy	Mitotic segregation (cyh^r)	Point mutation (adenine$^-$)	Point mutation (isoleucine$^-$)
HMPA							
2–1	D6	–	High glucose	+ +	–	–	
3	D61-M	–	High glucose	+ +	+ +		–
4–3	D61-M	–	Stationary	+ +	–		+ +
4–3	D61-M	+	Stationary	+	–		+ +
4–5	D61-M	–	High glucose	+ +	–		+ +
4–5	D61-M	+	High glucose	+ +	+ +		+ +
DES							
2–1	D6	–	High glucose	+	–	–	
3	D61-M	–	High glucose	+	–		–
4–3	D61-M	–	Stationary	+ +	–		–
4–3	D61-M	+	Stationary	+ +	–		–
4–5	D61-M	–	High glucose	+ +	–		–
4–5	D61-M	+	High glucose	+ +	–		–
CAP							
2–1	D6	–	High glucose	–	–	–	
3	D61-M	–	High	–	–		–
4–3	D61-M	–	Stationary	–	–		–
4–3	D61-M	+	Stationary	–	–		–
4–5	D61-M	–	High glucose	–	–		–
4–5	D61-M	+	High glucose	–	–		–
ZOIN							
2–1	D6	–	High glucose	–	–	–	
3	D61-M	–	High glucose	+ +	+ +		+ +
4–3	D61-M	–	Stationary	–	–		–
4–3	D61-M	+	Stationary	+ +	–		–
4–5	D61-M	–	High glucose	–	–		–
4–5	D61-M	+	High glucose	–	–		–
BEN							
2–1	D6	–	High glucose	+ +	+	–	
3	D61-M	–	High	+ +	–		–
4–3	D61-M	–	Stationary	–	–		–

TABLE 6 (continued)

Expt. No.	Strain	± S9	Cell stage	Aneuploidy	Mitotic segregation (cyh^r)	Point mutation (adenine$^+$)	Point mutation (isoleucine$^+$)
4–3	D61-M	+	Stationary	+ +	–		–
4–5	D61-M	–	High glucose	–	–		+
4–5	D61-M	+	High glucose	–	–		+
DEHP							
2–1	D6	–	High glucose	+ +	–	–	
3	D61-M	–	High glucose	+	–		–
4–3	D61-M	–	Stationary	–	–		–
4–3	D61-M	+	Stationary	+	–		–
4–5	D61-M	–	High glucose	+	–		+ +
4–5	D61-M	+	High glucose	+ +	–		–
ACN							
2–1	D6	–	High glucose	+ +	–	+ +	
3	D61-M	–	High glucose	+	–		–
4–3	D61-M	–	Stationary	+ +	–		–
4–3	D61-M	+	Stationary	+ +	–		+ +
4–5	D61-M	–	High glucose	–	+ +		+ +
4–5	D61-M	+	High glucose	+ +	+		+
TOL							
2–1	D6	–	High glucose	+ +	–	–	
3	D61-M	–	High glucose	+ +	+		+ +
4–3	D61-M	–	Stationary	+ +	–		–
4–3	D61-M	+	Stationary	+ +	–		–
4–5	D61-M	–	High glucose	+	–		–
4–5	D61-M	+	High glucose	+	–		–
SAF							
2–1	D6	–	High glucose	+ +	–	–	
3	D61-M	–	High glucose	–	–		–
4–3	D61-M	–	Stationary	–	–		–
4–3	D61-M	+	Stationary	+ +	+ +		+ +
4–5	D61-M	–	High glucose	+	–		+
4–5	D61-M	+	High glucose	+	+ +		+ +

TABLE 6 (continued)

Expt. No.	Strain	± S9	Cell stage	Aneuploidy	Mitotic segregation (cyh^r)	Point mutation ($adenine^+$)	Point mutation ($isoleucine^+$)
PB							
2–1	D6	–	High glucose	+ +	–	–	
3	D61-M	–	High glucose	+ +	–		–
4–3	D61-M	–	Stationary	–	–		–
4–3	D61-M	+	Stationary	+ +	–		–
4–5	D61-M	–	High glucose	–	–		+ +
4–5	D61-M	+	High glucose	–	–		–

indicates no inducing capacity.

Because of the number of experiments performed on the IPCS chemicals and the experimental variation found (particularly for maximum toxicity) the limits of integration were not comparable. We therefore determined which doses produced integral yields at least as large as the background yield. In the case of the calculation of the integral yields we subtract the spontaneous background from the induced level and thus an integral yield of at least the background yield is equivalent to the concept of a doubling in the plate counts.

For each of the test chemicals we classified the results into 3 groups on the following basis:

(1) +, significant induction at 3 more doses that produce yields at least as large as the background yield.

(2) ±, 2 doses produce yields at least as large as background yield.

(3) −, no induction, or yields smaller than background, or a single dose produces yield above background.

For comparative purposes these results were given the following numerical values, where + = 1, ± = 0.5 and − = 0. The overall results obtained are illustrated in Table 5. Our criteria for a positive response in the induction of any genetic endpoint is a value of 3 in the experiments described in this paper.

A further comparison of the data was performed using the criteria of a positive result being defined by the ability of a chemical to produce a 2-fold increase in plate counts for each genetic endpoint. For this analysis the results were classified in the following manner:

(1) + +, 2 × increase in plate counts for at least 3 dose points.

(2) +, 2 × increase in plate counts for 2 dose points.

(3) −, no increase in plate counts at 2 × increase produced at only 1 dose point.

The results of all 5 studies analysed in this manner are shown in Table 6. For comparative purposes these results were given the following

TABLE 7

COMPARISON OF RESPONSES OF 10 TEST CHEMICALS USING THE CRITERION OF A 2× INCREASE IN PLATE COUNTS

Chemical	Mitotic aneuploidy	Mitotic segregation	Mutation
SAF	6 [b]	4	5
PB	6 [b]	0	2
DEHP	7 [b]	0	2
ACN	9 [b]	3	7 [b]
HMPA	11 [b]	4	8 [b]
BEN	6 [b]	1	2
ZOIN	4	2	2
DES	10 [b]	0	0
TOL	10 [b]	1	2
CAP	0	0	0

[a] Numerical values as explained in text.

[b] Positive response for endpoint under test.

numerical values, where + + = 2, + = 1 and − = 0 and the overall results shown in Table 7. As 6 individual experiments were performed with each test chemical we have used the criteria of the value of 6 to indicate a positive result (a value of 12 indicating a class 1 positive response in all studies).

Conclusions

The results of this study of the 10 test chemicals using yeast strains D6 and D61-M are summarized in Table 5 for the analysis based upon the integral yield and in Table 7 for the analysis based upon at least 2-fold plate increases. The 2 sets of comparisons indicate that all 8 chemicals classified as carcinogens prior to this study i.e. HMPA, TOL, PB, SAF, ACN, BEN, DES and DEHP are capable of inducing mitotic chromosome aneuploidy in yeast. PB and BEN gave positive results that only just fell within our criteria for a positive response irrespective of the method of analysis.

In contrast to the results of the aneuploidy assay, only HMPA and ACN gave positive results for the induction of point mutation (base substitution) in strains D6 and D61-M (irrespective of the method of comparison). ACN gave a positive result in the assay for mitotic segregation when the comparison was made on the basis of integral yield.

ZOIN gave a number of positive results in experiments with strain D61-M. However using the criteria used here this chemical was classified as being nongenotoxic overall.

It is clear from the results described here that there was considerable variation in the responses obtained in the range of experiments described here with the 10 test chemicals. In the case of PB and BEN, the responses obtained for the induction of mitotic aneuploidy were low under conditions that failed to produce significant increase for point mutation and mitotic segregation. Such results indicate that both PB and BEN are capable of inducing mitotic aneuploidy but that the treatment protocols used were less than optimal. In view of the diversity of chemicals used in this study it is not surprising that such variations in the maximal genotoxic response for each chemical were found. However, it is clear from the overall comparisons of the data that the use of assays for the induction of mitotic chromosome aneuploidy has a valuable place in the assessment of the genotoxicity of potentially carcinogenic chemicals.

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Assays for forward mutation in *Schizosaccharomyces pombe* strain P1

N. Loprieno, G. Boncristiani, R. Forster [1] and B. Goldstein [2]

Istituto di Biochimica, Biofisica e Genetica, Università di Pisa, Pisa (Italy), [1] LSR Toma Toxicology Centre, Roma (Italy) and [2] Gruppo Lepetit S.p.A., Milano (Italy)

Summary

All 10 test chemicals were assayed for mutagenic activity in the *ade* forward-mutation system in *Schizosaccharomyces pombe* P1. This haploid strain carries mutant *ade 6-60* which accumulates a red pigment in the cell. The strain also carries the *rad 10-198* mutation, which enhances the sensitivity of the strain, since *rad* mutants are defective in a DNA-repair pathway. A mutation occurring at any one of 5 loci preceeding the *ade-6* block prevents the accumulation of pigment and the double mutants form white colonies. Using this assay system all 10 chemicals were tested and in no case were dose-related increases in mutation frequency found that exceeded the control frequencies.

Introduction

Our contribution to the IPCS study is based on the Ade forward mutation system in *Schizosaccharomyces pombe* P1. This haploid test strain carries a mutation in a gene involved in the adenine biosynthesis pathway (*Ade6-60)*; as a result it forms bright red colonies. A mutation occurring in any of the preceeding 5 genes in the pathway prevents the accumulation of the red pigment, and the double mutant thus formed produces white colonies that can readily be scored. Because of the large target available for mutations, this strain has a high spontaneous frequency of double-mutant formation. The strain also carries the *rad10-198* mutation, which enhances the sensitivity of the strain, since rad mutants are defective in a DNA-repair pathway.

This assay system has been validated with a wide range of chemical agents, as described in a recent report of the US EPA Gene-Tox program, (Loprieno et al., 1983), the Report of the International Collaborative Program for the Evaluation of Short-Term Tests for Carcinogens (Loprieno, 1981), and a number of recent publications.

We have assayed all 10 IPCS study chemicals for their ability to induce forward mutation in this test system. The assays were performed in the presence and absence of an exogenous S9 metabolic activation preparation, using a growth-phase procedure.

Materials and methods

The experimental work was performed in two laboratories, using common methods. Each laboratory assayed 5 of the test compounds: Lab I (BG) tested HMPA, CAP, ZOIN, SAF and TOL while Lab II (RF) tested BEN, PB, DES, ACN and DEHP.

Microbial strains

Schizosaccharomyces pombe P1 was originally obtained from Istituto di Biochimica, Biofisica e Genetica, Università di Pisa, but both laboratories used their own maintained stocks of this strain for the present study. YEA, YEL and YELD media were used for the growth of the strain as described by Barale et al. (1979); all incubations were at 32°C.

Test materials

The 10 test compounds were received from the IPCS Program and were stored in the dark at 4°C. Fresh solutions were prepared for each experiment. Dimethyl sulphoxide (DMSO) was used as the solvent in all cases except ACN, DES (ethanol), PB (water) and DEHP (DMSO/Tween 80).

Metabolic activation

S9 tissue homogenate. One common batch of S9 liver-tissue fraction was prepared for this study. Young male Sprague–Dawley/CD rats were induced with phenobarbital and β-naphthoflavone following the method of Matsushima et al. 1976. The 9000-*g* homogenate supernatant was prepared

TABLE 1

HMPA

		Expt. 1			Expt. 2		
		Survival	Mut/pl	Frequency	Survival	Mut/pl	Frequency
Zero time control		–	1/17	0.410			
Solvent control (DMSO)		100	0/18	0.000			
HMPA	20 μg/ml	127	3/18	1.866			
	78 μg/ml	112	0/18	0.000			
	196 μg/ml	97	1/18	0.813			
	782 μg/ml	29	1/16	3.024			
	1956 μg/ml	117	1/18	0.672			
EMS	20 μg/ml	80	18/18	17.600			
		Plating density: 705					
Presence of S9							
Zero time control		–	1/19	0.329	–	2/17	1.016
Solvent control (DMSO)		100	0/17	0.000	100	3/19	1.079
HMPA	20/21 μg/ml	71	1/20	0.427	65	2/16	1.316
HMPA	80/82 μg/ml	65	0/20	0.000	69	0/20	0.000
HMPA	200/205 μg/ml	91	2/19	0.698	60	1/14	0.807
HMPA	800/822 μg/ml	97	0/17	0.000	76	0/18	0.000
HMPA	2000/2054 μg/ml	74	2/17	0.956	62	2/16	1.377
CPA	250 μg/ml	92	39/17	15.068	10	8/18	29.144
		Plating density: 1657			Plating density: 1463		

Key to Tables 1–10.

Survival. Estimate of the toxicity of the test substance to the cells, presented as viable cell count on YEA plates expressed as percentage of solvent control value (HMPA, CAP, ZOIN, SAF, TOL) or plating efficiency on YEA plates expressed as percentage of solvent control value (BEN, PB, DES, ACN, DEHP). Values for the untreated controls are reference values for positive controls survival which are not expressed relative to solvent controls. Wherever possible, control values are referred to the relevant solvent control (or untreated control if the solvent was water).

Mut/pl. The number of mutant colonies (white or sectored) counted is given before the oblique stroke; the number of plates prepared is given after the stroke.

Frequency. The calculated mutation frequency per viable cell $\times 10^{-4}$.

Zero time control. Control culture plated for mutation frequency at outset of experiment prior to 16-h growth period.

Untreated control. Control culture plated for mutation frequency after 16-h growth period.

Solvent control. Control culture treated with solvent vehicle only.

Dose levels. Where two values are given before and after an oblique stroke, these refer to the dose levels used in Expts. 1 and 2 respectively.

Plating density. The estimated number of viable cells per YEA plate in the solvent control series for the experiment.

using standard methods (Ames et al., 1975).

S9 mix. The mixture of S9 tissue fraction and cofactors (S9 mix) was prepared as follows (per 10 ml):

Phosphate buffer (0.1 M, pH 7.4)	7.4 ml
$MgCl_2$ (0.4M) and KCl (1.65M)	0.1 ml
Glucose 6-phosphate	30.0 mg
NADP	30.0 mg
S9 tissue fraction	2.5 ml

Experimental design

Each experiment included negative and positive controls, and at least 5 doses of the test compound. Experiments were performed both in the presence and absence of an S9 metabolic activation mix. Two independent experiments were performed in the majority of cases.

Negative controls. Negative control cultures treated with appropriate solvent vehicles were prepared in each experiment. Untreated controls were prepared in most experiments, and in some experiments control cultures were plated to estimate mutation frequencies at the outset of the experiment (zero time controls).

Positive controls. In the absence of S9 metabolism, methyl methanesulphonate or ethyl methanesulphonate were used; in the presence of S9 metabolism cyclophosphamide was used as a postive control.

Test compound. Doses of the test compound were set according to the results of preliminary toxicity assays (data not presented).

Assay procedure (16-h growth phase procedure)

A liquid culture of *S. pombe* P1 was grown for

TABLE 2

o-TOLUIDINE

		Expt. 1			Expt. 2		
		Survival	Mut/pl	Frequency	Survival	Mut/pl	Frequency
Absence of S9							
Zero time control		–	2/20	0.644			
Solvent control (DMSO)		100	1/20	0.840			
TOL	10 μg/ml	94	0/20	0.000			
TOL	29 μg/ml	202	0/20	0.000			
TOL	96 μg/ml	79	1/19	1.126			
TOL	288 μg/ml	95	0/20	0.000			
TOL	960 μg/ml	153	0/18	0.000			
TOL	1920 μg/ml	44	0/20	0.000			
EMS	20 μg/ml	69	13/19	16.587			
EMS	40 μg/ml	86	37/20	36.275			
		Plating density: 595					
Presence of S9							
Zero time control		–	1/20	0.344			
Solvent control (DMSO)		100	2/20	0.416			
TOL	19 μg/ml	52	1/14	0.573			
TOL	96 μg/ml	73	1/15	0.382			
TOL	192 μg/ml	62	0/16	0.000			
TOL	960 μg/ml	49	1/19	0.444			
TOL	1920 μg/ml	10	1/20	2.174			
CPA	250 μg/ml	5	10/20	41.667			
		Plating density: 2403					

24 h to reach a cell density of $0.5–1.0 \times 10^7$ cells/ml. The culture was centrifuged for 5 min at 3200 *g*. The supernatant was discarded and the cells resuspended in fresh YELD medium. Incubation mixtures were then prepared for each test point as follows:

Absence of S9 metabolism

Cell suspension	1.0 ml
Phosphate buffer (0.01 M)	1.0 ml
Test solution, reference mutagen or solvent vehicle	0.1 ml
	2.1 ml

Presence of S9 metabolism

Cell suspension	1.0 ml
S9 mix	1.0 ml
Test solution, reference mutagen or solvent vehicle	0.1 ml
	2.1 ml

The incubation mixtures were prepared in sterile universal bottles and incubated for 16 h at 32°C in a shaking water bath. The contents of each universal bottle were then diluted with sterile distilled water and recentrifuged. The cells were resuspended in 2.1 ml of YEL medium, then plated onto YEA plates to estimate mutant numbers; plates were also set up to estimate toxicity. One laboratory measured surviving viable cells, while the other laboratory estimated the plating efficiency of the treated cells. Survival in terms of viable cells can be calculated from the plating efficiency and the dilution factor used.

The agar plates were incubated for 5 days at 32°C and then held at 4°C for 2 days to enhance the pigmentation of the colonies prior to scoring.

TABLE 3

BENZENE

		Expt. 1			Expt. 2		
		Survival	Mut/pl	Frequency	Survival	Mut/pl	Frequency
Absence of S9							
Untreated control		100	2/10	0.659	100	2/10	0.591
Solvent control (DMSO)		100	3/10	1.073	100	4/10	1.094
BEN	62 μg/ml	124	0/10	0.000	105	0/10	0.000
BEN	125 μg/ml	104	0/10	0.000	77	0/10	0.000
BEN	250 μg/ml	93	0/10	0.000	96	1/10	0.284
BEN	500 μg/ml	39	0/10	0.000	46	4/10	2.358
BEN	1000 μg/ml	27	0/10	0.000	64	0/10	0.000
MMS	0.07 mM	94	21/10	7.360	93	15/10	4.762
MMS	0.14 mM	100	40/10	13.201	88	72/10	24.161
		Plating density: 2797			Plating density: 3657		
Presence of S9							
Untreated control		100	2/10	0.604	100	2/10	0.626
Solvent control (DMSO)		100	5/10	1.070	100	1/10	0.262
BEN	62 μg/ml	65	0/10	0.000	21	0/10	0.000
BEN	125 μg/ml	63	0/10	0.000	25	0/10	0.000
BEN	250 μg/ml	11	0/10	0.000	5	1/10	5.085
BEN	500 μg/ml	10	0/10	0.000	3	0/10	0.000
BEN	1000 μg/ml	15	0/10	0.000	24	0/10	0.000
CPA	1.25 mM	94	31/10	9.947	79	22/10	8.707
CPA	2.5 mM	89	44/10	14.966	77	40/10	16.194
		Plating density: 4673			Plating density: 3813		

Interpretation of results

The criterion we have used is that an agent will be considered mutagenic if it induces reproducible dose-related increases in mutation frequency, 3-fold greater than control values, at 2 dose levels. The solvent control provides the reference control value for mutation frequency. Where no mutants were found on the solvent control plates, and thus no value is available for the control mutation frequency, the mean values for this series of experiments may be used. (Mean solvent control frequencies: in absence of S9 metabolism 0.542×10^{-4}, in presence of S9 metabolism 0.538×10^{-4}.)

Results

The results are presented in summary form in Tables 1–10, where the survival levels, number of mutant colonies scored, and calculated mutation frequency are presented for each experiment. The full set of data has been stored on the computer with the other IPCS study contributions, and is available on request from the study organizers.

Increases in mutation frequency that were reproducible, 3-fold greater than controls, and dose-related, were not observed with any of the 10 chemicals. In the absence of S9 metabolism, 3-fold increases in mutation frequency were obtained over 3 consecutive doses with DEHP, but in a second experiment this finding was not confirmed. The result is therefore regarded as equivocal. Sporadic increases that were greater than 3-fold but were not dose-related, were seen in a number of experiments. We conclude, therefore, that negative results were obtained with the test chemicals although equivocal results were obtained with DEHP

TABLE 4

SAFROLE

		Expt. 1			Expt. 2		
		Survival	Mut/pl	Frequency	Survival	Mut/pl	Frequency
Absence of S9							
Zero time control		–	2/20	0.644			
Solvent control (DMSO)		100	1/20	0.840			
SAF	7 μg/ml	121	3/20	2.076			
SAF	66 μg/ml	117	3/20	2.158			
SAF	125 μg/ml	129	1/20	0.654			
SAF	209 μg/ml	190	2/20	0.885			
SAF	626 μg/ml	13	0/20	0.000			
EMS	20 μg/ml	69	13/19	16.587			
EMS	40 μg/ml	86	37/20	36.275			
		Plating density: 595					
Presence of S9							
Zero time control		–	1/19	0.520	–	1/18	0.629
Untreated control		–	0/19	0.000	–	–	–
Solvent control (DMSO)		100	0/16	0.000	100	0/18	0.000
SAF	20 μg/ml	47	0/20	0.000	74	0/18	0.000
SAF	66 μg/ml	55	0/20	0.000	67	0/18	0.000
SAF	125 μg/ml	120	3/20	3.922	86	2/18	0.673
SAF	209 μg/ml	90	0/20	0.000	111	4/18	1.048
SAF	626μg/ml	194	1/20	0.806	10	0/18	0.000
CPA	250 μg/ml	70	17/17	44.444	32	24/17	19.791
		Plating density: 320			Plating density: 1917		

TABLE 5

CAPROLACTAM

		Expt. 1			Expt. 2		
		Survival	Mut/pl	Frequency	Survival	Mut/pl	Frequency
Absence of S9							
Zero time control		–	0/20	0.000			
Untreated control		–	1/20	0.382			
Solvent control (DMSO)		100	0/20	0.000			
CAP	8 μg/ml	148	1/19	0.877			
CAP	19 μg/ml	105	0/20	0.000			
CAP	76 μg/ml	136	0/20	0.000			
CAP	190 μg/ml	108	0/19	0.000			
CAP	762 μg/ml	133	0/19	0.000			
CAP	1900 μg/ml	86	0/19	0.000			
EMS	20 μg/ml	141	44/20	38.428			
EMS	40 μg/ml	157	63/20	49.606			
		Plating density: 405					
Presence of S9							
Zero time control		–	0/18	0.000	–	1/19	0.351
Untreated control		–	1/19	0.403	–	–	–
Solvent control (DMSO)		100	0/19	0.000	100	0/17	0.000
CAP	19/20 μg/ml	106	0/20	0.000	139	1/18	0.492
CAP	76/80 μg/ml	85	0/20	0.000	78	0/19	0.000
CAP	190/200 μg/ml	130	0/20	0.000	157	1/20	0.393
CAP	762/800 μg/ml	685	0/20	0.000	133	5/18	2.572
CAP	1900/2000 μg/ml	728	1/19	0.616	339	4/19	0.765
CPA	500 μg/ml	40	13/19	144.04	43	22/15	41.905
		Plating density: 1175			Plating density: 812		

TABLE 6

ACRYLONITRILE

		Expt. 1			Expt. 2		
		Survival	Mut/pl	Frequency	Survival	Mut/pl	Frequency
Absence of S9							
Untreated control		100	1/10	0.292	100	3/10	0.989
Solvent control (ethanol)		100	3/10	0.709	100	2/10	0.706
ACN	16 μg/ml	75	6/10	1.887	113	0/10	0.000
ACN	31.2 μg/ml	80	6/10	1.772	80	0/10	0.000
ACN	62.5 μg/ml	91	2/10	0.520	66	1/10	0.532
ACN	125 μg/ml	59	3/10	1.211	41	1/10	0.855
ACN	250 μg/ml	28	3/10	2.550	44	1/10	0.798
MMS	0.07 mM	90	22/10	7.127	98	19/10	6.390
MMS	0.14 mM	88	40/10	13.216	104	40/10	12.658
		Plating density: 4233			Plating density: 2833		

TABLE 6 (continued)

	Expt. 1			Expt. 2		
	Survival	Mut/pl	Frequency	Survival	Mut/pl	Frequency
Presence of S9						
Untreated control	100	2/10	0.661	100	3/10	0.912
Solvent control (ethanol)	100	4/10	1.162	100	3/10	0.953
ACN 16 μg/ml	97	3/10	0.903	110	0/10	0.000
ACN 31.2 μg/ml	106	3/10	0.826	35	1/10	0.901
ACN 62.5 μg/ml	134	6/10	1.299	83	3/10	1.144
ACN 125 μg/ml	64	3/10	1.366	71	5/10	2.242
ACN 250 μg/ml	72	3/10	1.208	51	0/10	0.000
CPA 1.25 mM	93	40/10	14.168	87	23/10	8.033
CPA 2.5 mM	100	36/10	11.947	94	45/10	14.579
	Plating density: 3443			Plating density: 3147		

TABLE 7

DIETHYLHEXYLPHTHALATE

	Expt. 1			Expt. 2		
	Survival	Mut/pl	Frequency	Survival	Mut/pl	Frequency
Absence of S9						
Untreated control	100	1/10	0.292	100	3/10	0.989
Solvent control (DMSO/Tween 80)	100	1/10	0.336	100	3/10	0.989
DEHP 369 μg/ml	93	1/10	0.359	87	2/10	0.759
DEHP 738 μg/ml	122	8/10	2.192	85	1/10	0.389
DEHP 1467 μg/ml	79	10/10	4.261	137	2/10	0.481
DEHP 2935 μg/ml	108	6/10	1.865	109	0/10	0.000
DEHP 5870 μg/ml	152	2/10	0.441	17	0/10	0.000
MMS 0.07 mM	90	22/10	7.127	98	19/10	6.390
MMS 0.14 mM	88	40/10	13.216	104	40/10	12.658
	Plating density: 2980			Plating density: 3033		
Presence of S9						
Untreated control	100	2/10	0.661	100	3/10	0.912
Solvent control (DMSO Tween 80)	100	2/10	0.600	100	3/10	0.983
DEHP 369 μg/ml	74	6/10	2.439	160	3/10	0.614
DEHP 738 μg/ml	90	1/10	0.332	51	2/10	1.296
DEHP 1467 μg/ml	90	1/10	0.334	143	0/10	0.000
DEHP 2935 μg/ml	100	3/10	0.901	120	2/10	0.545
DEHP 5870 μg/ml	145	7/10	1.451	67	2/10	0.971
CPA 1.25 mM	93	40/10	14.168	87	23/10	8.033
CPA 2.5 mM	100	36/10	11.947	94	45/10	14.579
	Plating density: 3333			Plating density: 3053		

TABLE 8

BENZOIN

		Expt. 1			Expt. 2		
		Survival	Mut/pl	Frequency	Survival	Mut/pl	Frequency
Absence of S9							
Zero time control		–	0/20	0.000			
Untreated Control		–	1/20	0.382			
Solvent control (DMSO)		100	0/20	0.000			
ZOIN	8 μg/ml	106	0/20	0.000			
ZOIN	19 μg/ml	133	0/20	0.000			
ZOIN	76 μg/ml	104	0/19	0.000			
ZOIN	190 μg/ml	99	1/18	1.389			
ZOIN	762 μg/ml	98	1/19	1.324			
ZOIN	1900 μg/ml	89	0/19	0.000			
EMS	20 μg/ml	141	44/20	38.428			
EMS	40 μg/ml	157	63/20	49.606			
		Plating density: 405					
Presence of S9							
Zero time control		–	0/18	0.000	–	1/19	0.487
Untreated control		–	1/19	0.403	–	–	–
Solvent control (DMSO)		100	0/19	0.000	100	3/19	1.729
ZOIN	19/20 μg/ml	155	0/20	0.000	181	2/19	0.638
ZOIN	76/80 μg/ml	472	0/20	0.000	68	1/20	0.810
ZOIN	190/200 μg/ml	279	1/19	1.607	84	1/19	0.686
ZOIN	762/800 μg/ml	1390	2/19	0.644	109	1/19	0.529
ZOIN	1900/2000 μg/ml	732	1/19	0.612	148	2/20	0.741
CPA	500/250 μg/ml	40	13/19	144.04	116	31/17	17.285
		Plating density: 1175			Plating density: 913		

TABLE 9

DIETHYLSTILBESTROL

		Expt. 1			Expt. 2		
		Survival	Mut/pl	Frequency	Survival	Mut/pl	Frequency
Absence of S9							
Untreated control		100	1/10	0.329	100	1/10	0.331
Solvent control (ethanol)		100	1/10	1.333	100	1/10	0.308
DES	0.08 μg/ml	84	1/10	0.395	88	0/10	0.000
DES	0.4 μg/ml	99	5/10	1.687	129	1/10	0.239
DES	2 μg/ml	105	1/10	0.317	114	2/10	0.541
DES	10 μg/ml	80	0/10	0.000	99	0/10	0.000
DES	50 μg/ml	0	0/10	0.000	–	–	–
MMS	0.07 mM	92	20/10	7.160	108	21/10	6.448
MMS	0.14 mM	105	39/10	12.251	102	41/10	13.355
		Plating density: 3007			Plating density: 3247		

TABLE 9 (continued)

	Expt. 1			Expt. 2		
	Survival	Mut/pl	Frequency	Survival	Mut/pl	Frequency
Presence of S9						
Untreated control	100	1/10	0.310	100	1/10	0.319
Solvent control (ethanol)	100	2/10	0.536	100	1/10	0.274
DES 0.08 μg/ml	86	1/10	0.312	67	2/10	0.820
DES 0.4 μg/ml	70	0/10	0.000	124	2/10	0.443
DES 2 μg/ml	76	0/10	0.000	103	4/10	1.070
DES 10 μg/ml	65	1/10	0.412	88	1/10	0.311
DES 50 μg/ml	152	3/10	0.530	92	2/10	0.598
CPA 1.25 mM	100	57/10	17.629	95	41/10	13.851
CPA 2.5 mM	82	84/10	31.899	89	39/10	13.945
	Plating density: 3730			Plating density: 3643		

TABLE 10

PHENOBARBITONE

	Expt. 1			Expt. 2		
	Survival	Mut/pl	Frequency	Survival	Mut/pl	Frequency
Absence of S9						
Untreated control	100	1/10	0.329	100	1/10	0.331
Solvent control (water)	100	1/10	0.329	100	1/10	0.331
PB 2.5 μg/ml	90	2/10	0.734	143	1/10	0.231
PB 5 μg/ml	85	1/10	0.389	124	0/10	0.000
PB 10 μg/ml	102	0/10	0.000	97	0/10	0.000
PB 20 μg/ml	81	0/10	0.000	133	0/10	0.000
PB 40 μg/ml	98	1/10	0.336	136	1/10	0.244
MMS 0.07 mM	92	20/10	7.160	108	21/10	6.448
MMS 0.14 mM	105	39/10	12.251	102	41/10	13.355
	Plating density: 3037			Plating density: 3017		
Presence of S9						
Untreated control	100	1/10	0.310	100	1/10	0.319
Solvent control (water)	100	1/10	0.310	100	1/10	0.319
PB 2.5 μg/ml	87	0/10	0.000	161	0/10	0.000
PB 5 μg/ml	72	0/10	0.000	110	3/10	0.870
PB 10 μg/ml	141	0/10	0.000	104	0/10	0.000
PB 20 μg/ml	75	0/10	0.000	119	3/10	0.808
PB 40 μg/ml	84	3/10	1.111	145	3/10	0.660
CPA 1.25 mM	100	57/10	17.629	95	41/10	13.851
CPA 2.5 mM	82	84/10	31.899	89	39/10	13.945
	Plating density: 3223			Plating density: 3130		

in the absence of S9 metabolism.

One laboratory recorded reduced survival in the presence of S9 mix, which was alleviated by increasing doses of test substances, giving rise to inverted survival curves (first replicate tests with CAP and ZOIN) in some experiments.

Discussion

The sensitivity of the *S. pombe* P1 forward mutation system is limited by the numbers of cells screened (20000–40000) for mutants. Unlike reverse mutation systems, it is also limited by the fact that in forward-mutation systems a small increase in induced mutants of one specific type must compete for detection with spontaneous mutations of all kinds. The advantage of the forward-mutation system, therefore, is not in the detection of weak mutagens, since the resolution of the system is limited, but in the detection of point mutagens with diverse mechanisms.

In the set of 10 IPCS study chemicals, there were 2 agents considered to be point mutagens (ACN and PB), which we might have expected *S. pombe* P1 to detect, but which did not demonstrate mutagenicity in the test system. The performance of the assay system with the other 8 study chemicals reflects the conclusions of the study regarding these chemicals, namely that other genetic end points are appropriate for their detection. Some appropriate end points (e.g. mitotic aneuploidy) are readily available in other yeast test systems. This set of study chemicals did not offer an opportunity for the careful assessment of the protocol improvements for the same reason. To evaluate the performance of the revised methods that have been used, it would be better to use a number of known and previously studied point mutagens.

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Assays in *Aspergillus nidulans* for the induction of forward-mutation in haploid strain 35 and for mitotic nondisjunction, haploidization and crossing-over in diploid strain P1

A. Carere, G. Conti, L. Conti and R. Crebelli

Istituto Superiore di Sanità, Rome (Italy)

Summary

4 chemical carcinogens selected for the IPCS collaborative study have been assayed for their ability to induce forward mutations in *Aspergillus nidulans* by the growth-mediated technique. All 4 carcinogens, HMPA, TOL, SAF and BEN, together with the negative control CAP, were unable to increase in a significant way the frequency of methionine suppressors in treated cultures.

The same chemicals, together with ACN, DEHP, ZOIN, DES and PB were also assayed for the induction of mitotic segregation (mitotic non-disjunction, haploidization and crossing-over) in the diploid heterozygous strain P1. The results obtained applying the test chemicals to growing colonies (plate-incorporation assay) show that SAF significantly increases the frequency of haploid sectors; an increased frequency of haploid and non disjunctional segregants was also observed by applying ACN to germinating conidia (liquid test).

The *Istituto Superiore di Sanità* participated in the IPCS collaborative study on short-term tests for genotoxicity and carcinogenicity (first phase: in vitro assays).

The genotoxic characteristics of the 8 carcinogens and 2 noncarcinogens selected by the Working Group were evaluated in the mould *Aspergillus nidulans* with respect to 3 endpoints: gene mutation, mitotic crossing-over and mitotic aneuploidy.

Materials and methods

Induction of gene mutations in A. nidulans: growth mediated assay

4 of the 8 carcinogens selected for the CSSTT have been tested for the ability to induce forward mutations (methionine suppressors) employing a technique previously developed in our laboratory that exploits the endogenous metabolic activity of the test organism (Bignami et al., 1981). These chemicals, HMPA, TOL, SAF and BEN, are known to be insufficiently or inadequately activated by the usual exogenous metabolic system employed in in vitro short-term tests (i.e. mammalian microsomes). The rationale of the choice of the *A. nidulans* growth-mediated assay as the test system for evaluating their in vitro activity rests (i) on the observed ability of *A. nidulans* to activate, together with classical promutagens (e.g. nitrosamines), also chemicals like dimethylhydrazine and natulan (Bignami et al., 1981), known false negatives in other short-term tests; and (ii) on the observed sensitivity of the *A. nidulans sumeth* system to mutagens of both the missense and frameshift type (Bignami et al., 1982).

The *A. nidulans* haploid strain 35 (*anA1, pabaA1, yA2, methG1, s12, nicA2, nicB8*) of Glasgow origin was used for the detection of methionine suppressors (forward mutations in at least 5 loci). Usually spontaneous mutation frequencies are in the order of $0.2–1.0 \times 10^{-6}$.

The protocol for the growth-mediated assay was

as follows: 10^7 conidia were inoculated into 20 ml of molten complete medium supplemented with various amounts of the test chemicals and poured in one petri dish. 3 plates were usually prepared to test each concentration. Plates were then incubated at 37°C in the dark for 5 days to allow full vegetative growth and sporulation of cultures. Afterwards conidia from these primary cultures grown in the presence of the test chemical were collected, washed, and seeded at appropriate dilutions on selective and supplemented media to evaluate the frequency of methionine-independent mutants. Each chemical was tested at 3 concentrations: the highest not interfering with the cultures' sporulation (in the case of TOL and CAP) or allowed by the chemical availability (in the case of SAF, HMPA and BEN) and 2 lower ones to detect possible dose–effect relationships.

Diethylnitrosamine was used as positive control through the whole work. Results were statistically evaluated by the *t* test and the nonparametric Mann–Witney *U* test.

Induction of somatic segregation in A. nidulans

The 8 carcinogens (and 2 negative controls) selected for the CSSTT have been tested for their ability to induce somatic segregation (mitotic non disjuction, haploidization and crossing-over) in *A. nidulans* employing a genetic system (segregation of the *yA2* marker in the heterozygous strain P1)

TABLE 1
INDUCTION OF GENE MUTATIONS IN *A. nidulans*: GROWTH MEDIATED ASSAY

Compound	μg/ml	*su methA1*/10^6 [a]					
HMPA	0	0.96 (8/8.3) [b]	2.99 (29/9.7)	5.71 (48/8.4)			
	513.5	0.89 (7/7.8)	3.82 (26/6.8)	5.89 (43/7.3)			
	1027	0.51 (6/11.7)	2.33 (20/8.6)	1.75 (18/10.3)			
	2567.5	0.82 (5/6.1)	2.03 (25/12.3)	2.19 (16/7.3)			
Diethylnitrosamine	475	13.63 (169/12.4)					
TOL	0	0.50 (4/8.0)	1.51 (13/8.6)	2.32 (19/8.2)			
	126	2.53(21/8.3)	2.05 (25/12.2)	0.96 (12/12.5)			
	252	1.95 (23/11.8)	1.96 (18/9.2)	2.23 (21/9.4)			
	504	6.59 (60/9.1)	0.69 (9/13.1)	1.52 (24/15.8)			
Diethylnitrosamine	475	19.17 (278/14.5)					
BEN	0	0.36 (3/8.4)	0.39 (5/12.9)	1.46 (19/13)			
	439.5	0.59 (4/6.8)	0.68 (5/7.4)	0.71 (8/11.3)			
	879	0.22 (2/9.3)	0.62(7/11.3)	0.66 (5/7.6)			
	2197.5	0.14 (1/6.9)	1.82 (16/8.8)	2.15 (23/10.7)			
Diethylnitrosamine	475	14.37 (102/7.1)					
SAF Expt. 1	0	1.70 (23/13.5)	3.33 (30/9)				
	1096	0.49 (7/14.3)	0.11 (0/9.4)				
	2740	4.67 (43/9.2)	1.76 (15/8.5)				
Expt. 2	0	5.25 (62/11.8)	0.39 (2/5.1)	2.39 (17/7.1)	1.21 (9/7.4)	1.46 (23/15.8)	3.33 (24/7.2)
	5480	4.32 (67/15.5)	5.53 (69/12.5)	4.88 (79/16.2)	1.28 (11/8.6)	1.33 (15/11.3)	0.52 (5/9.7)
DEN Expt. 1	2375	43.65 (227/5.2)					
Expt. 2	2375	32.99 (320/9.7)					
CAP	0	0.86 (7/8.1)	0.81 (6/7.4)	2.26 (38/16.8)			
	250	1.74 (28/16.1)	3.54 (62/17.5)	1.24 (21/16.9)			
	500	1.41 (32/22.7)	1.95 (23/11.8)	1.60 (21/13.1)			
	1000	4.00 (34/8.5)	0.30 (6/20.1)	0.34 (5/14.7)			
Diethylnitrosamine	2375	64.81 (350/5.4)					

[a] *su methA1* frequencies among conidial suspensions of independent cultures of strain 35 grown in the presence of the test chemical. Numbers against each concentration represent replicates of the same treatment.

[b] In parentheses *su methA1* colonies/plated conidia $\times 10^6$.

previously validated by us and others with many chemical mutagens/carcinogens (Bignami et al., 1977; Bellincampi et al., 1980; Carere et al., 1981; Gualandi et al., 1981; Morpurgo et al., 1979).

The rationale of the choice of these genetic end points (in particular the induction of aneuploidies) to evaluate these chemical carcinogens rests on (i) the proposed role of aneuploidies in the initiation or progression of cancer (Klein, 1979; Onho, 1979), (ii) the observed aneuploidizing activity of tumor promoters in fungal systems (Parry et al., 1981), (iii) the paucity of data on the induction of aneuploids by these chemical carcinogens.

The *A. nidulans* diploid strain P1(*suA1adE20, riboA1, fpaA1, anA1, proA1, pabaA1, yA2, adE20, biA1, s12, phenA2, methA1, pyroA4, nicA2, lysB5, nicB8*) was used for the detection of induced somatic segregation.

Homozygous (*yA2/yA2*) or hemizygous(*yA2*) yellow sectors arising in heterozygous pale green-colonies growing on complete medium in the presence of the test chemicals were analyzed for their nitritional requirements and classified as mitotic crossing-overs, non disjunctional diploids and haploids (Morpurgo et al., 1979). In most instances, the mean frequency of yellow sectors starting from the center of the colony in strain P1 is about 10^{-2}. Most of these (more than 90%) are cross-overs, the other non-disjunctional diploids and haploids arising from non-disjunction or misdistibution of chromosomes or, in the case of haploid segregants, also from large chromosomal damages or semi-dominant lethals (Käfer et al., 1982).

Two different technical approaches were used to detect induced somatic segregation: plate incorporation assay and liquid test procedure, performed according to the protocols described below.

Plate-incorporation assay. Complete agarized molten (45 °C) medium is inoculated with the required number of conidia (to obtain 15–20 viable colonies/plate) and a known amount of the test chemical immediatly before pouring it into petri dishes (20 ml/plate). Plates are incubated 3 days at 37 °C, then colonies inspected for the presence

TABLE 2
INDUCTION OF SOMATIC SEGREGATION IN *A. nidulans*: PLATE-INCORPORATION ASSAY

Chemical	μg/ml	Survival %	Analyzed colonies	Colonies with *n* sectors [a]		Colonies with 2*n* – ND sectors		Colonies with 2*n* – CO sectors	
				n	%	*n*	%	*n*	%
Untreated control		100	2228	6	0.27	0		19	0.85
HMPA	2567.5	100	275	0		0		2	0.73
TOL	2520	100	164	2	1.22	0		3	1.83
	3780	20	42	1	2.38	0		0	
BEN	2197.5	100	255	2	0.78	0		2	0.78
SAF	548	100	140	0		0		0	
	5480	35	236	7	2.97 ***	0		1	0.42
CAP	500	100	256	0		0		1	0.39
ACN	806	50	281	0		0		8	2.85 **
	2015	10	656	1	0.15	2	0.30	8	1.22
DEHP	2465	100	243	0		0		6	2.47 *
ZOIN	500	100	207	2	0.64	1	0.32	2	0.96
DES	500	100	451	1	0.22	1	0.22	3	0.66
PB	500	100	843	6	0.71	0		7	0.83
MMS	100	50	97	0		0		12	12.37 ***
Chloral hydrate	825	80	125	5	4.00 ***	3	2.4 **	0	
Benomyl	0.5	30	86	15	17.55 ***	0		2	2.32

[a] *n* haploid sectors; 2*n* – ND diploid non-disjunctional sectors; 2*n* – CO, mitotic cross-overs. *$P < 0.05$; ** < 0.01; ***$P < 0.001$.

of segregated sectors which are phenotypically analyzed. If the test chemical inhibits colony sporulation or if low growing abnormal colonies are observed, these are transferred with a needle onto drug-free medium and incubated 3–4 additional days to complete sporulation.

Liquid test procedure. Conidia are incubated in semi-liquid (0.2% agar) supplemented medium at 37 °C with gentle shaking. After 4.5 h samples of swollen, pre-germinating conidia are treated with the chemical under test at various concentrations and/or for variable times. Treatment is stopped with serial dilutions with sterile water. Conidia are then plated on complete medium at a density able to provide 15–20 colonies/plate and incubated 3 days at 37 °C. Afterwards sporulated colonies are inspected for segregated sectors which are isolated and analyzed.

The first technique was initially preferred because growth of colonies in the presence of the test chemicals may exploit with greater efficiency the endogenous activation of *A. nidulans* metabolism. This technique could not give information on inhibition or cytotoxicity usually associated with the induction of aneuploids, because of the low toxicity and the limited availability of test chemicals. For this reason, when new samples of chemicals were delivered, a few (ACN, DES, DEHP and HMPA) were retested by the liquid test procedure. This technique allows an acute exposure to the chemicals that may be required to exceed the threshold value inside the cell necessary to induce aneuploidies (Gualandi and Morpurgo, 1984).

Results and discussion

Induction of Gene Mutations in A. nidulans: Growth Mediated Assay

The data in Table 1 show the results obtained by assaying HMPA, TOL, BEN and SAF by the growth-mediated assay for the induction of methionine suppressors in strain 35.

TOL and CAP were assayed at one dose that partially inhibited the sporulation of cultures and two lower ones; in the case of HMPA, BEN and SAF, no similar inhibition was observed and the upper dose was decided on the basis of the chemicals' availability.

Comparing mutant frequencies in conidial suspensions of treated and control cultures it may be observed that, with two exceptions, the former all fell within the range of spontaneous frequencies. Two slightly higher values were observed in two cultures grown in the presence of TOL(505 μg/ml) and HMPA (513.5 μg/ml). Parallel cultures, however, did not show similar high mutants frequencies. Only the diethylnitrosamine, used as a positive control in all the experiments, always gave significant dose-related increases in the frequency of methionine suppressors.

In the case of very weak mutagens, it is conceivable that a consistent positive response may not be observed in all cultures due to the genetic drift during the growth of the cultures. A better evaluation of the mutagenic potential of the test compound may be obtained by comparing the distribution of mutant frequencies in control and treated cultures by means of a nonparametric test, such as the Mann–Witney U test. The analysis performed by this test failed to show any significant excess in mutant frequencies among treated cultures over controls. In conclusion, all chemicals can be evaluated as negative in this test system.

Induction of Somatic Segregation in A. nidulans

The data in Table 2 show the results obtained by assaying by the plate incorporation technique the 10 chemicals selected for the CSSTT for their ability to induce somatic segregation in *A. nidulans.*

The same suspension of quiescent conidia was used to inoculate all control and treated plates; therefore all treatments share the same spontaneous and positive controls. In the absence of any cytotoxic or inhibiting effect, HMPA, BEN, CAP, DEHP, ZOIN, DES and PB were assayed at just one dose, the highest allowed by the chemical availability or, in the case of DES, by its solubility in the medium. TOL, ACN and SAF were tested at two concentrations with different survival levels.

Statistical analysis of data shows a highly significant excess of haploid sectors in colonies growing on SAF supplemented medium, in comparison with untreated colonies ($P < 0.001$). All the other carcinogens were unable to significantly increase the frequency of aneuploids. This genetic event is usually observed after treatments associated with some cytotoxicity or inhibition of colonies: there-

TABLE 3
INDUCTION OF SOMATIC SEGREGATION IN *A. nidulans*: LIQUID TEST PROCEDURE

Chemical	Dose (μg/ml)	Survival (%)	Analyzed colonies	Colonies with *n* sectors [a]		Colonies with 2*n* − ND sectors		Colonies with 2*n* − CO sectors		Lethals yellow segregants	
				No.	%	No.	%	No.	%	No.	%
ACN	0	100	303					4	(1.3)		
(90 min treated)	806	63	312	7	(2.24)	1	(0.32)	3	(0.96)	7	(2.24)
	2418	16	398	6	(1.51)	6	(1.51)	4	(1.00)		
	4030	1.6	104	4	(3.85)	1	(0.96)				
Benomyl	200	5	355	4	(1.13)	2	(0.56)	2	(0.56)		
ACN	0	100	354					1	(0.28)		
(150 min treated)	806	36	292	6	(2.05)	2	(0.68)	5	(1.71)	3	(1.03)
	2418	1.8	224	2	(0.89)	2	(0.89)	2	(0.89)	1	(0.45)
	4030	0.06	17								
Benomyl	200	1.5	220	8	(3.64)	4	(1.82)	2	(0.91)		
DEHP	0	100	373	2	(0.54)			2	(0.54)		
(150 min treated)	2465	100	427					2	(0.47)	1	(0.23)
	4930	88	404	1	(0.25)			5	(1.24)		
	9860	78	382	1	(0.26)			1	(0.26)		
Benomyl	200	1.0	131	6	(4.58)			1	(0.76)		
HMPA	0	100	336					3	(0.89)		
(150 min treated)	5135	100	345	1	(0.29)			4	(1.16)		
	10270	100	385					4	(1.04)	1	(0.26)
	25675	100	338	2	(0.59)			2	(0.59)	1	(0.29)
4-NQO	0.5	23	78	4	(5.12)			7	(8.97)	1	(1.28)
DES	0	100	336					3	(0.89)		
(150 min treated)	2.5	100	335	1	(0.30)			2	(0.60)		
	5	100	360	1	(0.28)			2	(0.55)		
	10	33	165	2	(1.21)			1	(0.61)	3	(1.82)
	25	8	79	1	(1.27)			1	(1.27)		
4-NQO	0.5	23	78	4	(5.12)			7	(8.97)	1	(1.28)

[a] *n*, haploid yellow sectors; 2*n* − ND, diploid non-disjunctional yellow sectors; 2*n* − CO, diploid cross-overs yellow sectors; yellow lethal segregants: yellow segregants with poor viability unable to growth on minimal supplemented medium.

Historical control values of spontaneous somatic segregation (the same conidial suspension used in liquid test experiments): in 2024 analyzed colonies (10 Expts.) 12 2*n* − CO (0.6%), 4*n* (0.2%), 1 2*n* − ND (0.05%).

fore, where these conditions could not be achieved, results should more properly be evaluated as inconclusive.

An excess of mitotic cross-overs with lower statistical significance was observed among colonies grown in the presence of DEHP and ACN (only at the lower dose tested for the latter). To confirm these preliminary results DEHP and ACN, together with HMPA and DES, were also assayed by the liquid test procedure to get more effective treatment conditions.

The data in Table 3 show that ACN efficiently induced haploid and diploid non disjunctional sectors in treated colonies, while DEHP, HMPA and DES were unable to induce any kind of mitotic segregants.

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The Drosophila somatic recombination and mutation assay (SRM) using the *white-coral* somatic eye color system

Ekkehart W. Vogel

Department of Radiation Genetics and Chemical Mutagenesis, State University of Leiden (The Netherlands)

Summary

10 samples of carcinogenic and noncarcinogenic chemicals were tested for activity in a Drosophila mitotic recombination and somatic mutation assay (SRM) using *white* and *white-coral* as genetic markers. After treatment of larvae, hatching females were scored for genetic changes which produced *w* clones (ML) or $w^{co}//w$ twin spots (TS) in their eyes. The carcinogens HMPA, TOL, SAF, ACN and the noncarcinogen CAP increased the frequency of genetic damage in somatic tissue. ZOIN and PB gave negative results. The remaining 3 chemicals — BEN, DEHP and DES — induced a significant frequency of mosaic spots, but these are regarded as inconclusive because more data are needed for their final classification.

The assay for somatic mutation and mitotic recombination (SRM), utilizing the *white* locus, was originally devised by Becker (1957, 1966) as a tool for genetic analysis in developmental biology. With X-rays and TEM as the mutagens, this technique was successfully used to study mechanisms of mitotic recombination in *Drosophila melanogaster*. With the exception of two studies on the effects of MMS, captan, formaldehyde and malondialdehyde (Mollet an Würgler, 1974; Szabad et al., 1983) the SRM assay has not yet been applied for screening potential mutagenic substances.

The method is based on the determination of twin mosaic spots (TS) and single mosaic light spots (ML) in the eyes of females that had been treated as embryos, first instar or second instar larvae (Mollet and Würgler, 1974; Becker, 1957, 1966, 1975; Mollet and Weilemann, 1976; Vogel et al., 1980). These clones may arise from many types of genetic alterations, i.e., as a consequence of somatic crossing-over, chromosomal aberrations (unequal recombination between sister-strands or the two homologous X-chromosomes), aneuploidy, chromosome loss, deletions and gene mutations. Mitotic recombination and chromosome breakage events will result in mosaic twin spots, whereas deletions and gene mutations will give rise to mosaic single light spots. Thus a broad sprectrum of genotoxic chemicals, if they interact with DNA in the target cells, should be picked up as mutagens by this assay.

Materials and methods

Compounds were prepared in distilled water or a mixture of ethanol and Tween 60, to yield a final concentration of 3% ethanol and 1% Tween 60. Virgin females of the genotype w^{co} sn^2; *se h* were crossed in vials with *w*; *se h* males at a ratio of 6 females and 6–8 males.

Genetic markers	Location	Phenotype
white; w	1–1.5	white eyes
white-coral; w^{co}	1–1.5	eyes deep ruby
sepia; se	3–26.0	eyes dark brown
hairy; h	3–26.5	extra hairs on scutellum, along veins, and on top of head

The females were permitted to lay eggs for a period of 4 days on food supplemented with the

TABLE 1
EVALUATION OF TOXICITY AFTER TREATMENT OF DROSOPHILA LARVAE WITH 9 COMPOUNDS [a]

Compound	Concentration range tested (mM)	Solvent	Toxic concentration (mM)	Effect
HMPA	1.2–4.8	H_2O	4.8	25% reduction in survival
TOL	2.0–8.0	1%T/3%E	4.0	Cytotoxic
BEN	No test			
SAF	1.0–4.0	1%T/3%E	2.0	Cytotoxic
CAP	20.0	H_2O	–	None
ACN	5.0–20.0	H_2O	10.0/20.0	LC_{50}/sterile
DEHP	2.9–5.8	1%T/3%E	–	None
ZOIN	4.0–16.0	1%T/3%E	–	None
DES	2.0–8.0	1%T/3%E	8.0	50% reduction in survival
PB	0.8–3.2	1%T/3%E	3.2	30% reduction in survival

T, Tween 60; E, ethanol.
[a] (Permanent exposure of growing cultures for 9–11 days.)

test substance that had been dissolved (in 1 ml water or the ethanol/Tween 60 mixture) before they were mixed into the food. The usual procedure was to run in one experiment 3–4 treated groups (different compounds) and the solvent control(s). Developing cultures were incubated at 25 ± 0.5 °C for 10–11 days. Hatching females were scored for genetic changes which produced *w* clones (ML) or $w^{co}//w$ twin spots (TS) in their eyes. The scoring was carried out in an ethanol mixture (90% ethanol, 1% Tween 60, 9% water) at a magnification power of 75 ×.

It was tried to obtain an approximation of the possible toxicity of the test compound. For this test, groups of flies were fed on food supplemented with different concentrations of the compounds, using the procedure already described. On day 12, the number of hatching flies was counted and compared with control cultures kept under identical conditions but without the test compound. The criteria applied for toxicity measurements were no survival (LC_{100}) or reduction in survival (Table 1). No toxic effects were noted, under the condition of test, for some of the chemicals. For several reasons — solubility problems, limited amount of sample available — we were unable to fully evaluate their toxicity.

Results and discussion

Due to the short time period (3 months) we had to evaluate the chemicals, it was decided to carry out single-dose experiments. To be scored positive a chemical had to induce a repeatable increase in the number of mosaic spots in 3 separate experiments. HMPA is a very effective mutagen in Drosophila and therefore only one replicate experiment was carried out with this compound. A test substance was not considered to be satisfactorily negative until 2000 eyes had been screened for spots; and it had to be repeatedly ineffective in at least 3 separate experiments. Scoring 2000 eyes corresponds to the testing of 40000 to 400000 primordial pigment cells exposed to the chemical, depending on the time point (first or late second instar) of induction (Vogel, unpublished).

For the statistical evaluation of the raw data, the Fisher exact test was applied to discriminate among the following 3 categories:

+, mutagenic. *P*-values < 0.01 are obtained when the poooled data from the treated groups are compared with the pooled results from control runs.

M, weakly mutagenic. This means that small but reproducible effects (about a doubling of the spontaneous mutation frequency) were found in separate experiments, with $0.01 < P < 0.05$.

TABLE 2
RAW DATA FROM EXPERIMENTS WITH 10 CHEMICALS IN THE EYE MOSAIC (SRM) ASSAY

Compound	Code number	Concentration (mM)	Eyes Tested	Twin spots (TS)		Single spots (ML)	
				n	%	*n*	%
HMPA	953	0.56 (H_2O)	504	4	0.79	9	1.79
	955	0.56	794	2	0.25	8	1.01
TOL	959	2.0 (T/E)	260	1	0.38	3	1.15
	963	2.0	134	1	0.75	2	1.49
	968	2.0	60	0		2	3.33
	970	1.0	1116	0		11	0.99
	971	1.0	472	1	0.21	3	0.64
BEN	961	2.0 (T/E)	274	1	0.36	7	2.55
	969	2.0	528	0		1	0.19
	971	2.0	486	1	0.21	3	0.62
	970	2.0	828	0		4	0.48
SAF	953	0.5 (T/E)	430	1	0.23	4	0.93
	955	0.5	558	0		8	1.43
	963	0.5	800	3	0.38	7	0.88
	968	0.5	570	1	0.18	3	0.53
CAP	959	5.0 (H_2O)	968	1	0.10	10	1.03
	961	5.0	800	2	0.25	9	1.13
	970	5.0	162	0		1	0.62
ACN	955	5.0 (H_2O)	606	2	0.33	9	1.49
	959	5.0	320	0		3	0.94
	968	5.0	602	1	0.17	7	1.16
DEHP	961	2.0 (T/E)	320	0		3	0.94
	969	2.0	564	0		3	0.53
	970	2.0	446	0		5	1.12
ZOIN	970	2.0 (T/E)	222	0		1	0.45
	971	2.0	1160	1	0.09	4	0.34
	972	2.0	840	2	0.24	3	0.36
DES	955	1.4 (T/E)	816	6	0.74	3	0.37
	959	2.0	274	1	0.36	4	1.46
	968	2.0	666	0		5	0.75
	969	2.0	502	1	0.20	3	0.60
PB	969	0.8 (T/E)	752	1	0.13	5	0.66
	970	0.8	498	0		2	0.40
	971	0.8	742	1	0.13	3	0.40
Control 1		(H_2O)	2798	1	0.04	9	0.38
Control 2		(T/E)	5892	5	0.08	20	0.34
Controls 1 + 2			8690	6	0.07	29	0.33

T/E, 1% Tween 60, 3% Ethanol.

–, Nonmutagenic in 3–4 separate experiments for the concentration tested.

The raw data are summarized in Table 2 where the frequencies of mosaic TS and ML are recorded. Of the 10 compounds evaluated, 4 carcinogens and 1 noncarcinogen gave positive responses in replicate experiments. These are hexamethylphosphoramide, *o*-toluidine, safrole,

TABLE 3
SUMMARY RESULTS ON 10 COMPOUNDS TESTED IN THE EYE MOSAIC (SRM) ASSAY

Compound	Conc. (mM)	Separate experiment	Eyes tested	Mosaic twin spots			Mosaic single spots			Twina and single spots		
				n	%	Act.	*n*	%	Act	*n*	%	Act.
HMPA	0.56	2	1298	6	0.46	+	17	1.31	+	23	1.77	+
TOL	1.0	2	1588	1	0.06	–	14	0.88	+	15	0.94	+
TOL	2.0	3	454[a]	2	0.44	–	7	1.54	+	9	1.98	+
BEN	2.0	4	2116	2	0.09	–	15	0.71	M	17	0.80	M
SAF	0.5	4	2358[a]	5	0.21	–	22	0.93	+	27	1.15	+
CAP	5.0	3	1930	3	0.16	–	20	1.04	+	23	1.19	+
ACN	5.0	3	1528	3	0.20	–	19	1.24	+	22	1.44	+
DEHP	2.0	3	1330	0		–	11	0.83	M	11	0.83	M
ZOIN	2.0	3	2222	3	0.14	–	8	0.36	–	11	0.50	–
DES	1.4/2.0	4	2258	8	0.35	M	15	0.66	M	23	1.02	M
PB	0.8	3	1992[a]	2	0.10	–	10	0.50	–	12	0.60	–
Controls		25	8690	6	0.07		29	0.33		35	0.40	

[a] Reduced fertility
+, Mutagenic, $P < 0.01$; M, weakly active, $0.01 < P < 0.05$; –, Not mutagenic.

acrylonitrile and caprolactam (noncarcinogenic). The noncarcinogen benzoin and the carcinogen phenobarbital had no apparent genotoxic activity in the eye mosaicism testing. With 3 chemicals, benzene diethylstilbestrol and diethylhexylphthalate, the effects were rather inconsistent, based on my opinion that there was a difference between replicate experiments. More experimental data are needed for a final classification of these 3 carcinogens.

Table 3 is a compilation of all the results, using for the classification the 3 categories definite positive (+), questionable positive (M) and ineffective (–). Of the 5 compounds giving a definite positive effect, i.e., producing 2–5-fold increases above the spontaneous level, 4 are carcinogens. These mutagens were merely identified as a clear positive on the basis of induced mosaic single spots (ML), whereas only HMPA produced significant numbers of TS. In other words, most of the compounds caused effects resulting in a loss of gene function at the *white*-locus (gene mutations, deletions). Thus the class of ML seems to correlate best to that of recessive lethals in the germ line.

One problem associated with the SRM-assay is the correct classification into TS and ML of small spots, which is open to personal bias. It therefore seems a better solution to put the two classes (TS and ML) into one category, and accept a compound as positive only if it gives at least a "doubling effect" in separate experiments. This procedure should prevent misclassification resulting from errors in the judgement of small spots.

Conclusions

One objective of this study was to determine the ability of this eye mosaic assay to identify carcinogens/mutagens, using a chronic application procedure. The results presented here, together with data on 40 reference mutagens (Vogel et al., 1980; Vogel, 1984; and unpublished), support the view that the SRM-assay may be more sensitive than the conventional sex-linked recessive lethal method, in particular after chronic exposure of Drosophila larvae. The validity of this tentative conclusion remains to be confirmed by further experimental work. The higher sensitivity of this new screening method seems the result of the larger number of cells scored. Moreover, a selective elimination mechanism comparable to the phenomenon of 'germinal selection' in germ cells, which puts severe limitations on the use of X-linked recessive lethal assays after larvae treatment (Vogel, 1984), does apparently not take place in somatic tissue.

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The Drosophila reversion assay using the unstable *zeste-white* somatic eye color system

Kazuo Fujikawa, Haruko Ryo [1] and Sohei Kondo [1]

Central Research Division, Takeda Chemical Industries Ltd., Osaka (Japan) and [1] Faculty of Medicine, Osaka University, Osaka (Japan)

Summary

Newly hatched male larvae carrying the unstable *white-zeste* system and an excision-repair deficient gene *mei-9^a* were treated with 10 chemicals of IPCS-Part I. Red spots in adult eyes were scored as somatic mutations. Significant dose-dependent increases of the mutation frequency were observed with HMPA, ACN and CAP. On the other hand, TOL, SAF, BEN, ZOIN, DES and PB showed no mutagenic activity, although, excepting ZOIN, they caused dose-dependent reduction in survival of the larvae. DEHP produced significant increase in the mutations only at 20 mM after treatment with 6 doses from 10 to 320 mM. Thus, among 10 chemicals tested, 3 were positive 6 negative, while one was marginally positive.

10 "Part I" chemicals were tested for mutagenicity in vivo in somatic cells of *Drosophila melanogaster* using a transposon-bearing, excision-repair-deficient, eye-color mutation system.

Materials and methods

Chemicals

The following chemicals were obtained from M.H. Draper (WHO, Geneva, Switzerland): hexamethylphosphoramide (HMPA), *o*-toluidine (TOL), benzene (BEN), safrole (SAF), caprolactam (CAP), acrylonitrile (ACN), diethylhexylphthalate (DEHP), benzoin (ZOIN), diethylstilbestrol (DES), phenobarbital (PB).

The vehicles used to prepare the test solutions and the dates of treatment are summarized in Table 1.

Drosophila stock

The stock used has the genotype *C(1)DX, y f/Y/sc z w$^+$ mei-9^a*. Males from this stock have the somatic eye-color mutation system developed by Rasmuson (Rasmuson et al., 1978) in the X-chromosome; the system comprises the *zeste* mutation (*z*, I-1.0) and the unstable *white* locus (*w$^+$*, I-1.5). The instability of the *white* locus is caused by a transposable element inserted in or near the rightmost part of the regulatory region of the locus (Rasmuson et al., 1981; Rasmuson, personal communication, 1983). Interaction of the *zeste* mutation and the unstable *white* locus manifests the typical *zeste* phenotype, namely, lemon-yellow eye color. Mutational changes in the regulatory region and probably also in the transposable element act as suppressors of the *zeste* phenotype and result in the expression of red eye-color phenotype (Rasmuson et al., 1978).

In addition to the unstable *white-zeste* system, the X-chromosome carries an excision-repair-deficient gene *mei-9^a* (I-6, Boyd et al., 1976). Females from the same stock have attached X-chromosomes *C(1)DX* with marker *yellow* (*y*, I-0.0) and *forked* (*f*, I-56.7) mutations. In the stock, therefore, the transposon-bearing, excisionless, somatic eye-color mutation system is maintained in males only.

TABLE 1
TREATMENT CONDITIONS AND DATES OF TREATMENT

Chemicals	Vehicles	Concentrations	Dates of treatement
HMPA	D.W.	12.5, 0 mM	June 22, 1983
		6.3, 3.1, 0 mM	June 29, 1983
		12.5, 6.3, 3.1, 0 mM	July 1, 1983
TOL	20% Eth	100, 10, 0 mM	June 16, 1983
		50, 25, 0 mM	June 30, 1983
		100, 50, 25, 0 mM	July 1, 1983
BEN	40% Eth	1, 0.5, 0%	June 19, 1983
		2, 1, 0.5, 0%	June 22, 1983
SAF	20% Eth	2, 1, 0.5, 0 mM	June 16, 1983
		1, 0.5, 0 mM	June 19, 1983
		2, 1, 0.5, 0 mM	July 1, 1983
CAP	D.W.	800, 400, 0 mM	May 29, 1983
		400, 200, 0 mM	June 10, 1983
		400, 200, 0 mM	June 22, 1983
ACN	D.W.	2, 1, 0 mM	June 16, 1983
		8, 4, 0 mM	June 30, 1983
		8, 4, 0 mM	July 1, 1983
DEHP	20% Eth	20, 10, 0 mM	May 13, 1983
		20, 10, 0 mM	May 31, 1983
		320, 160, 80, 40, 0 mM	June 30, 1983
ZOIN	Tw/Eth/D.W. (1:1:2)	10, 0 mg/ml	June 20, 1983
		40, 20, 10, 0 mg/ml	June 21, 1983
		80, 0 mg/ml	June 22, 1983
DES	20% Eth	50, 25, 12.5, 6.3, 0 mg/ml	June 19, 1983
		12.5, 6.3, 0 mg/ml	June 22, 1983
		50, 25, 0 mg/ml	June 28, 1983
PB	20% Eth	10, 5, 0 mg/ml	June 19, 1983
		5, 2.5, 0 mg/ml	June 22, 1983
		2.5, 0 mg/ml	June 28, 1983

D.W., distilled water; Eth, ethanol; Tw, Tween 60.

Treatment of larvae

Newly emerged males and females from the above-mentioned stock were mass mated in culture bottles for 4–5 days and transferred, at a fixed number ratio of 40 males to 40 females, into fresh culture bottles (180 ml in volume with 40 ml medium, Fig. 1A-1) to oviposit for 24 h. After the oviposition, parental flies were discarded and the resultant eggs were allowed to develop for 24 h (Fig. 1A-2).

Culture bottles containing newly hatched larvae were divided into 2–5 groups. One group served as the control and the rest for treatment with the test chemicals. To test a chemical, a 1-ml sample of the test solution at an appropriate concentration was pipetted onto the surface (18 cm^2) of the culture medium (Fig. 1A-3). Control bottles were treated in the same way with vehicles. After the larvae were treated with test solution or vehicle, they were allowed to develop in the same bottles throughout the larval period (Fig. 1B).

All the experiments were carried out at 25 ± 1°C.

Detection of somatic mutations

Eyes of adult males were surveyed for the color

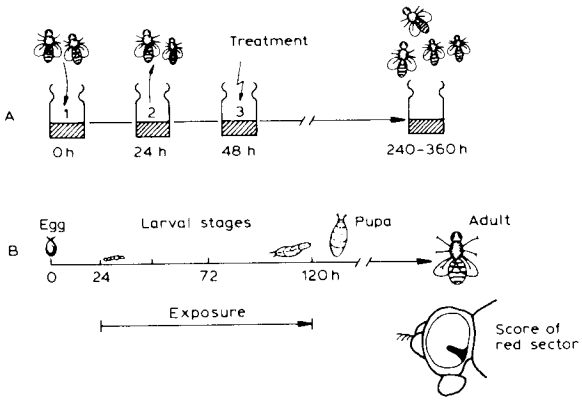

Fig. 1. (A) Procedures for treatment with chemical. (B) A scheme of potential exposure period and of a red spot scored as somatic mutation.

mutations. Red sectors consisting of 4 or more ommatidia were scored as somatic mutations (Fig. 1B). The frequency of somatic mutations is defined as the number of males with red spots divided by the number of males examined.

Toxicity test

The killing effect of test chemicals is defined as follows:

$$\text{Lethality}\,(\%) = (1 - S) \times 100 \qquad (1)$$

where S (surviving fraction) is the ratio of the average number of males emerged per treated bottles to that per control bottles.

Statistics

Necessary statistical tests were performed using Fisher's exact test.

Results

The results are summarized in Table 2.

Statistical analyses

Statistical analyses were carried out in two ways. First, we used different control data obtained for each set of experiments of a test chemical. As shown in Table 2, the frequencies of somatic eye-color mutations observed at doses of 3.1–12.5 mM HMPA, 8 mM ACN, and 20 mM DEHP are significantly higher than those observed in concurrent controls. The remaining 7 chemicals produced no significant increases in mutation frequency over the control levels, although, excepting ZOIN, they showed dose-dependent toxic effects on the survival of male larvae (Table 2).

Second, we used control data pooled among the experiments where distilled water (D.W.) was used as solvent for chemicals. When compared with the average control frequency, CAP at 400 mM become positive, that is, mutagenic, at the 5% level (Table 3). Control data obtained with 20% ethanol in different sets of experiments were also pooled (Table 3). Reanalyses of the mutagenicity data with the pooled 20% ethanol as controls confirm the nonsignificant effects of TOL, SAF, DES and PB.

Dose–response relations

As depicted in Fig. 2, HMPA, CAP and ACN increased mutation yields with increasing doses faster than linearly. On the other hand, within a wide dose range (10–320 mM), DEHP caused an increase of the frequency only at a low dose of 20 mM (Fig. 2). No marked changes in lethality around the positive dose of DEHP were observed (Table 2).

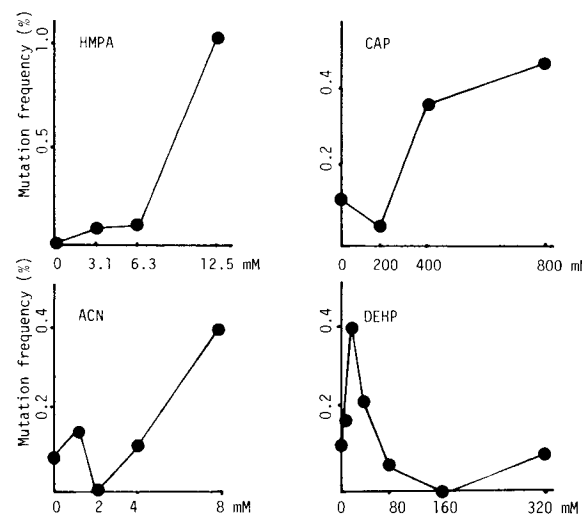

Fig. 2. Dose–response relations for somatic eye-color mutations observed after treatement with HMPA, CAP, ACN and DEHP.

TABLE 2
FREQUENCIES OF SOMATIC EYE-COLOR MUTATIONS OBSERVED AFTER TREATMENT WITH PART I CHEMICALS AT LARVAL STAGE OF *Drosophila melanogaster*

Treatment	Lethality (%)	Males (A)	Males with red spots (B)	Frequency (B/A × 100)	Fisher's exact test *P* values
HMPA					
12.5 mM (2)	30	1950	21	1.077 **	2.205×10^{-6}
6.3 mM (2)	10	2111	10	0.474 **	8.700×10^{-3}
3.1 mM (2)	0	1956	9	0.460 *	0.012
Control (3)	–	2522	2	0.079	
TOL					
100 mM (2)	50	1131	2	0.177	0.268
50 mM (2)	30	2201	2	0.091	0.173
25 mM (2)	20	2489	4	0.161	0.221
10 mM (1)	10	726	1	0.137	0.447
Control (3)	–	3324	2	0.060	
BEN					
2% (1)	50	673	0	–	0.339
1% (2)	30	1753	4	0.228	0.482
0.5% (2)	30	2127	3	0.141	0.517
Control (2)	–	2787	5	0.179	
SAF					
2 mM (2)	70	498	0	–	0.486
1 mM (3)	20	2430	3	0.123	0.521
0.5 mM (3)	10	2718	7	0.258	0.281
Control (3)	–	3206	5	0.156	
CAP					
800 mM (1)	60	215	1	0.465	0.263
400 mM (3)	10	1663	6	0.361	0.070
200 mM (2)	0	2158	1	0.046	0.360
Control (3)	–	3417	4	0.117	
ACN					
8 mM (2)	0	2837	11	0.388 *	0.018
4 mM (2)	0	2731	3	0.110	0.587
2 mM (1)	0	647	0	–	0.571
1 mM (1)	0	652	1	0.153	0.529
Control (3)	–	3153	3	0.095	
DEHP					
320 mM (1)	20	1240	1	0.081	0.639
160 mM (1)	20	1229	0	–	0.312
80 mM (1)	10	1464	1	0.068	0.568
40 mM (1)	20	1229	3	0.244	0.201
20 mM (2)	20	2266	9	0.397 *	0.010
10 mM (2)	20	2627	4	0.152	0.388
Control (3)	–	5740	6	0.105	
ZOIN					
80 mg/ml (1)	0	1194	1	0.084	0.375
40 mg/ml (1)	0	1112	2	0.180	0.646
20 mg/ml (1)	0	1760	6	0.341	0.222
10 mg/ml (2)	0	2388	7	0.293	0.296
Control (3)	–	3647	7	0.192	

TABLE 2 (continued)

Treatment	Lethality (%)	Males (A)	Males with red spots (B)	Frequency (B/A × 100)	Fisher's exact test *P* values
DES					
50 mg/ml (2)	80	324	1	0.309	0.354
25 mg/ml (2)	40	1377	3	0.218	0.307
12.5 mg/ml (2)	0	2246	5	0.223	0.241
6.3 mg/ml (2)	0	1876	3	0.160	0.458
Control (3)	–	3547	4	0.113	
PB					
10 mg/ml (1)	50	284	0	–	0.622
5 mg/ml (2)	10	1985	3	0.151	0.571
2.5 mg/ml (2)	10	2191	2	0.091	0.334
Control (3)	–	4047	7	0.173	

* Significant from the control at 5% level.
** Significant from the control at 1% level.
( ) Number of replicates.

TABLE 3
POOLING OF CONTROL DATA OBTAINED WITH DISTILLED WATER OR 20% ETHANOL IN DIFFERENT SETS OF EXPERIMENTS AND REANALYSES OF MUTAGENICITY DATA. [a]

Treatment	B/A	Frequency (%)	Fisher's exact test *P* values
D.W. control			
for HMPA exp.	2/2522	0.079	
for CAP exp.	4/3417	0.117	
for ACN exp.	3/3153	0.095	
Pooled	9/9092	0.099	
HMPA			
12.5 mM	21/1950	1.077 **	3.892×10^{-10}
6.3 mM	10/2111	0.474 **	9.636×10^{-4}
3.1 mM	9/1956	0.460 **	1.750×10^{-3}
CAP			
400 mM	6/1663	0.361 *	1.931×10^{-2}
ACN			
8 mM	11/2837	0.388 **	2.561×10^{-3}
20% ethanol control			
for TOL exp.	2/3324	0.060	
for SAF exp.	5/3206	0.156	
for DEHP exp	6/5740	0.105	
for DES exp.	4/3547	0.113	
for PB exp.	7/4047	0.173	
Pooled	24/19865	0.121	
DEHP			
20 mM	9/2266	0.397 **	4.808×10^{-3}

TABLE 4
CONCLUSIONS: MUTAGENICITY OF PART I CHEMICALS IN THE TRANSPOSON-BEARING, EXCISION-REPAIR DEFICIENT, SOMATIC EYE-COLOR MUTATION SYSTEM OF *Drosophila melanogaster*

Chemicals	Mutagenicity
HMPA	+
TOL	–
BEN	–
SAF	–
CAP	+
ACN	+
DEHP	M
ZOIN	–
DES	–
PB	–

+, positive; M, marginally positive (needs further tests); –, negative.

* Significant from the pooled control at 5% level.
** Significant from the pooled control at 1% level.
[a] The data showing nonsignificant difference from the average control data are excluded from the tabulation.

Conclusion

From the above results, we may conclude that HMPA, CAP and ACN were mutagenic, whereas TOL, BEN, SAF, ZOIN, DES and PB were non-genotoxic under the present experimental conditions.

When the strange picture of the dose–response relation (Fig. 2) is taken into account, no clear-cut decision could be made in the case of DEHP. Further experiments and analyses would be desirable to make a final judgment about the mutagenicity of this chemical.

These conclusions are summarized in Table 4.

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Somatic mutation and recombination test in wings of *Drosophila melanogaster*

F.E. Würgler, U. Graf and H. Frei

Institute of Toxicology, Swiss Federal Institute of Technology and University of Zurich (Switzerland)

Summary

10 compounds were tested for genotoxic activity in the somatic mutation and recombination test in wings of *Drosophila melanogaster*. The compounds were administered to larvae which were trans-heterozygous for two recessive wing cell markers and which in addition were either DNA-repair-proficient or excision-repair-defective. The treatments were either acute or chronic feeding, or gas exposure. The wings of the surviving flies were analyzed for single and twin mosaic spots expressing the mutant phenotype(s). HMPA, TOL, BEN, SAF, CAP and ACN were mutagenic in this test system.

A new test system for the detection of genotoxic activity of chemicals in somatic cells of *Drosophila melanogaster* has been developed. The test system makes use of genetic markers expressed on the wing blade: Larvae trans-heterozygous for the mutations multiple wing hairs (*mwh*, 3-0.0) and flare (*flr*, 3-39.0) are exposed to the test compounds for various periods of time. Induced mutations (point mutations, deletions, etc.) lead to single mosaic spots on the wings of surviving adults which express either the *mwh* or the *flr* phenotype. Induced mitotic recombination between *flr* and the centromere results in *mwh*//*flr* twin spots, whereas recombination between the two markers produces *mwh* single spots. The test system is described in detail in Graf et al. (1983, 1984), Würgler et al. (1983, 1984) and Szabad et al. (1983).

Materials and methods

Chemicals

The chemicals prepared for the collaborative study were shared with the group of P. Arni, Ciba-Geigy, Basel (Switzerland), so we were not able to do all the experiments with the official samples. We purchased commercial samples for all compounds from Fluka (Buchs, Switzerland) with the exception of DES which was bought from Sigma (St. Louis, U.S.A.). Experimental data obtained with the official samples are marked with an asterisk in the Appendix.

Stocks

For the standard cross with DNA-repair-proficient larvae we used the following flies:

Females: *y; mwh jv*
Males: *y; Dp(1;3)sc*^{J4}*, flr/TM1, Me ri sbd*^{2}

In this cross, 50% of the progeny have the desired genotype (*mwh* + / + *flr*) and can be distinguished from their sibs by a non-yellow phenotype.

For the test with excision-repair-defective larvae we crossed:

Females: *y mei-9*^{L1} *cv; mwh jv*
Males: *flr*^{3}/*TM2, Ser*

From this cross two types of progeny result: repair-proficient heterozygous (+ / *mei-9*) and excision-repair-defective (*mei-9*) larvae.

The genetic symbols are explained in Lindsley and Grell (1968), Garcia-Bellido and Dapena (1974) and Graf et al. (1979).

Treatments

Larvae from the above crosses are exposed to the chemicals as follows:

(A) Acute feeding for 2 h or 6 h at a larval age of 48 h or 72 h.

(B) Chronic feeding of larvae until pupation; beginning of feeding at a larval age of 48 h (duration of feeding 72 h) or 72 h (duration of feeding 48 h). In some cases, larvae were treated during the entire larval life (96 h treatments).

(C) Exposure of 48 h or 72 h old larvae to the gaseous chemicals for 0.5 h or 1 h.

For the acute treatments, eggs are collected for 8 h from optimally fertile flies. After 2 or 3 days, when the larvae are 48 h or 72 h old, respectively, they are collected from the bottles using a 20% NaCl solution (Nöthiger, 1970). The larvae are placed into plastic tubes which have the lower end covered with a fine nylon gauze. The tube is placed into a 100-ml beaker containing 0.3 g powdered cellulose and 1.5 ml mutagen solution. The larvae start immediately to feed through the gauze on the wet powdered cellulose. At the end of the feeding, the plastic tube with the larvae is removed and the larvae are rinsed with tap water and then flushed into a culture vial containing 5 ml of dry Instant Medium (Carolina Biological Supply Company, Burlington NC, U.S.A.). The cultures are kept at 25 °C until the adult flies hatch.

For 72 h and 48 h feeding the larvae are collected as described above. They are placed into culture vials containing 5 ml Instant Medium and 5 ml mutagen solution. For the treatment of larvae during their entire development (96 h), culture vials with Instant Medium and mutagen solution are prepared. Several pairs of flies are put into each vial where they are allowed to lay eggs for 24 h. With all chronic treatments the treated individuals remain in the vials until the emergence of the surviving adult flies. For the gas treatment, 48 h or 72 h old larvae are collected as described above. They are introduced into a 1150-ml plasma bottle with an airtight seal. The mutagen is then injected into the bottle where it evaporates immediately. After 0.5 h or 1 h the larvae are flushed from the bottle with tap water and transferred to normal culture vials with Instant Medium.

Analysis of wings

Wings of surviving adults are mounted in Faure's solution. They are scored under a compound microscope for the presence of single spots (*mwh* or *flr*) and for the presence of twin spots (one part *mwh*, the other *flr*). The number of wings scored and the frequency and size of the spots are recorded. For the evaluation of the data the spots are grouped into 4 categories: small single spots (1 or 2 cells), large single spots (3 and more cells), total single spots, and twin spots.

Results and discussion

The various treatment conditions used with the 10 compounds are given in Table 1. The toxicity of the chronic treatments is listed in Table 2. The detailed spot-frequency and spot-size data together with the results of the statistical tests are compiled in the Appendix.

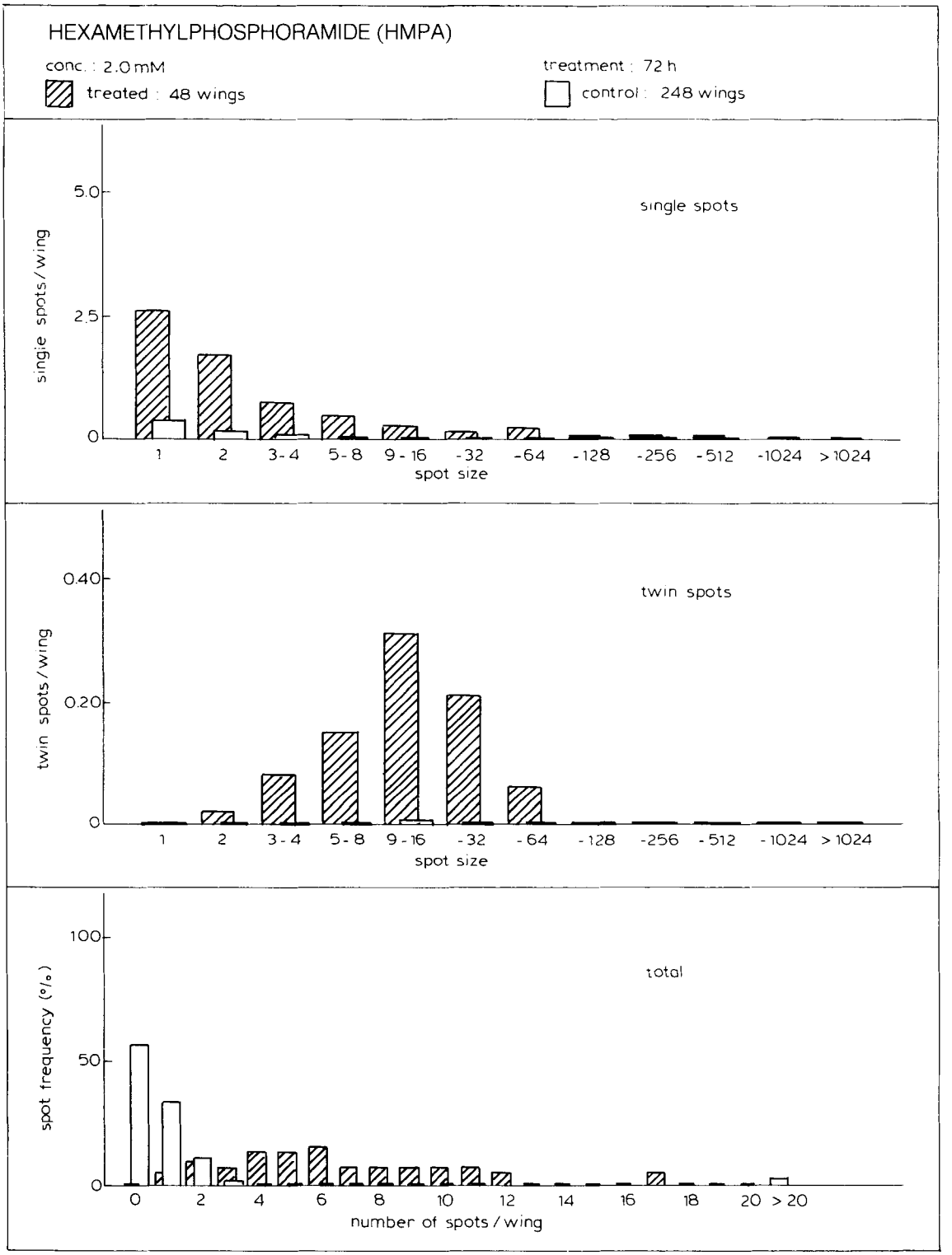
The interpretation of the test results is primarily based on the frequency of wings with spots in a treated and a control series and on the spot-density distribution considering the number of spots observed per wing. The spot-size distribution gives only additional information in the case of acute treatments with strong mutagens, in particular if larvae treated at different stages are to be compared. For chronic treatments we do not expect a pronounced effect of the mutagen on the spot-size distribution. In evaluating spot induction for each spot category in each experiment, we considered those outcomes statistically most reliable in which a congruent diagnosis was provided by the Selby–Olson analysis (considering the frequency of wings with spots) as well as by the chi-square and/or the Kolmogorov–Smirnov tests in their application to the spot-density data (see Graf et al., 1984). It is our experience that for general screening purposes, chronic treatments yield better results than acute treatments. Chronic treatments may go over 1, 2, or all 3 larval stages and thus allow for the time needed to induce the metabolic activation processes when these are required to render a compound mutagenically active. Therefore, negative results obtained after chronic exposure have more weight in the evaluation of the data in comparison to negative outcomes of acute exposures. There are a few instances in which acute exposure might be indicated, e.g. in testing of short-lived compounds (Graf et al., 1984) or volatile compounds such as ACN and BEN for example.

TABLE 1

TREATMENT CONDITIONS

Compound	Vehicle [a]	Concentration (mM)	Larval age at beginning (h)	Duration of treatment (h)
Cross with repair-proficient larvae				
HMPA	2% DMSO	0.57	72	6
	5% T-E	10	72	6
	5% T-E	2	48	72
TOL	5% E	9.41	48	6
		47.0	72	6
		94.0	72	6
		4.7	48	72
BEN	gas exposure	0.2 ml/1150 ml	72	1
		0.3 ml/1150 ml	72	1
SAF	5% T-E	6.76	48	6
		6.76	72	6
		0.68	48	72
		0.68	1	96
CAP	water	442	48	6
		663	48	6
		884	48	6
		442	72	6
		1768	72	6
		8.84	48	72
		44.2	48	72
ACN	gas exposure	1 μl/1150 ml	72	1
DEHP	5% T-E	200	72	48
		200	48	72
		200	1	96
ZOIN	5% T-E	200	72	48
		200	48	72
		200	1	96
DES	5% T-E	3.73	48	72
		18.65	48	72
PB	5% T-E	20	72	48
		20	48	72
Cross with excision-repair-defective larvae				
TOL	5% E	0.94	72	48
		0.94	48	72
BEN	gas exposure	0.05 ml/1150 ml	48	1
	5% T-E	11.25	72	2
		56.25	48	2
		56.25	1	96
SAF	5% T-E	0.34	1	96
		0.68	1	96
ACN	gas exposure	1 μl/1150 ml	48	0.5
		1 μl/1150 ml	48	1
		0.5 μl/1150 ml	48	1
	5% T-E	15.19	48	2
	water	1.52	1	96
DEHP	5% T-E	200	1	96

[a] Vehicle: 2% DMSO, 2% dimethyl sulfoxide.
5% E, 5% ethanol.
5% T-E, 5% Tween 80 + 5% ethanol.
For gas exposures the volume of liquid added to a bottle (1150 ml air) containing the larvae is given.


HEXAMETHYLPHOSPHORAMIDE (HMPA)
conc. : 2.0 mM
treated : 48 wings
treatment : 72 h
control : 248 wings
single spots
singie spots / wing
5.0
2.5
0
1
2
3-4
5-8
9-16
-32
-64
-128
-256
-512
-1024
>1024
spot size
twin spots
twin spots / wing
0.40
0.20
0
spot size
total
spot frequency (%)
100
50
0
0
2
4
6
8
10
12
14
16
18
20
>20
number of spots / wing

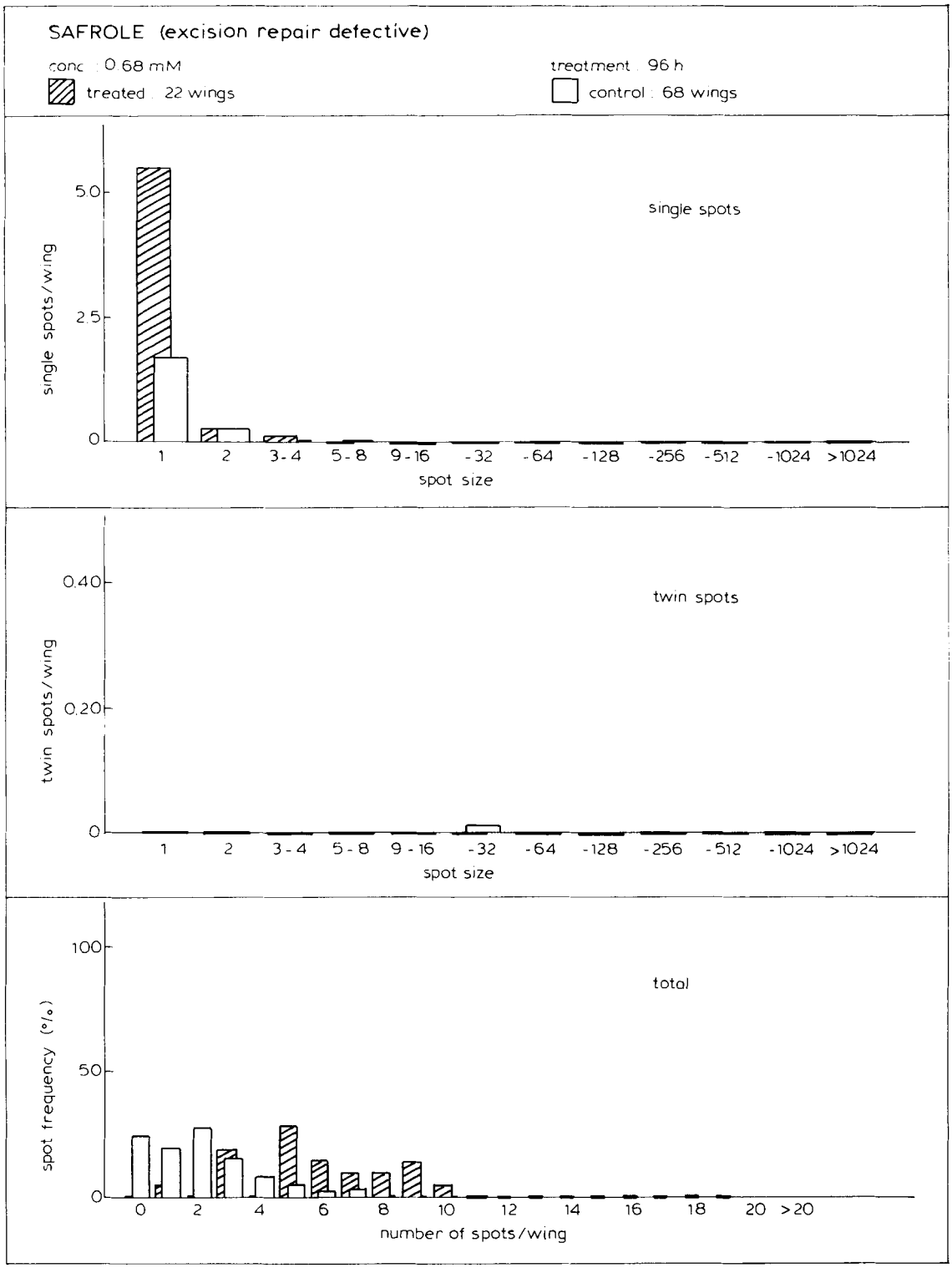


Fig. 1. Spot-size distributions for single and twin spots and spot-density distributions for all spots after chronic feeding of HMPA (72 h) and SAF (96 h). HMPA was tested in the standard cross, whereas with SAF the cross with excision-repair-defective (*mei-9*) larvae is represented.

TABLE 2

CHRONIC EXPOSURES: TOXICITY

Compound	Vehicle [a]	Treatment (h)	Concentration (mM)	Diagnosis
HMPA	5% T-E	72	5.0	lethal
			2.0	toxic
			1.0	not toxic
TOL	5% E	72	9.4	lethal
			4.7	not toxic
		96	4.7	lethal
			1.9	not toxic
BEN	5% T-E	96	112.5	lethal
			56.3	toxic
SAF	5% T-E	72	3.4	lethal
			2.0	toxic
			0.7	not toxic
		96	3.4	lethal
			0.7	not toxic
CAP	water	72	88.4	lethal/few flies
			44.2	not toxic
		96	44.2	lethal
			8.8	not toxic
ACN	5% T-E	96	7.6	lethal
			1.5	toxic
DEHP	5% T-E	72	200.0	not toxic
		96	200.0	not toxic
ZOIN	5% T-E	72	200.0	not toxic
		96	200.0	not toxic
DES	5% T-E	72	37.3	lethal
			18.7	not toxic
		96	18.7	toxic
PB	5% T-E	72	200.0	lethal
			50.0	toxic
		96	5.0	lethal

[a] Vehicle: 5% E, 5% ethanol.
5% T-E, 5% Tween 80 + 5% ethanol.

The evaluation of the data leads to the following conclusions which are summarized in Table 3.

HMPA

Acute as well as chronic treatments gave strong positive results. The result of the chronic treatment is shown in Fig. 1.

TOL

With the excision-proficient standard cross, the acute exposures carried out with relatively high concentrations gave a positive response in one case. With chronic exposures only sensibly lower concentrations could be tested. The chronic exposures with both progeny types (+/*mei-9* as well as *mei-9*) in the *mei-9* cross did not yield clear-cut positive results. The only result showing a seemingly significant difference from the control was obtained after feeding 0.94 mM for 48 h to +/*mei-9* larvae; but the frequency of 50.0% wings with small single spots is within the range of variation of ethanol control samples. We consider the acute treatments with high concentrations as more conclusive and therefore classify TOL as positive in acute treatments. The effects of chronic feeding need additional experiments, and TOL is therefore classified as marginally positive for this type of treatment.

BEN

Overall, a significant increase of the frequency of small single spots and/or total single spots was observed in 4 experimental series. The effects are not strong because only one case shows a positive outcome in the Kolmogorov–Smirnov test comparing spot-density distributions (SP/W). All the other decisions are based on the Selby–Olson analysis. It is worthwile to note that 3 of the 4 positive series were obtained within the set of 4 series conducted with excision-repair-defective larvae. With repair-proficient larvae, only one experiment at the higher concentration of the gas exposure series provided a positive result. All the other treatments were ineffective. With excision-repair-defective larvae, however, chronic feeding and gas treatment gave positive results. Acute feeding is considered as marginally positive in outcome because of a positive result in one of the experiments which could not be confirmed in a second assay with higher exposure of younger larvae.

SAF

The experimental series with the standard cross showed a few statistically positive responses. The strongest effect was seen with the chronic treatment during 96 h. Since the Selby–Olson analysis is positive but the chi-square and the Kolmogorov–Smirnov tests for the density distribution

TABLE 3

INTERPRETATION OF DATA

Compound	Excision-repair-proficient			Excision-repair-defective			Consensus		
	A	B	C	A	B	C	A	B	C
HMPA	+	+					+	+	
TOL	+	M			M		+	M	
BEN	−	−	+	M	+	+	M	M	+
SAF	M	M			+		M	+	
CAP	+	M					+	M	
ACN	−	+	M	−	M	+	−	M	M
DEHP		M			−			−	
ZOIN		−						−	
DES		−						−	
PB		−						−	

A, acute feeding (2 h, 6 h).
B, chronic feeding (48 h, 72 h, 96 h).
C, gas treatment (0.5 h, 1 h).
+, positive.
M, marginally positive.
−, negative.

are significant for the pooled single spots only (not for the small single spots), the overall result with these crosses is only marginally positive. The acute treatments are also considered marginally positive, because of the weak positive result in one of the two experiments. By contrast, both concentrations tested with excision-repair-defective (*mei-9*) larvae gave a clear positive response for the small single spots. An example is shown in Fig. 1. Therefore, we consider SAF to be positively mutagenic in the tests with excision-repair-defective larvae.

CAP

This compound is not a strong mutagen and has a low toxicity for Drosophila larvae in acute treatments. In consequence, high concentrations were tested. In two experimental series with acute treatments not only the frequency of small single spots but also the frequency of large single spots were significantly higher than in the control according to the Selby–Olson analysis. This is a good indication for mutagenic activity in the wing spot test. With chronic treatments a positive response was obtained at a low concentration, but a negative one at a higher exposure. Until the exposure–effect relationship of this compound is analyzed in detail, we classify the effect of chronic treatments as marginally positive.

ACN

Compared to the situation encountered with BEN, ACN is an even weaker mutagen in the Drosophila wing spot test. With ACN gas exposure of larvae of the standard cross a positive result was obtained as in the case of BEN. A statistically positive result for twins spots in the series with heterozygous (+/*mei-9*) larvae clearly appears as a false positive result (see also below for DEHP). On the other hand an exposure to 1.52 mM for the whole larval period was positive in the heterozygous, excision-repair-proficient as well as in the excision-repair-defective larvae. In this case the frequencies of wings with spots are near to but above the upper sample variation limit estimated from the corresponding controls. Overall, ACN seems to be a weak mutagen. We classify ACN as marginally positive, because of a limited reproducibility of the positive outcome in gas-exposure experiments with excision-repair-proficient larvae, and also because in the experiments with excision-repair-defective larvae the different statistical analyses did not provide internally consistent positive diagnoses throughout.

DEHP

Although most outcomes with DEHP were negative, the Selby–Olson statistical analysis gave a positive diagnosis for the twin spots in the experimental series with 200 mM fed for 48 h, whereas the diagnosis for the total of single spots was negative. This is uncommon and also theoretically not expected. Hitherto, all the chemicals that had shown strong recombinogenic activity (positive result in the twin spot category), had always also induced single spots (recombination between the two markers *mwh* and *flr*). Because of this observation and because the data evaluation for the twin spots is based on only 1 twin spot in the control and 2 twin spots in the treated series, we classify DEHP as nongenotoxic in our test.

ZOIN

Overall the results obtained with ZOIN are negative. There is one case with a significant chi-square in the comparison of spot-density distributions. Obviously this is due to chance variation and represents a false positive result.

DES

The DES studies gave only negative results. The Selby–Olson diagnosis for the small single spots seen in the series fed 3.73 mM for 72 h appears as a false positive result, because a similarly positive result does not appear at the higher exposure level and also because the frequency of wings with spots is within the range of corresponding control samples.

PB

Two experimental series gave clearly negative results. A long chronic exposure was not possible because of an increased toxic effect in young larvae, as compared to older larvae. We may consider the possibility that PB could be mutagenic, but only at exposure levels that are impractical because of the toxic effects. But no genotoxic effects were observed after chronic treatment of 48 h and 72 h old larvae (72 h and 48 h treatment, respectively), i.e. at stages where we believe that the balance of toxic versus mutagenic effects would be more favorable for the detection of mutagenic activity.

Conclusion

We conclude that of the 10 chemicals tested, HMPA, TOL, BEN, SAF, CAP and ACN were genotoxic in our assay. Depending on the physical properties of the chemicals, acute and chronic feeding and/or gas treatment of the larvae proved to be effective. 5 compounds were tested with *mei-9* excision-repair-defective larvae. The cases of SAF and BEN show that the use of this mutant type of larvae can improve the detection capacity of the somatic mutation and recombination test in wings of *Drosophila melanogaster.*

Acknowledgement

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Appendix

SOMATIC MUTATION AND RECOMBINATION TEST IN *Drosophila melanogaster*

Codes

Controls		Standard cross	
	C1		water
	C2		5% ethanol (72 h)
	C3		5% Tween 80 + 5% ethanol (72 h)
	C4		5% Tween 80 + 5% ethanol (96 h)
	C5		2% DMSO (6 h; > 72 h)
		Heterozygous (+ /*mei-9*)	
	Ch1		water (or air)
	Ch2		5% ethanol (72 h)
	Ch3		5% Tween 80 + 5% ethanol (2 h)
	Ch4		5% Tween 80 + 5% ethanol (96 h)
		Excision repair defective (*mei-9*)	
	Ce1		water (or air)
	Ce2		5% ethanol (72 h)
	Ce3		5% Tween 80 + 5% ethanol (2 h)
	Ce4		5% Tween 80 + 5% ethanol (96 h)
Treatments	f		feeding
	g		gas exposure
	2 h		exposure period 2 h
	(> 48 h) (> 72 h)		treatment of 48 h or 72 h old larvae, respectively
	*		official IPCS sample of compound used
Wings with spots			
Type	s		total of single spots
	s 1–2		small single spots
	s > 2		large single spots
	t		twin spots
Statistics	Conclusion		Selby–Olson analysis
	m		induced frequency no less than m times control m = 1 for small single spots (s 1 – 2) and for total single spots (s) m = 4 for large single spots (s > 2) and for twin spots (t)
	NEG		negative
	INC		inconclusive
	POS		positive
	WEAK		weak positive Selby–Olson analysis not possible
	SIG		if m too large, but Fisher exact test significant
	NS		if m too large, but Fisher exact test not significant
	Size distribution/density distribution		
	SP/W		mean number of spots per wing
	C		Chi-square test at 5% level of significance (2-sided)
	K		Kolmogorov–Smirnov-test at 5% level of significance (2-sided for size distribution, 1-sided for density distribution)
	+		significant
	–		not significant
	(blank)		calculation not possible (sample too small)

Appendix (continued)

Chemical concentration (mM)		Treatment	Control	Wings with spots			Conclusion	Size-distribution			Density-distribution		
				N	Type	%		Mean class	C	K	SP/W	C	K
C1	f	72 h		176	s	27.27		1.33			0.31		
					s 1–2	25.57		1.18			0.29		
					s > 2	1.70		4.00			0.02		
					t	1.14		5.50			0.01		
C2	f	72 h		80	s	38.75		1.70			0.54		
					s 1–2	36.25		1.41			0.49		
					s > 2	5.00		4.50			0.05		
					t	3.75		5.00			0.04		
C3	f	72 h		208	s	45.19		1.62			0.58		
					s 1–2	40.38		1.26			0.50		
					s > 2	7.21		4.13			0.07		
					t	0.48		5.00			0.005		
C4	f	96 h		40	s	35.00		1.26			0.47		
					s 1–2	35.00		1.17			0.45		
					s > 2	2.50		3.00			0.03		
					t	0		0			0		
C5	f	72 h		162	s	29.63		1.48			0.32		
					s 1–2	26.54		1.28			0.29		
					s > 2	3.09		3.40			0.03		
					t	1.85		5.33			0.02		
		heterozygous (+/*mei-9*)											
Ch1	f	2 h		136	s	28.68		1.48			0.34		
					s 1–2	28.68		1.34			0.32		
					s > 2	1.47		4.50			0.01		
					t	1.47		6.00			0.01		
Ch2	f	72 h		26	s	11.54		4.33			0.12		
					s 1–2	3.85		1.00			0.04		
					s > 2	7.69		6.00			0.08		
					t	0		0			0		
Ch3	f	2 h		62	s	29.03		1.36			0.35		
					s 1–2	29.03		1.36			0.35		
					s > 2	0		0			0		
					t	1.61		6.00			0.02		
Ch4	f	96 h		62	s	17.74		1.57			0.23		
					s 1–2	16.13		1.23			0.21		
					s > 2	1.61		6.00			0.02		
					t	0		0			0		
		excision-repair-defective (*mei-9*)											
Ce1	f	2 h		100	s	74.00		1.25			1.66		
					s 1–2	74.00		1.15			1.60		
					s > 2	6.00		4.00			0.06		
					t	0		0			0		
Ce2	f	72 h		50	s	76.00		1.28			2.00		
					s 1–2	74.00		1.11			1.94		
					s > 2	6.00		6.67			0.06		
					t	0		0			0		

Appendix (continued)

Chemical concentration (mM)		Treatment	Control	Wings with spots			Conclusion	Size-distribution			Density-distribution		
				N	Type	%		Mean class	C	K	SP/W	C	K
Ce3	f	2 h		56	s	71.43		1.23			1.38		
					s 1–2	71.43		1.17			1.36		
					s > 2	1.79		6.00			0.02		
					t	0		0			0		
Ce4	f	96 h		68	s	76.47		1.17			1.96		
					s 1–2	76.47		1.13			1.93		
					s > 2	2.94		3.50			0.03		
					t	1.47		6.00			0.01		
Hexamethylphosphoramide (HMPA)													
0.57	f	6 h (> 72 h)	C5	48	s	64.58	POS	3.08	+	+	1.31	+	+
					s 1–2	39.58	NEG	1.21	−	−	0.58	+	−
					s > 2	50.00	POS	4.57			0.73	+	+
					t	12.50	POS	5.14			0.15		−
10.0	f	6 h (> 72 h)	C3	48	s	100.00	POS	1.48	+	−	3.67	+	+
					s 1–2	100.00	POS	1.10	+	−	3.29	+	+
					s > 2	29.17	POS	4.83	−	−	0.37	+	+
					t	6.25	POS	6.67			0.06		−
2.0	f	72 h	C3	48	s	100.00	POS	2.29	+	+	6.04	+	+
					s 1–2	97.92	POS	1.39	+	−	4.27	+	+
					s > 2	79.17	POS	4.47	−	−	1.77	+	+
					t	54.17	POS	4.95			0.83	+	+
o-Toluidine (TOL)													
9.41	f	6 h (> 48 h)	C2	40	s	50.00	NEG	1.59	−	−	0.68	−	−
					s 1–2	47.50	NEG	1.25	−	−	0.60	−	−
					s > 2	5.00	NEG	4.33			0.08		−
					t	2.50	NEG	7.00			0.03		−
47.0 *	f	6 h (> 72 h)	C2	34	s	73.53	POS	1.67	−	−	1.35	+	+
					s 1–2	64.71	POS	1.20	−	−	1.03	+	+
					s > 2	32.35	POS	3.18			0.32	+	+
					t	0	NS	0			0		−
4.70	f	72 h	C2	30	s	56.67	NEG	1.70	−	−	0.67	−	−
					s 1–2	50.00	NEG	1.35	−	−	0.57	−	−
					s > 2	10.00	NEG	3.67			0.10		−
					t	0	NEG	0			0		−
		Heterozygous (+/*mei-9*)											
0.94	f	48 h	Ch2	20	s	50.00	POS	2.08			0.65	+	+
					s 1–2	50.00	POS	1.30			0.50		+
					s > 2	15.00	NEG	4.67			0.15		−
					t	0	NS	0			0		−
0.94	f	72 h	Ch2	36	s	22.22	INC	2.13			0.22		−
					s 1–2	19.44	INC	1.14			0.19		−
					s > 2	2.78	NEG	9.00			0.03		−
					t	2.78	NS	5.00			0.03		−

Appendix (continued)

Chemical concentration (mM)		Treatment	Control	Wings with spots			Conclusion	Size-distribution			Density-distribution		
				N	Type	%		Mean class	C	K	SP/W	C	K
		excision-repair-defective (*mei-9*)											
0.94	f	48 h	Ce2	22	s	72.73	NS	1.20	–	–	1.59	–	–
					s 1–2	68.18	NS	1.06		–	1.50	–	–
					s > 2	9.09	NEG	3.50			0.09		–
					t	0	NS	0			0		–
0.94	f	72 h	Ce2	48	s	89.58	NS	1.27	–	–	2.69	–	+
					s 1–2	87.50	NS	1.14	–	–	2.60	–	+
					s > 2	8.33	NEG	5.50			0.08		–
					t	0	NS	0			0		–
Benzene (BEN)													
0.2 ml/1150 ml	g	1 h (> 72 h)	C1	20	s	30.00	NEG	1.83			0.30	–	–
					s 1–2	25.00	NEG	1.20			0.25	–	–
					s > 2	5.00	INC	5.00			0.05		–
					t	0	INC	0			0		–
0.3 ml/1150 ml	g	1 h	C1	20	s	55.00	POS	1.23			0.65	+	–
					s 1–2	55.00	POS	1.23			0.65	+	+
					s > 2	0	INC	0			0		–
					t	5.0	INC	5.00			0.05		–
		heterozygous (+/*mei-9*)											
11.25	f	2 h (> 72 h)	Ch3	30	s	3.33	NEG	2.00			0.03	+	–
					s 1–2	3.33	NEG	2.00			0.03	+	–
					s > 2	0	NS	0			0		–
					t	0	INC	0			0		–
56.25	f	2 h (> 48 h)	Ch3	40	s	32.50	NEG	1.39	–	–	0.45	–	–
					s 1–2	32.50	NEG	1.39	–	–	0.45	–	–
					s > 2	0	NS	0			0		–
					t	2.50	INC	5.00			0.03		–
56.25 *	f	96 h	Ch4	70	s	28.57	INC	1.63	–		0.43	–	–
					s 1–2	25.71	INC	1.43			0.40	–	–
					s > 2	2.86	INC	4.50			0.03		–
					t	1.43	NS	5.00			0.01		–
		excision-repair-defective (*mei-9*)											
0.05 ml/1150 ml	g	1 h (> 48 h)	Ce1	102	s	90.20	SIG	1.24	–	–	2.09	–	–
					s 1–2	89.22	SIG	1.14	–	–	2.02	–	–
					s > 2	6.86	NEG	4.14			0.07	–	–
					t	0.98	NS	4.00			0.01		–
11.25	f	2 h (> 72 h)	Ce3	26	s	92.31	SIG	1.26	–	–	2.77	+	+
					s 1–2	92.31	SIG	1.16	–	–	2.65	+	+
					s > 2	7.69	INC	3.67			0.12		–
					t	0	NS	0			0		–
56.25	f	2 h (> 48 h)	Ce3	52	s	67.31	NS	1.26	–	–	1.62	+	–
					s 1–2	67.31	NS	1.11	–	–	1.54	+	–
					s > 2	7.69	INC	4.25			0.08		–
					t	0	NS	0			0		–

Appendix (continued)

Chemical concentration (mM)		Treatment	Control	Wings with spots			Conclusion	Size-distribution			Density-distribution		
				N	Type	%		Mean class	C	K	SP/W	C	K
56.25 *	f	96 h	Ce4	42	s	95.24	SIG	1.26	–	–	1.64	+	–
					s 1–2	90.48	NS	1.14	–	–	1.57	+	–
					s > 2	7.14	INC	4.00			0.07		–
					t	2.38	INC	4.00			0.02		–
Safrole (SAF)													
6.76	f	6 h (> 48 h)	C3	76	s	59.21	WEAK	1.73	–	–	0.99	+	–
					s 1–2	55.26	WEAK	1.23	–	–	0.84	+	–
					s > 2	11.84	NEG	4.64	–		0.14	–	–
					t	0	INC	0			0		–
6.76	f	6 h (> 72 h)	C3	40	s	45.00	NEG	1.76	–	–	0.52	–	–
					s 1–2	42.50	NEG	1.47	–	–	0.47	–	–
					s > 2	5.00	NEG	4.50			0.05		–
					t	2.50	INC	3.00			0.03		–
0.68	f	72 h	C3	40	s	40.00	NEG	1.61	–	–	0.57	–	–
					s 1–2	35.00	NEG	1.24	–	–	0.52	–	–
					s > 2	5.00	NEG	5.50			0.05		–
					t	2.50	INC	4.00			0.03		–
0.68 *	f	96 h	C4	40	s	67.50	POS	1.60	–	–	1.05	+	+
					s 1–2	60.00	POS	1.25		–	0.90	–	–
					s > 2	12.50	INC	3.67			0.15		–
					t	0	NS	0			0		–
		heterozygous (+ /*mei-9*)											
0.34 *	f	96 h	Ch4	36	s	22.22	INC	1.56			0.25	–	–
					s 1–2	19.44	INC	1.25			0.22	–	–
					s > 2	2.78	INC	4.00			0.03		–
					t	2.78	NS	5.00			0.03		–
0.68 *	f	96 h	Ch4	34	s	17.65	NEG	1.83			0.18	–	–
					s 1–2	14.71	NEG	1.40			0.15	–	–
					s > 2	2.94	INC	4.00			0.03		–
					t	0	NS	0			0		–
		excision-repair-defective (*mei-9*)											
0.34 *	f	96 h	Ce4	38	s	92.11	SIG	1.09	–	–	3.92	+	+
					s 1–2	92.11	SIG	1.07	–	–	3.87	+	+
					s > 2	5.26	INC	3.00			0.05		–
					t	0	INC	0			0		–
0.68 *	f	96 h	Ce4	22	s	100.00	SIG	1.08	+	–	5.82	+	+
					s 1–2	100.00	SIG	1.05	+	–	5.73	+	+
					s > 2	9.09	INC	3.00			0.09		–
					t	0	INC	0			0		–
Caprolactam (CAP)													
442	f	6 h (> 48 h)	C1	40	s	57.50	POS	1.45	–	–	0.82	+	+
					s 1–2	55.00	POS	1.27	–	–	0.75	+	+
					s > 2	7.50	INC	3.33			0.08		–
					t	0	INC	0			0		–

Appendix (continued)

Chemical concentration (mM)		Treatment	Control	Wings with spots			Conclusion	Size-distribution			Density-distribution		
				N	Type	%		Mean class	C	K	SP/W	C	K
663	f	6 h (> 48 h)	C1	40	s	57.50	POS	1.53	−	−	0.75	+	+
					s 1–2	55.00	POS	1.15		−	0.65	+	+
					s > 2	10.00	POS	4.00			0.10		−
					t	5.00	INC	5.00			0.05		−
884 *	f	6 h (> 48 h)	C1	40	s	47.50	POS	1.42	−	−	0.65	+	−
					s 1–2	47.50	POS	1.17		−	0.57	+	+
					s > 2	7.50	INC	3.33			0.08		−
					t	2.50	INC	6.00			0.03		−
442	f	6 h (> 72 h)	C1	20	s	55.00	POS	1.50			0.60	+	−
					s 1–2	50.00	POS	1.36			0.55	+	−
					s > 2	5.00	INC	3.00			0.05		−
					t	5.00	INC	5.00			0.05		−
1768	f	6 h (> 72 h)	C1	46	s	60.87	POS	1.66	+	−	0.76	+	+
					s 1–2	54.35	POS	1.31	−	−	0.63	+	+
					s > 2	13.04	POS	3.33			0.13		−
					t	2.17	INC	4.00			0.02		−
8.84	f	72 h	C1	20	s	60.00	POS	1.31			0.65	+	+
					s 1–2	60.00	POS	1.31			0.65	+	+
					s > 2	0	INC	0			0		−
					t	0	INC	0			0		−
44.2	f	72 h	C1	38	s	36.84	NEG	1.38	−	−	0.55	−	−
					s 1–2	34.21	NEG	1.30		−	0.53	−	−
					s > 2	2.63	INC	3.00			0.03		−
					t	0	INC	0			0		−
Acrylonitrile (ACN)													
1 µl/1150 ml	g	1 h (> 72 h)	C1	40	s	47.50	POS	1.19	−	−	0.65	+	−
					s 1–2	47.50	POS	1.19		−	0.65	+	+
					s > 2	0	NEG	0			0		−
					t	0	INC	0			0		−
		heterozygous (+ / *mei-9*)											
1 µl/1150 ml	g	1 h (> 48 h)	Ch1	59	s	33.90	NEG	1.80	−		0.42	−	−
					s 1–2	28.81	NEG	1.38			0.36	−	−
					s > 2	6.78	NEG	4.00			0.07		−
					t	1.69	NEG	5.00			0.02		−
1 µl/1150 ml	g	0.5 h (> 48 h)	Ch1	30	s	43.33	NEG	2.00	−		0.53	−	−
					s 1–2	33.33	NEG	1.46			0.43	−	−
					s > 2	10.00	INC	4.33			0.10	−	−
					t	3.33	NEG	6.00			0.03		−
75.95	f	2 h (> 72 h)	Ch3	20	s	25.00	NEG	2.89	−		0.45	−	−
					s 1–2	20.00	NEG	1.00			0.20	−	−
					s > 2	15.00	SIG	4.40			0.25		−
					t	15.00	POS	5.60			0.25		−
15.19	f	2 h (> 48 h)	Ch3	30	s	23.33	NEG	2.25			0.27	−	−
					s 1–2	16.67	NEG	1.20			0.17	−	−
					s > 2	6.67	NS	4.00			0.10		−
					t	0	INC	0			0		−

Appendix (continued)

Chemical concentration (mM)		Treatment	Control	Wings with spots			Conclusion	Size-distribution			Density-distribution		
				N	Type	%		Mean class	C	K	SP/W	C	K
1.52 *	f	96 h	Ch1	52	s	51.92	POS	1.45	–	–	0.63	+	+
					s 1–2	46.15	POS	1.17	–	–	0.56	–	–
					s > 2	7.69	INC	3.50			0.08		–
					t	5.77	INC	5.33			0.06		–
		excision-repair-defective (*mei-9*)											
1 µl/1150 ml	g	1 h (> 48 h)	Ce1	26	s	73.08	NS	1.32	–	–	1.92	–	–
					s 1–2	73.08	NS	1.19	–	–	1.81	–	–
					s > 2	11.54	NEG	3.33			0.12		–
					t	0	NS	0			0		–
1 µl/1150 ml	g	0.5 h (> 48 h)	Ce1	54	s	85.19	NS	1.29	–	–	2.48	–	–
					s 1–2	85.19	NS	1.17	–	–	2.35	+	–
					s > 2	7.41	NEG	3.43			0.13		–
					t	1.85	NS	4.00			0.02		–
0.5 µl/1150 ml	g	1 h (> 48 h)	Ce1	66	s	75.76	NS	1.17	–	–	2.02	+	–
					s 1–2	75.76	NS	1.14	–	–	1.98	+	–
					s > 2	3.03	NEG	3.50			0.03		–
					t	1.52	NS	6.00			0.02		–
15.19	f	2 h (> 48 h)	Ce3	30	s	76.67	NS	1.27	–	–	1.33	–	–
					s 1–2	73.33	NS	1.16	–	–	1.27	–	–
					s > 2	6.67	INC	3.50			0.07		–
					t	0	NS	0			0		–
1.52 *	f	96 h	Ce1	50	s	90.00	SIG	1.19	–	–	2.36	–	–
					s 1–2	90.00	SIG	1.12	–	–	2.28	+	–
					s > 2	8.00	NEG	3.25			0.08		–
					t	4.00	NS	6.00			0.04		–
Di(ethylhexyl)phthalate (DEHP)													
200	f	48 h	C3	37	s	59.46	NEG	1.47	–	–	0.92	+	–
					s 1–2	56.76	WEAK	1.26	–	–	0.84	–	–
					s > 2	8.11	NEG	3.67			0.08		–
					t	5.41	POS	4.00			0.05		–
200	f	72 h	C3	48	s	37.50	NEG	1.57	–	–	0.44	–	–
					s 1–2	31.25	NEG	1.18		–	0.35	–	–
					s > 2	8.33	NEG	3.25			0.08		–
					t	0	INC	0			0		–
200	f	96 h	C4	48	s	56.25	WEAK	1.21		–	0.79	–	–
					s 1–2	54.17	NEG	1.14		–	0.77	–	–
					s > 2	2.08	NEG	4.00			0.02		–
					t	0	NS	0			0		–
		heterozygous (+/*mei-9*)											
200	f	96 h	Ch4	18	s	27.78	INC	1.67			0.33		–
					s 1–2	27.78	INC	1.40			0.28		–
					s > 2	5.56	INC	3.00			0.06		–
					t	0	NS	0			0		–

Appendix (continued)

Chemical concentration (mM)		Treatment	Control	Wings with spots			Conclusion	Size-distribution			Density-distribution		
				N	Type	%		Mean class	C	K	SP/W	C	K
		excision-repair-defective (*mei-9*)											
200	f	96 h	Ce4	22	s	86.36	NS	1.14	–	–	2.00	–	–
					s 1–2	86.36	NS	1.09	–	–	1.95	–	–
					s > 2	4.55	INC	3.00			0.05		–
					t	0	INC	0			0		–
Benzoin (ZOIN)													
200	f	48 h	C3	48	s	43.75	NEG	1.28	–	–	0.75	–	–
					s 1–2	43.75	NEG	1.28	–	–	0.75	+	–
					s > 2	0	NEG	0			0		–
					t	2.08	INC	5.00			0.02		–
200	f	72 h	C3	50	s	44.00	NEG	1.29	–	–	0.56	–	–
					s 1–2	44.00	NEG	1.22	–	–	0.54	–	–
					s > 2	2.00	NEG	3.00			0.02		–
					t	2.00	INC	4.00			0.02		–
200	f	96 h	C4	48	s	35.42	NEG	1.73	–	–	0.46	–	–
					s 1–2	33.33	NEG	1.32		–	0.40	–	–
					s > 2	6.25	INC	4.33			0.06		–
					t	0	NS	0			0		–
Diethylstilbestrol (DES)													
3.73	f	72 h	C3	40	s	57.50	NEG	1.45	–	–	0.82	–	–
					s 1–2	57.50	WEAK	1.32	–	–	0.77	–	–
					s > 2	5.00	NEG	3.50			0.05		–
					t	0	INC	0			0		–
18.65	f	72 h	C3	34	s	23.53	NEG	1.73	–		0.32	–	–
					s 1–2	20.59	NEG	1.30			0.29	–	–
					s > 2	2.94	NEG	6.00			0.03		–
					t	0	INC	0			0		–
Phenobarbitone (PB)													
20	f	48 h	C3	48	s	45.83	NEG	1.41	–	–	0.56	–	–
					s 1–2	41.67	NEG	1.04		–	0.50	–	–
					s > 2	6.25	NEG	4.33			0.06		–
					t	0	INC	0			0		–
20	f	72 h	C3	38	s	28.95	NEG	1.31	–	–	0.42	–	–
					s 1–2	26.32	NEG	1.20			0.39	–	–
					s > 2	2.63	NEG	3.00			0.03		–
					t	0	INC	0			0		–

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Tests with the rat hepatocyte primary culture/DNA-repair test

Gary M. Williams, Charles Tong and S. Ved Brat

Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, NY (U.S.A.)

Summary

10 chemicals selected by the International Program on Chemical Safety were evaluated in the hepatocyte primary culture/DNA-repair test using hepatocytes from the livers of adult male Fischer strain rats. The induction of DNA-repair synthesis was determined by quantification in emulsion autoradiography of [^{3}H]thymidine incorporation in non-S phase cells. The carcinogen HMPA induced DNA-repair synthesis while the carcinogens TOL, BEN, SAF, ACN, DEHP, DES and PB were negative. The noncarcinogens ZOIN and CAP were negative. The collective assessment of all participants was that only HMPA, TOL, SAF and ACN were proven genotoxic. Thus, the high specificity of the hepatocyte primary culture/DNA-repair test for DNA-reactive carcinogens is confirmed in this test series, although the sensitivity for the chemicals selected was not high.

The International Program on Chemical Safety (IPCS) selected for study 10 chemicals consisting of 8 carcinogens that were negative in the Ames salmonella/microsome test and 2 noncarcinogens. The objective of the study was to determine whether any other in vitro system had the capacity to detect the carcinogens that were not bacterial mutagens, while not scoring false positives for the noncarcinogens.

The hepatocyte primary culture (HPC)/DNA repair test developed in this laboratory (Williams, 1976, 1977) uses freshly isolated hepatocytes for the detection of DNA-reactive chemicals by the autoradiographic measurement of DNA-repair synthesis. Using rat hepatocytes, the test has displayed a high sensitivity and specificity in the detection of carcinogens for both the liver and other tissues (Williams, 1980, 1981). Since the organotropism and species selectivity of many carcinogens is determined by the biotransformation capability of different organs and species (Weisburger and Williams, 1982), the HPC/DNA-repair test has been extended to the use of hepatocytes from other species, including mouse, hamster and rabbit (Maslansky and Williams, 1981; McQueen et al., 1983).

Several chemicals included in the IPCS test series, such as phenobarbital and diethylstilbestrol have already been studied in rat hepatocytes and found negative (Williams, 1979; Williams et al., 1982). Acrylonitrile was shown to be genotoxic in rat hepatocyte-mediated sister-chromatid exchange (Ved Brat and Williams, 1982) and safrole was found to induce DNA repair in mouse and hamster hepatocytes (McQueen and Williams, 1983). Nevertheless, since the objective of this study was to test the study chemicals in a defined system without manipulation, the 10 chemicals were processed in this laboratory in the rat HPC/DNA-repair test as unknowns.

Materials and Methods

Initiation of hepatocyte primary cultures

HPCs are initiated according to the modified procedures of Williams et al. (1977). Adult male F344 rats are anesthetized with 50 mg/kg body weight nembutal sodium solution (Abbott Laboratories). Perfusion is performed with a peristaltic pump which is sterilized by circulation of 70% ethanol followed by sterile water.

A ventral midline incision is made from the xiphisternum to the pubic bone and the liver is exposed. A 21-gauge butterfly needle is inserted in the portal vein and clamped in place with a serafine forcep (Arista Surgical, New York). Perfusion is then commenced with a 37 °C ± 1 °C sterile Solution I, which consists of 0.5 mM ethylene glycol-bis-(β-aminoethyl ether) *N,N'*-tetracetic acid (EGTA) (Sigma) in Ca^{2+}, Mg^{2+} free Hanks' balanced salt solution buffered with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (Calbiochem), pH adjusted to 7.35 with 1 N NaOH. A perfusion rate of 8 ml/min is maintained for 1.5 min.

Immediately following the start of perfusion with Solution I, ligation of the infrahepatic inferior vena cava is completed and the vein is severed distally to permit the perfusate to run to waste. At this point uniform blanching of the liver should be evident. While this first perfusion is being performed, the thoracic inferior vena cava is cannulated by puncturing the right atrium and the perfusate is collected via this return cannula. At this point, the proximal segment of the subhepatic inferior vena cava is clamped to close the system and the pump speed is then increased for 2.5 min to 40 ml/min.

Completion of perfusion with Solution I is followed by perfusion with 250 ml of a sterile Solution II, containing 100 unit/ml type 1 collagenase (Sigma) in Williams medium E (WME) buffered with 10 mM Hepes, pH adjusted to 7.35. This solution at 37 °C ± 1 °C is perfused for 10 min at 20 ml/min in the rat, without recirculation of the return perfusate. The liver is covered with sterile gauze and a 40-W bulb positioned 6 cm above the liver for warming.

Following perfusion, the liver is removed to a sterile petri dish containing warm WME and in a sterile hood, is trimmed of extraneous fat and connective tissues. The liver is then removed to a fresh petri dish containing Solution II. The portis hepatis is grasped with forceps and the capsule of the liver is opened at numerous points on the inferior surface with small scissors and removed. Cells are detached by gentle combing with a stainless steel comb, leaving a fibrous plug of hepatic connective tissue to be discarded. Using a wide bore pipet, 25-ml aliquots of the hepatocyte suspension are pipetted into 50-ml centrifuge tubes, and the volume brought to 50 ml per tube with WME supplemented with 10% calf serum and 50 μg/ml gentamycin (WMES). Cells are sedimented at 50 × *g* for 2.5 min, resuspended in WMES and gently mixed by inverting each tube several times. A 20-fold dilution of the rat-cell suspension is prepared; 0.5 ml of the diluted rat hepatocytes is added to 0.1 ml of 0.4% trypan blue stain (Gibco) for determination of viability using a hemocytometer.

The perfusion technique of Williams et al. (1977) regularly yields hepatocyte suspensions with viabilities of 90% in the rat. Cell yields of 200×10^6 per 100 g body weight are regularly obtained.

For HPC/DNA-repair studies, dilutions containing 5×10^5 cells in 1 ml WMES are immediately seeded onto 25-mm round coverslip (Thermanox) in 35-mm 6-well dishes (Linbro) containing 2 ml of WMES and placed in a 95% air, 5% CO_2 humidified 37 °C ± 1 °C incubator. 2 h after seeding, coverslips are washed with 1 ml of WME leaving only attached viable cells.

HPC / DNA-repair assay

The HPC/DNA-repair test was performed according to the methods developed by Williams (1976, 1977). After washing the cultures, 2 ml of WME containing the test chemical and 10 μCi/ml tritiated thymidine ([^{3}H]TdR), 60–80 Ci/mM, is added. The test chemicals and their abbreviations are shown in Table 1. Five logarithmically decreasing concentrations are tested on triplicate coverslips. Solids are dissolved in DMSO. The highest dose of solids is determined by their solubility in DMSO in order that the concentration of DMSO does not excede 1%. Liquids are tested up to a concentration of 1% in the medium. A positive control, benzo[*a*]pyrene, a negative control, pyrene, a solvent control, and an untreated cell control are run in parallel with the test chemicals.

HPC are incubated at 37 °C ± 1 °C for a total of 18–20 h in presence of the test chemical in [^{3}H]TdR-WME. Each coverslip is removed from its well and rinsed in three successive 100-ml washes of phosphate-buffered saline. Coverslips are then immersed with the cell surface up in 2 ml of 1% sodium citrate (Fisher) in a clean well dish for 10 min to allow the nuclei to swell, which

TABLE 1

SUMMARY OF RESULTS FOR IPCS TEST CHEMICALS IN THE RAT HEPATOCYTE/DNA-REPAIR TEST

Chemical	Dose (μg/ml)	Result [a] (net grains/nucleus)
Hexamethylphosphorimide (HMPA)	1×10^{1}	0 ±0
	1×10^{2}	0.5±0.4
	1×10^{3}	0.2±0.1
	5×10^{3}	0.1±0.1
	1×10^{4}	9.4±6.4
o-Toluidine (TOL)	1×10^{-1}	0 ±0
	1	0.3±0.6
	1×10^{1}	0.2±0.2
	1×10^{2}	Toxic
	1×10^{3}	Toxic
	1×10^{4}	Toxic
Benzene (BEN)	1×10^{-1}	0.2±0.3
	1	0 ±0
	1×10^{1}	0 ±0
	1×10^{2}	0.1±0.1
	1×10^{3}	0.1±0.1
	1×10^{4}	0 ±0
Safrole (SAF)	1×10^{-3}	0.6±1.0
	1×10^{-2}	0 ±0
	1×10^{-1}	0 ±0
	1	0.2±0.4
	1×10^{1}	Toxic
	1×10^{2}	Toxic
Caprolactam (CAP)	1×10^{-2}	0 ±0
	1×10^{-1}	0.4±0.7
	1	0.7±0.8
	1×10^{1}	1.1±2.0
	1×10^{2}	0 ±0
	1×10^{3}	0 ±0
Acrylonitrile (ACN)	1×10^{-1}	0.3±0.4
	1	0.1±0.2
	1×10^{1}	0.2±0.2
	1×10^{2}	0 ±0
	1×10^{3}	Toxic
	1×10^{4}	Toxic
Di(2-ethylhexyl)phthalate (DEHP)	1×10^{-1}	0.6±0.5
	1	1.0±0.9
	1×10^{1}	0.2±0.2
	1×10^{2}	0 ±0
	1×10^{3}	0 ±0
	1×10^{4}	0.6±0.7
Benzoin (ZOIN)	1×10^{-2}	0 ±0
	1×10^{-1}	0.1±0.1
	1	2.8±4.9
	1×10^{1}	0 ±0
	1×10^{2}	Toxic
	1×10^{3}	Toxic
Diethylstilbestrol (DES)	1×10^{-2}	0.4±0.6
	1×10^{-1}	0.2±0.2
	1	0.8±0.6
	1×10^{1}	Toxic
	1×10^{2}	Toxic
	1×10^{3}	Toxic
Phenobarbital (PB)	1×10^{-2}	0.4±0.5
	1×10^{-1}	0 ±0
	1	0 ±0
	1×10^{1}	0 ±0
	1×10^{2}	Toxic
	1×10^{3}	Toxic

[a] The results presented are those of a typical assay. Each chemical was assayed more than once. If the test chemical was a liquid, the specific gravity was taken as 1 g/ml.

permits better quantification of nuclear grains. Finally, the cells are fixed in three 30-min changes of ethanol–glacial acetic acid (3 : 1), air-dried, and mounted cell surface up on glass slides with Permount (Fisher). Slides are placed in slide grip holders (Peel-a-way Scientific) and dipped, in total darkness, into NTB emulsion (Eastman Kodak), prewarmed for 1 h at 45 °C ± 1 °C. Slides are dried overnight by suspending the slides, in their holders, from a rack in a light-tight box. Slides are placed in cardboard slide boxes which are wrapped in foil and stored at 3 °C to 10 °C.

After 10 days, autoradiographs are developed in D19 (Eastman Kodak) for 4 min, placed in a stop bath of acidified tap water for 30 sec, immersed in fixer (Eastman Kodak) for 10 min and washed in running tap water for 5 min.

Slides are stained in Harris' alum hematoxylin followed by dipping successively in tap water, acid alcohol, tap water, ammonia water and tap water prior to counterstaining with eosin. The cells are then dehydrated through 100% ethanol, air-dried and coverslipped with Permount.

Results of the HPC/DNA-repair test are quantified by determining the net increase in nuclear grains induced by a test chemical. An Artek Model 880 electronic counter with microscopic attachment is used for grain counting. This counter is able to make counts in either a grain mode or an area mode. The area mode is used because it

allows aggregates to be resolved into discrete grains. To convert area units into grain counts, the count/area ratio is determined by counting several discrete grains in both modes. This conversion factor is entered into a Ti59 calculator that has been programmed to determine a net grain count for each nucleus.

Nuclear counts of unscheduled DNA synthesis in viable cells are obtained in the area created by opening the instrument's aperture to coincide with the nuclear area, adjusting the focus and sensitivity until the perimeters of the grains or aggregates of grains are outlined in white indicating detection by the instrument. Cytoplasmic counts of areas of the same size are made by moving the aperture to positions adjacent to each counted nucleus. Digital counts are automatically recorded by an Artek Compu-print 700 and a Ti59 calculator.

Only those cells which were viable at the time of fixation, indicated by swollen nuclei (as a result of incubation in the hypotonic sodium citrate), and those evenly coated with emulsion are scored. Between 5 and 20 cells randomly selected from each quadrant of the coverslip are counted. The numbers of cells to be scored depends on the nuclear/cytoplasmic ratio obtained (Rogers, 1973). Counts are obtained for each nucleus; background is determined by counting 3 nuclear sized areas adjacent to the nucleus. Net nuclear grain counts are calculated by subtracting the highest cytoplasmic count from the nuclear count.

Interpretation of results

The scoring method is designed to avoid false positives by choosing the highest cytoplasmic count of each cell as a background. Results of individual experiments are tabulated as the mean ± standard deviation of net grain counts for triplicate coverslips. A test compound is reported positive when the minimum net grain count of 5 per nucleus is consistently observed in triplicate coverslips throughout experiment. Negative results below this level are considered valid only when the positive control is positive.

Cytotoxicity of the test compound is identified by the absence of S-phase cells in the autoradiograph and by general morphology. The compound is reported negative in this assay if the net nuclear count is less than 5 at the highest nontoxic dose.

Results

Each chemical was tested initially over a dose range to find either the highest nontoxic dose or the limit of solubility. In the case of liquid chemicals, the maximum tested dose was set at 1% (v/v). Chemicals were then retested over a more narrow dose range in the region of either positive results or maximum testable doses.

Of the 10 compounds tested, only hexamethylphosphorimide induced repair synthesis (Table 1). The others were negative in tests in which the positive control benzo[*a*]pyrene induced repair synthesis.

Discussion

The rat HPC/DNA-repair test detected 1 of the 8 test carcinogens and was negative for the 2 noncarcinogens. The one positive carcinogen, hexamethylphosphorimide, was 1 of the 4 carcinogens (the others were *o*-toluidine, safrole and acrylonitrile) concluded to be genotoxic from the results from all systems in the collaborative program. Thus, the high specificity of the HPC/DNA-repair test for DNA-reactive carcinogens (Williams, 1980, 1981) is confirmed. The positive result with hexamethylphosphorimide provides further support for the recommendation that chemicals found to be reliably positive in a comprehensive battery of in vitro tests need not be tested for carcinogenicity in animals (Weisburger and Williams, 1981).

Of the other 3 genotoxic carcinogens, safrole has been positive in the mouse and hamster HPC/DNA-repair test (McQueen and Williams, 1983) and acrylonitrile was positive for rat HPC-mediated sister-chromatid exchange (Ved Brat and Williams, 1982). These findings with Ames-negative carcinogens extend previous results with other structural types such as aminoazo dyes, nitrosamines and pyrrolizidine alkaloids showing that the HPC/DNA-repair test is a useful compliment to the Ames test (Williams, 1977; Williams and Laspia, 1979; Williams et al., 1980).

5 carcinogens (*o*-toluidine, di(2-ethylhexyl) phthalate, diethylstilbestrol, phenobarbital and benzene) were negative in both the Ames test and the rat HPC/DNA-repair test. A carcinogen can be negative in an in vitro system for a variety of

reasons, including lack of appropriate metabolism, insensitivity of the endpoint, and, importantly, the possibility that the carcinogen does not produce the biological effect being measured. The carcinogens that were negative using rat hepatocytes have not been tested in the HPC/DNA-repair test using hepatocytes from other species, so it is possible that some of them, particularly the one established genotoxin, *o*-toluidine, might be positive under other circumstances. Nevertheless, for at least 3 of these 5, i.e. di(2-ethylhexylphthalate, diethylstilbestrol and phenobarbital, there is evidence that they may be carcinogenic through a nongenotoxic mode of action (Williams and Weisburger, 1984) and thus the results from the in vitro tests may be appropriate.

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Assays for unscheduled DNA synthesis in HeLa S3 cells

R.H. Barrett

The Boots Company PLC, The Priory, Thurgarton, Nottingham (Great Britain)

Summary

The first 5 compounds of the IPCS collaborative study on short-term tests (C.S.S.T.), namely HMPA, TOL, BEN, SAF and CAP, were assayed for their ability to induce unscheduled DNA synthesis (UDS) in HeLa S3 cells. Both TOL and SAF induced reproducible positive responses, whereas HMPA, BEN and CAP were assessed as negative in this test system.

Introduction

The detection of potentially carcinogenic chemicals by induction of UDS in cultured mammalian cells relies upon those chemicals being electrophiles or capable of conversion to such by metabolism, which then react with nucleophilic centres in DNA to form covalently-bound adducts. A consequence of such DNA–carcinogen interaction is the removal of adducts and the synthesis of replacement DNA by an excision-repair process. A proportion of the DNA lesions produced by most carcinogens are removed in this way; however, compounds causing other types of DNA damage, or those acting by epigenetic mechanisms, are unlikely to be detected by UDS assays.

The method described is based upon that of Martin et al. (1977, 1978) but incorporates modifications of scale. Measurement of UDS in HeLa S3 cells, after treatment with test compounds or known carcinogens, is achieved by monitoring the uptake of [^{3}H]thymidine into the DNA of treated cultures. Replicative DNA synthesis in these cells is minimized by growth-limiting culture conditions, i.e. arginine-deficient medium, and by the use of hydroxyurea.

Materials and methods

Culture conditions

HeLa S3 cells were obtained from Gibco Europe Ltd., Paisley (Scotland). Master stock cultures are held in liquid nitrogen; test stocks were maintained by subculture at confluency and were replaced every 6–8 weeks from the master stock.

Cells were grown in monolayer culture, in Eagles' minimal essential medium (EMEM) buffered with 20 mM Hepes, supplemented with glutamine (2 mM), nonessential amino acids (1%) and 15% foetal calf serum; no antibiotics were added.

For testing fo the trial compounds, HeLa S3 cells were seeded at approximately 1×10^6 cells/flask into 75-cm^2 plastic tissue-culture flasks (LUX) in 20 ml of growth medium and incubated at 37 °C for 4 days without a change of medium. The resultant monolayers were then incubated in 30 ml of growth-limiting medium comprising: EMEM with Hepes without arginine, supplemented with glutamine (2 mM), nonessential amino acids (1%) and 2.5% dialysed foetal calf serum (foetal calf serum was dialysed against 4 changes of 10 volumes of normal saline over 4 days at 0–4 °C). The cells were incubated at 37 °C for 72 h in this arginine-deficient medium which was changed after 24 h.

Preparation of rat-liver homogenate (S9) and cofactor / S9 mix

Male Wistar rats (100 ± 5 g) obtained from Charles River, U.K. Ltd. were given a single intraperitoneal injection of Aroclor 1254 (500

TABLE 1

RESULTS OF TESTS ON 5 IPCS TRIAL COMPOUNDS IN AN ASSAY FOR DETECTION OF UDS IN HeLa S3 CELLS

Concentration (μl/ml)	Test 1 [^{3}H]Thymidine incorporation into DNA DPM·μg^{-1} ± S.D. [b]		Concentration (μl/ml)	Test 2 [^{3}H]Thymidine incorporation into DNA DPM·μg^{-1} ± S.D. [b]	
	+ S9	− S9		+ S9	− S9
Compound 1. Hexamethylphosphoramide (HMPA)					
0 [a]	167.1 ± 17.6	173.2 ± 18.7	0 [a]	130.1 ± 15.5	141.1 ± 15.3
0.0001	156.6 ± 5.4	158.6 ± 6.2	0.5	130.4 ± 11.7	142.5 ± 18.2
0.001	164.9 ± 10.9	154.3 ± 2.1	1.0	139.1 ± 12.7	152.4 ± 21.4
0.01	175.8 ± 9.3	177.9 ± 22.8	1.5	126.0 ± 7.6	148.8 ± 19.5
0.1	180.6 ± 41.4	161.9 ± 15.5	2.0	154.6 ± 10.9 [c]	136.6 ± 15.7
1.0	157.5 ± 16.1	166.4 ± 5.3	2.5	153.5 ± 8.3 [c]	160.6 ± 11.2
B(α)P [c] (10^{-5} M)	241.7 ± 11.2		B(α)P [c] (10^{-5} M)	344.4 ± 36.4	
MNNG [d] (6.6×10^{-5} M)		2584.1 ± 58.9	MNNG [d] (6.6×10^{-5} M)		1336.3 ± 26.5
Compound 2. o-Toluidine (TOL)					
0 [a]	169.0 ± 9.6	165.1 ± 7.9	0 [a]	100.8 ± 12.0	NT
0.01	177.9 ± 6.0	161.8 ± 9.0	0.01	109.1 ± 11.7	NT
0.05	214.1 ± 13.1 [c]	138.8 ± 19.6	0.05	121.2 ± 22.8 [c]	NT
0.1	241.4 ± 17.5 [c]	176.0 ± 21.9	0.1	128.7 ± 7.4 [c]	NT
0.5	220.6 ± 33.6 [c]	135.9 ± 12.0	0.5	138.1 ± 4.2 [c]	NT
1.0	217.1 ± 14.7 [c]	134.2 ± 6.8	1.0	139.0 ± 9.2 [c]	NT
B(α)P [c] (10^{-5} M)	354.6 ± 55.4		B(α)P [c] (10^{-5} M)	290.1 ± 24.4	
MNNG [d] (6.6×10^{-5} M)		1825.3 ± 130.7			
Compound 3. Benzene (BEN)					
0 [a]	121.7 ± 11.0	124.5 ± 12.2	0 [a]	114.4 ± 11.5	119.3 ± 14.3
0.188	133.4 ± 2.4	137.3 ± 7.4	0.05	105.2 ± 12.1	110.8 ± 8.1
0.375	113.2 ± 4.6	103.9 ± 10.6	0.075	111.6 ± 17.4	105.7 ± 8.7
0.750	102.6 ± 8.1	110.1 ± 16.8	0.10	112.2 ± 4.5	109.2 ± 15.5
1.5	NR	NR	0.15	108.2 ± 8.6	99.8 ± 3.9
3.0	NR	NR	0.20	109.0 ± 20.5	101.3 ± 17.0
2-AAF [c] (10^{-4} M)	145.7 ± 5.0		2-AAF [c] (10^{-4} M)	147.0 ± 2.2	
4-NQO [d] (10^{-6} M)		2387.5 ± 132.8	MNNG [d] (6.6×10^{-5} M)		1989.5 ± 151.7

Compound 4. Safrole (SAF)					
0 [a]	119.9 ± 14.2	133.8 ± 13.5	0 [a]	106.5 ± 14.7	126.1 ± 18.6
0.01	140.2 ± 2.0 [c]	130.6 ± 8.7	0.001	114.7 ± 21.6	116.5 ± 4.2
0.05	177.6 ± 18.6 [e]	82.3 ± 8.9	0.005	166.3 ± 20.7 [e]	128.6 ± 16.6
0.1	92.4 ± 8.9	50.9 ± 5.7	0.01	156.4 ± 18.5 [e]	89.3 ± 3.2
0.5	NR	NR	0.05	130.2 ± 11.5 [e]	77.6 ± 7.9
1.0	NR	NR	0.1	101.1 ± 33.7	49.3 ± 6.9
Aflatoxin B1 [c] (10^{-4} M)	785.5 ± 167.9		Aflatoxin B1 [c] (10^{-4} M)	688.7 ± 43.7	
MNNG [d] (6.6×10^{-5} M)		2347.1 ± 180.6	MNNG [d] (6.6×10^{-5} M)		1869.1 ± 366.5
Compound 5. Caprolactam (CAP) [f]					
0 [a]	141.0 ± 19.2	141.3 ± 8.4	0 [a]	121.0 ± 10.0	115.5 ± 10.4
10^{-7} M	126.9 ± 7.9	146.4 ± 19.0	10^{-6} M	98.8 ± 12.4	101.3 ± 12.8
10^{-6} M	133.3 ± 9.0	149.3 ± 14.8	10^{-5} M	129.3 ± 16.5	132.7 ± 11.9
10^{-5} M	127.9 ± 21.5	137.5 ± 26.7	10^{-4} M	116.4 ± 2.2	85.8 ± 6.4
10^{-4} M	137.5 ± 19.0	139.3 ± 4.8	10^{-3} M	130.9 ± 10.0	101.3 ± 13.8
10^{-3} M	139.3 ± 4.8	142.2 ± 20.7	10^{-2} M	115.1 ± 18.9	79.3 ± 1.6
B(α)P [c] (10^{-5} M)	324.2 ± 16.0		B(α)P [c] (10^{-5} M)	189.3 ± 11.3	
MNNG [d] (6.6×10^{-5} M)		2295.9 ± 160.1	MNNG [d] (6.6 ± 10^{-5} M)		1135.8 ± 190.2

[a] DMSO solvent control.
[b] Mean values of at least two replicates (or at least four replicates for solvent controls).
[c] + S9 positive control.
[d] − S9 positive control.
[e] Results significantly different from solvent control by *F*-test, with $P < 0.05$.
[f] Concentration expressed as molarity.
NT, not tested.
NR, not recorded due to cytotoxicity.

mg/kg) in corn oil. Food was withheld from the animals for approximately 16 h before they were killed 5 days after dosing, and the livers were removed aseptically and homogenised in 0.15 M KCl. A postmitochondrial supernatant (S9) was prepared by the method of Ames et al. (1973), and stored in screw-capped glass containers and held at −80 °C. Batches of S9 were reprepared monthly.

The cofactor/S9 mix was prepared immediately before use; it comprised (per ml): 0.4 ml S9; 5.0 mg NADP (BCL); 36.0 mg glucose 6-phosphate (BCL); 50 mM KCl; 30 mM $MgCl_2$ in phosphate-buffered saline. Cofactor-only mix was prepared as above but with 0.4 ml of saline substituted for the S9 homogenate.

Test procedure

Treatment. At the end of the 72-h incubation period, 0.5 ml of an aqueous solution of hydroxyurea was added to all flasks, giving a final concentration of 10 mM, to maximise inhibition of "background" DNA synthesis. The flasks were incubated for 1 h at 37 °C, and then 1.5 ml of S9 mix (equivalent to a final flask concentration of 2% S9), or cofactor-only mix, was added to appropriate flasks. All flasks were then dosed with [6-^{3}H]thymidine (Amersham International PLC, England) to a final concentration of 3.33 μCi/ml (spec. act. ca. 20–30 Ci/mmole).

Test agent solutions were added to appropriate flasks to give a range of concentrations. A set of negative control flasks received solvent only (dimethyl sulphoxide); positive control flasks received an appropriate concentration of either a direct- or indirect-acting carcinogen. The flasks were then incubated at 37 °C for 2.5 h to allow test agent reaction with DNA and subsequent DNA-repair activity with the associated incorporation of [^{3}H]thymidine. Test group assignment was as follows:

DMSO (negative) control + S9	6 flasks
DMSO (negative) control − S9	6 flasks
Test agent concentrations 1–5 + S9	3 flasks/concentration
Test agent concentrations 1–5 − S9	3 flasks/concentration
Positive control (indirect) + S9	3 flasks
Positive control (direct) − S9	3 flasks

Following incubation, the test medium was discarded and further ^{3}H uptake prevented by washing the cell monolayers twice with 20-ml volumes of ice-cold phosphate-buffered saline containing an excess of "cold" thymidine (2 mM). The cells were removed from the flasks by treatment with 10 ml of warm phosphate-buffered saline containing 200 mg/l of EDTA and glucose. The resultant cell suspensions were decanted into 48 polypropylene test tubes and pelleted by centrifugation at 1000 *g* for 5 min at 4 °C. The supernatants were carefully discarded and the pellets stored at −20 °C until the DNA was extracted.

DNA-extraction. All DNA extraction procedures were conducted at 0–4 °C, unless specified otherwise.

The cell pellets were thawed, and 5 ml of ice-cold 10% trichloroacetic acid (TCA) added to each tube. The tubes were mixed thoroughly and centrifuged at 5000 *g* for 5 min. The supernatants were discarded, and the pellets dissolved in 2 ml 1 M KOH and incubated at 37 °C for 1 h to hydrolyse RNA. Following neutralisation with 0.4 ml of 6 M HCl, protein and nucleic acid were reprecipitated by the addition of 2.5 ml of 10% TCA. The resultant suspensions were centrifuged as before and the supernatants discarded. The pellets were washed by resuspension in a further 5 ml of 5% TCA, centrifuged and the supernatants discarded. The pellets were finally resuspended in 2.5 ml of 5% TCA and heated at 90 °C for 20 min to hydrolyse DNA; precipitated protein was removed by centrifugation. Aliquots (1 ml) of the supernatants were counted for radioactivity in 10 ml of Packard P299 scintillation fluid, using a Packard 300C liquid scintillation counter. Correction for counting efficiency and calculation of DPM values was carried out using an on-line Apple II microcomputer.

Additional 1-ml aliquots of the DNA-hydrolysates were assayed for DNA content using a modified Burton colorimetric diphenylamine assay. The colour was developed by heating at 100 °C for 10 min and, after cooling, absorbances were read at 600 nm using a Pye-Unicam SP30 spectrophotometer.

A set of standard DNA solutions was assayed concurrently witht he test samples, and absolute values for the samples obtained from the resultant standards graph. Final results for each treatment were expressed as DPM/μg DNA. Means and

standard deviations were calculated for each replicate group.

Test agent and controls

Test and positive control compounds were stored in tightly stoppered glass bottles, in the dark at 0–4 °C.

Test solutions and dilutions were prepared immediately prior to use; "spectrograde" dimethyl sulphoxide (Fisons PLC, Loughborough, England) was used as the solvent for test agents, positive control carcinogens and as the negative control.

Maximum levels of test agents were determined by reference to available data on solubility and toxicity, or were limited by the quantity of material available. The concentrations of the positive control compounds were determined from the results of previous assays.

Test criteria

The results of the assay are expressed as mean DPM/μg DNA $\pm$standard deviations for each treatment group. The data were evaluated by one-way analysis of variance (F-test) (Armitage, 1971) using a programmable calculator.

A positive result in this assay is indicated by the demonstration of a dose-related, statistically significant increase in the incorporation of [^{3}H]thymidine into the DNA of treated groups compared with the solvent controls. Experience has shown that the background level within a specific test is subject to only very small standard deviations, though considerably greater intertest variation is seen in this parameter. This variability probably derives from slight differences in the conditions of the test cell stocks and, for this reason, the use of historical control data is considered not valid. Each assay must be evaluated individually by comparing concurrent test and control groups.

Results and discussion

Detailed results for each of the compounds tested in this assay are presented in Table 1. The conclusions based on these data are summarised in Table 2, as a simple + or − for a positive or negative response, respectively. Equivocal data or data considered inadequate for definitive assessment are regarded as negative for the purposes of this trial, but are discussed in the text.

TABLE 2

SUMMARY OF RESPONSES OF 5 TRIAL COMPOUNDS IN AN ASSAY FOR DETECTION OF UDS IN HeLa S3 CELLS

Compound	Abbreviation	Carcinogenicity [a]	Response of compound in this assay
Hexamethylphosphoramide	HMPA	+	−
o-Toluidine	TOL	+	+
Benzene	BEN	+	−
Safrole	SAF	+	+
Caprolactam	CAP	−	−

[a] As defined by the IPCS for the purposes of this trial.

Neither the noncarcinogen CAP nor the suspect human carcinogen BEN induced any increase in UDS in this test system and are therefore, regarded as having given clear negative responses. The absence of BEN-induced DNA-repair activity in this study, may derive from: (i) insensitivity to the type of genotoxicity shown by BEN; (ii) inadequate or inappropriate metabolic activation for the production of a DNA-reactive metabolite; (iii) inability of any reactive metabolite to live long enough to reach the DNA of the target cells.

In the initial assay on HMPA, there was no evidence of any significant compound-induced increase in DNA-repair activity but, in a second assay conducted over a higher range of concentrations, a weak positive effect was observed at 2.0 and 2.5 μl/ml in the presence of S9; this was statistically significant at the 5% level. In the absence of S9, increases in ^{3}H- incorporation were not significantly different from the control values.

On the basis of these data, HMPA must be classified as negative for the purpose of this trial. In contrast, both TOL and SAF induced reproducible statistically significant increases in UDS in the presence of S9, although the positive result with SAF contrasts with that of IPESTTC studies, in which SAF was assessed as negative in assays of this type (Robinson and Mitchell, 1981; Agrelo and Amos, 1981, Martin and McDermid, 1981). The increase in sensitivity evident in this study may derive from the choice of dose levels and

intervals, and/or the metabolic profile of the cells and S9 used.

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Measurement of DNA single-strand breaks by alkaline elution in rat hepatocytes

Matthews O. Bradley

Merck Institute for Therapeutic Research, West Point, PA (U.S.A.)

Summary

The alkaline elution/rat hepatocyte assay was used to test whether 10 selected chemicals make DNA single-strand breaks in rat hepatocytes. The assay identified the correct carcinogenic activity for 5 of the 10 compounds. This report discusses rationales for some of the results and protocol modifications that might increase the accuracy of the method.

The alkaline elution/rat hepatocyte assay sensitively measures single-strand breaks in the DNA of isolated rat hepatocytes that are caused by chemical, physical or enzymatic attack (Bradley et al., 1982; Sina et al., 1983). Hepatocytes actively metabolize most precarcinogens into electrophilic intermediates capable of attacking DNA, RNA and protein; in addition, direct-acting agents also attack hepatocyte DNA. Therefore, this assay should readily detect carcinogens that damage DNA in hepatocytes but might not detect other carcinogens that do not damage DNA or that are not active in hepatocytes.

The alkaline elution assay detects small changes in DNA molecular weight by measuring the rate at which single alkali-denatured strands of DNA pass through (elute) 2 μm pore-size filters. The rate of elution increases directly as a function of X-ray dose with as little as 30 rad being detectable. We measure the amount of DNA in each elution fraction by a chemical method utilizing the fluorometric product of diaminobenzoic acid with deoxysugars. The assay is rapid so that one person working half-time was able to screen the 10 chemicals in this study within 2 weeks.

Materials and methods

Compounds

The compounds arrived at our laboratory on 27 January 1983, and were kept at room temperature until assayed 10–12 weeks later. The compounds were weighed and then dissolved in water, DMSO or ethanol (70% weight/volume) immediately before use. The final concentration of the solvent in the hepatocyte medium was 1% by volume.

Hepatocytes

Rat hepatocytes were prepared by collagenase perfusion as previously described (Bradley et al., 1982; Bradley and Sina, 1984). Cells were seeded into 100-mm bacteriological plates at 10^5 cell/ml (11.0 ml/plate) in Leibovitz's L15 medium (Grand Island Biological Co., Grand Island, NY) without phenol red, with 10% fetal bovine serum. Bacteriological plates were used so that the cells would not adhere to them, thus (1) avoiding damage due to trypsinization, (2) simplifying the harvest procedure, and (3) obtaining more uniform control elution rates. Exposure was initiated by adding 110 μl of 100X compound to the appropriate plates. After 3 h, cells were scraped gently into a uniform suspension with a rubber policeman. A 1.0-ml sample was set aside for viability testing and 5.0

ml was dispensed into each of two tubes for the elution procedure.

Alkaline elution procedure

Duplicate samples from each test plate were eluted from filters as previously described (Bradley et al., 1982; Bradley and Sina, 1984). Briefly, 5×10^5 cells were loaded onto a 2-μm polycarbonate filter (Nucleopore) held in an elution column (Millipore) and 1.5 ml of lysis solution (50 mM Tris, 50 mM glycine, 25 mM Na_2EDTA, 2% SDS, pH 9.6 plus 0.5 mg/ml freshly added proteinase K) was added to lyse the cells and to remove protein from the DNA. After incubation at room temperature for 30 min, the lysis solution was allowed to drip through the filters. Filters were rinsed by allowing 0.5 ml of lysis solution without SDS or proteinase K to drip through. The pumping lines were connected to each column, tetrapropyl ammonium hydroxide eluting solution (0.2% SDS in 20 mM H_4EDTA plus enough tetrapropyl ammonium hydroxide to give a pH of 12.1) was slowly added down the sides of the column at about 3 ml/min and the pump was started at a rate of 0.035 ml/min. A fraction collector was set to collect 3×180-min fractions and one 90-min fraction. When this was completed, the material remaining in the lines of the elution apparatus was pumped into the fourth fraction. The filter was removed to a scintillation vial and the elution unit was washed by pumping through 6.4 ml of 0.4 N NaOH. The amount of DNA in each fraction, in the wash, and on the filter was determined fluorometrically as described (Bradley et al., 1982; Bradley and Sina, 1984). The total of these amounts was the initial amount of DNA on the filter. The fraction of DNA remaining on the filter at each time point was the fraction of DNA in that sample subtracted from the fraction of DNA on the filter at the previous time point. The rate of elution was calculated from a semilogarithmic plot of the fraction of DNA retained (log scale) against the time of elution (arithmetic scale). The mean slope of the plotted line from 3 to 9 h of elution was taken as the elution rate and was calculated as: $(\log(A:B) + \log(B:C)):2$, where A is the fraction of DNA remaining on the filter at 3 h B is the fraction remaining a 6 h and C is the fraction remaining at 9 h.

Cytotoxicity measurements

Hepatocyte viability after the 3 h exposure to the chemical was measured by trypan blue dye exclusion. The method was to dilute 0.5 ml of chemically treated and harvested cell suspension with 0.1 ml of 0.4% trypan blue. After 5 min at room temperature, the number of viable dye-excluding cells and the number of nonviable dye-stained cells was determined.

Controls

Dimethylnitrosoamine (0.3 mM) was used as a positive control for the activity of the metabolizing system. γ-Irradiation (450 rad of ^{137}Cs to cells on ice) was used as a positive control for the elution system itself.

The negative controls were treated with only the solvent.

Criterion for a positive result

We consider a 3-fold increase in elution rate compared to the concurrent negative control to be a biologically significant increase in DNA single-strand breaks and a positive result.

Results

Table 1 presents the data obtained in this study. We have included the solvent used for each chemical and whether the chemical visibly precipitated when added to the medium containing the cells. The mean elution slopes were calculated between 3 and 9 h of elution time. The mean slope for each concentration of test chemical is divided by the mean elution slope of the concurrent negative control. Greater than 3-fold increases in elution slope are considered to represent biologically meaningful numbers of DNA single-strand breaks. A qualitative evaluation of results for the compounds is presented in the last column: a + means that the treated elution slope was 3 times greater than the control, while a − means that the treated slope was less than 3 times the control slope.

The carcinogenic compounds that induced DNA single-strand breaks in rat hepatocytes included TOL, SAF, ACN and DES. The carcinogens that were inactive in this study included HMPA, BEN, DEHP and PB. The noncarcinogen ZOIN induced DNA single-strand breaks although at high toxic-

TABLE 1

DNA SINGLE-STRAND BREAKS INDUCED BY 10 CHEMICALS — TEST RESULTS

Compound	Dose (μg/ml)	Solvent	Precipitate at time of dosing	Percent survival relative to control	[a] Mean elution slope, control	[b] Mean elution slope, treated	Increase in slope relative to control	Qualitative evaluation of results
HMPA	522	H_2O	–	101	0.014	0.006	0.31	–
	1736		–	101	0.014	0.015	0.83	
	5218		–	105	0.014	0.020	1.08	
TOL	319	EtOH	–	59	0.010	0.043	4.30	+
	1063		–	72	0.010	0.048	4.80	
	3190		+	0	0.010	0.190	19.0	
SAF	44.4	EtOH	–	96	0.010	0.012	1.20	+
	148		+	80	0.010	0.055	5.50	
	444		+	1	0.010	0.228	22.80	
BEN	2.77	EtOH	–	88	0.012	0.015	1.25	–
	26.7		–	70	0.012	0.023	1.92	
	267		–	87	0.012	0.013	1.08	
CAP	339	H_2O	–	99	0.014	0.013	0.93	–
	1146		–	106	0.014	0.008	0.57	
	3390		–	104	0.014	0.005	0.36	
ACN	65.8	EtOH	–	61	0.012	0.068	5.67	+
	197		–	3	0.012	0.309	25.75	
	658		–	2	0.012	0.362	30.17	
DEHP	391	DMSO	+	121	0.018	0.014	0.78	–
	1172	+1%	+	94	0.018	0.019	1.06	
	3907	Tween	+	94	0.018	0.016	0.89	
ZOIN	63.7	DMSO	–	88	0.018	0.019	1.06	+
	212		+	–	0.018	0.025	1.39	
	637.0		+	45	0.018	0.482	26.78	
DES	8.51	EtOH	–	96	0.014	0.013	0.93	+
	26.84		–	92	0.014	0.020	1.43	
	80.51		–	0	0.014	0.236	16.86	
PB	232	EtOH	–	106	0.010	0.008	0.80	–
	581		–	110	0.010	0.007	0.70	
	1161		+	88	0.010	0.014	1.40	

[a] Mean of 4 determinations of the solvent negative control.

[b] Mean of 2 determinations of the treated sample.

Freshly isolated hepatocytes in Leibovitz's L15 media with 10% fetal bovine serum were treated for 3 h with solvent or test compound and then lysed on polycarbonate filters for alkaline elution.

ity and at precipitable doses, whereas the other noncarcinogen CAP did not. Thus, the assay correctly identified 5 of the 10 compounds. As discussed below, some simple alterations to the protocol used here may improve the overall accuracy of this method.

Discussion

Cytotoxicity

Cells that exclude the dye trypan blue possess some aspects of intact membranes and metabolism. Although other components of toxicity may

also be important, dye exclusion is a useful indicator of the state of the cells at the time they are lysed for elution. We previously evaluated, by the alkaline elution/rat hepatocyte assay, 64 carcinogens and 27 noncarcinogens from more than 25 diverse classes. In that study, cytotoxicity helped to distinguish different classes of chemicals (Sina et al., 1983). All 51 (100%) of the chemicals that induced DNA single-strand breaks at nontoxic doses were carcinogens, while only 75% of the chemicals that induced strand breaks correlated with cytotoxicity were carcinogens. Generally, the stronger carcinogens produced DNA strand breaks without toxicity whereas the weaker carcinogens produced DNA strand breaks with toxicity.

In the current study, only SAF and TOL induced strand breaks at less than 30% toxicity. ZOIN, ACN and DES produced breaks that were closely associated with significant cytotoxicity.

HMPA, CAP, DEHP and PB were not cytotoxic at the highest concentrations tested; this nontoxicity implies that these compounds are biologically inactive in isolated hepatocytes under the conditions of this study.

Incorrectly identified chemicals

One of the utilities of collaborative studies such as this, is that deficiencies in protocols may be identified so that improvements may be incorporated into future assays. In the alkaline elution/rat hepatocyte assay such problems might include: volatile compounds escaping from the treatment dish, too short a treatment time, 10% serum in the medium to adsorb compounds, too low a dose, or carcinogens that act in nonhepatocytes or by non-DNA mechanisms. Some of these possibilities are discussed below.

HMPA produced neither DNA strand breaks nor cytotoxicity during the 3-h treatment period. Since this compound was active in other mutation and DNA-damage assays, and should make DNA-damaging intermediates, this lack of biological effect is somewhat surprising. A number of explanations are possible and will require further investigation. We received HMPA on 27 January 1983 and maintained it at room temperature for 12 weeks. HMPA is relatively unstable so it may have become partially or completely inactive. Another explanation for the lack of any HMPA effect is that the cells were dosed in petri dishes and the HMPA may have evaporated too quickly. Other explanations may include: too short a treatment period, too much serum in the medium, and perhaps too low a dose. One suggestion that came out of our workshop discussion, was that HMPA may be inactivated inside hepatocytes and then transported outside to other cells where it becomes active. All of these possibilities will be explored in the future.

ZOIN-induced strand breaks at the highest dose (637 μg/ml) where much of the compound had precipitated at dosing. In addition, this dose reduced trypan blue exclusion to 45% of the control. There are probably few chemicals that will be an in vivo carcinogen at precipitable doses with high toxicity. Therefore, based on this rationale we would have predicted that ZOIN would be noncarcinogenic, although in the strictest sense it induced SSBs and thus was included as a positive compound in this study.

DEHP may be a carcinogen because it, like other hypolipidemic agents, induces peroxisomal proliferation that may be related to some unknown carcinogenic mechanism. DEHP precipitated at all doses and induced neither toxicity nor strand breaks. Since peroxisomal proliferation takes a number of days to occur, and since the other assay in which DEHP was positive in this study used 1–3-day treatment periods, perhaps longer treatment times would have shown a response.

PB appeared to be biologically inactive under the conditions of this assay inducing neither cytotoxicity nor SSBs. We will experiment with higher doses for longer treatment times to attempt to induce some biological activity.

BEN was just below our criterion for a positive result inducing a 1.9-fold increase ine lution slope. Many of the assay groups that found BEN positive used DMSO as a solvent, whereas we used ethanol. BEN was studied in glass flasks but with a large air volume above. We will repeat these experiments with higher doses for longer times and with a smaller air space in an effort to increase the effective concentration to the hepatocytes. It seems apparent that we have not optimized this system for testing volatile compounds.

It is possible that the protocol modifications

suggested by the results of this study will improve the overall accuracy of the alkaline elution/rat hepatocyte assay and will suggest modifications that should be incorporated into a screening system.

Correctly identified compounds

DES and SAF had been correctly identified by this assay in an earlier study (Sina et al., 1983). TOL and ACN were highly active in hepatocytes inducing large numbers of strand breaks. All of these compounds appear to interact with DNA, which is likely to be one of the major mechanisms of carcinogenesis.

Conclusions

It is apparent that if a chemical is carcinogenic but is not activated by hepatocytes or does not attack DNA, then the alkaline elution/rat hepatocyte assay will not detect it. On the other hand, this assay will detect the vast majority of carcinogens that are active in hepatocytes by a mechanism based on DNA damage. This assay also has the advantage that it can quickly and inexpensively screen large numbers of compounds.

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Alkaline sucrose sedimentation, sister-chromatid exchange and micronucleus assays in CHO cells

George R. Douglas, David H. Blakey, V.W. Liu-Lee, R.D.L. Bell and J.M. Bayley

Mutagenesis Section, Environmental Health Directorate, Department of National Health and Welfare, Ottawa (Canada)

Summary

The 10 chemicals selected for testing in the International Program on Chemical Safety (IPCS), Collaborative Study on Short-Term Tests were tested in Chinese hamster ovary (CHO) cells for the induction of alkaline sucrose gradient (ASG)-detectable DNA damage and micronuclei. In addition, 6 of the chemicals were tested for the ability to cause sister-chromatid exchange (SCE) in these cells. In the ASG assay, diethylstilbestrol (DES), and acrylonitrile (ACN) at very high concentrations produced dose-related increases in DNA damage, while *o*-toluidine (TOL) caused low levels of DNA damage at high concentrations. Only ACN caused an increase in micronuclei, and none of the 6 chemicals tested (HMPA, TOL, BEN, SAF, CAP or DES) induced SCE.

The genotoxic activity of the IPCS test chemicals was determined using a battery of in vitro mammalian tests for chromosome and DNA damage in Chinese hamster ovary (CHO) cells. Chromosome damage was measured by studying the induction of micronuclei (Raj and Heddle, 1980). Micronuclei are formed when chromosomal fragments are not included in either daughter nucleus following cell division (Heddle and Carrano, 1977). An increase in micronucleus frequency is therefore an indication of chromosome breakage. DNA damage was measured using alkaline sucrose gradient (ASG) sedimentation analysis (Douglas and Grant, 1980). The DNA lesions detected using ASG analysis include DNA-strand breaks induced directly by the DNA-damaging agent as well as alkali-labile lesions such as apurinic sites and phosphotriesters which hydrolyze under alkaline conditions to form strand breaks (Abbondandolo et al., 1982). In addition, this technique detects single-strand discontinuities produced during DNA-repair processes (Abbondandolo et al., 1982). The third assay in this battery measured the induction of SCE (Perry and Evans, 1975). The exact mechanisms of SCE formation are unknown at present; however, it appears that SCEs are the chromosomal manifestation of certain DNA lesions remaining on the DNA at the time of replication (Painter, 1980; Latt, 1982).

These assays were chosen because they detect presumably different types of DNA lesions or their effect on DNA and may thus complement each other, providing greater accuracy in the detection of genotoxic activity.

Materials and Methods

Cell culture

Mycoplasma-free monolayer cultures of wild-type CHO cells were maintained in minimal essential medium with nonessential amino acids and sodium pyruvate (MEM, Gibco). MEM was supplemented with 7.5% fetal calf serum (FCS). Cells were grown at 37 °C, 5% CO_2, and high relative humidity. All cell manipulations were carried out under illumination from gold fluorescent lamps (General Electric, F40G0).

Chemicals

The chemicals used as positive controls where appropriate were: methyl methanesulfonate (MMS, Eastman Kodak), dimethyl nitrosamine (DMN, Aldrich). Spectrophotometric grade dimethyl sulfoxide (DMSO, J.T. Baker) was used as a solvent to dissolve the test chemicals before addition to culture medium.

Treatment Mixtures

Where possible, stock solutions of 100× the highest final concentration were prepared so that the maximum final concentration of DMSO was 1%. Stock solutions were diluted in MEM without serum for treatments not requiring metabolic activation. For metabolic activation, Aroclor 1254-induced Sprague–Dawley rat-liver S9 was used. The S9 treatment mix was prepared in MEM according to the following:

Stock concentration	Vol. stock solution/100 ml	Final concentration
MEM (without serum)	85.3	85.3%
S9	4.0	4.0%
20 mM HEPES pH 7.3	4.9	0.98 mM
0.5 M $MgCl_2$	0.22	1.1 mM
3.3 M KCl	0.22	7.3 mM
50 mM glucose 6-phosphate	2.2	1.1 mM
40 mM NADP	2.2	0.88 mM
Concentrated test solution	1.0	1.0%
	100.0 ml	

All treatments were for 1 h.

Preliminary Cytotoxicity

Cytotoxicity was determined by observing inhibition of cell growth in 24-well cluster dishes (Linbro). 12 concentrations of each chemical were used, with 2 replicate wells per dish. Plates were set up at 4×10^4 cells/well in 0.5 ml MEM, 2 plates per chemical (i.e. with and without S9) with room temperature medium. After 45 min at room temperature, plates were incubated for 24 h at 37 °C. Following removal of the MEM, 0.5 ml of the final treatment mixture was dispensed to each well, and plates were incubated 1 h at 37 °C and washed with phosphate buffered saline (PBS) 3 times. After incubating 72 h at 37 °C, medium was removed, plates fixed in ethyl alcohol : glacial acetic acid, and stained with Giemsa (4% in H_2O).

To select a range of concentrations to be used in subsequent experiments, a cytotoxicity threshold concentration was determined. This value represents the lower limit of cytotoxicity as determined by visible inhibition of cell growth.

Alkaline Sucrose Gradient Analysis

CHO cells were labelled overnight in 60-mm tissue culture dishes containing 8×10^5 cells in MEM supplemented with 10% newborn calf serum and 0.5 μCi [^{3}H]thymidine (TdR)/ml (46 Ci/mM) or 1.0 μCi ^{14}C-TdR/ml (50 mCi/mM). Cells were chased with nonradioactive medium for 2 h prior to treatment and then treated with 1.4 ml of the treatment mix for 1 h. After removal of the mixture, the cells were washed 3 times in ice-cold, calcium- and magnesium-free, phosphate-buffered saline containing 10 mM EDTA (PBS). Cells were removed from the culture dishes by gentle scraping and resuspended in 1 ml ice-cold PBS.

Each gradient contained 4.8 ml composed of 4.5 ml 5–20% alkaline sucrose and a 0.3 ml lysis layer. Approximately 9×10^3 ^{14}C-TdR-labeled control cells and 9×10^3 ^{3}H-TdR-labeled treated cells were lysed on each gradient for 10.5 h at 20 °C and spun at 15000 rpm for 6 h in a Beckman SW50.1 rotor. Gradients were fractionated from the top into 0.2-ml fractions, collected in mini-scintillation vials, and counted in 5 ml Ready-Solv HP/b (Beckman) after neutralization with 0.2 ml 0.5 N HCl.

Calculation of molecular weights was carried out employing a sedimentation constant derived from the sedimentation characteristics of bacteriophage T4 DNA.

Sister-chromatid exchange

For each 60-mm plate, 2×10^5 cells were set up in MEM and incubated at 37 °C for 24 h. After removal of medium, 1.4 ml of treatment mixture was added to each plate. Following incubation for 1 h at 37 °C, cells were washed 3 times in Earle's balanced salt solution and incubated in 5 ml fresh MEM with 10 μM 5-bromodeoxyuridine (BrdUrd) per plate. Cultures were then incubated for 24 h at 37 °C, and 0.25 μg/ml colcemid added to each plate. After incubation for 2 h at 37 °C, cells were harvested by gentle scraping. Slides were stained in Hoechst 33258 (5.0 μg/ml in phosphate buffer,

pH 6.8) for 25 min, and rinsed in phosphate buffer (pH 6.8). Coverslips were mounted in the same buffer and exposed to black light (F15T8/BLB, Westinghouse) for 7–10 min at 55–60 °C on a slide warmer. After removal of coverslips, slides were stained with Giemsa 3% at pH 6.8 (BDH buffer powder) for 7 min.

After coding, two slides were scored (15 cells/slide) from each of the 2 duplicate cultures, giving a total of 30 cells per culture and 60 cells per treatment.

In vitro micronucleus assay

Cells were seeded in 60-mm culture dishes (2×10^5/dish) or in 8-well chamber slides (5000/well, Lab-Tek) and incubated at 37 °C for 24 h. Following a 1-h treatment, cultures were washed 3 times with Earle's BSS and incubated for a further 24 h at 37°C. For experiments performed in dishes, cells were removed by gentle scraping and treated with 0.075 M KCl for 10 min at 37 °C. Cells were fixed with 2 changes of ethanol : glacial acetic acid, 3 : 1, and slide preparations made. For chamber slides, these operations were performed in situ.

Nuclear fragments were scored as micronuclei only if: (a) the entire circumference of the fragment or the primary nucleus could be seen; (b) the diameter of the nuclear fragment was not greater than one-third that of the primary nucleus; and (c) the fragment exhibited a texture and staining intensity similar to that of the primary nucleus.

Slides and wells were coded such that the scorer had no knowledge of the treatment; 500 cells were scored from each of 2 cultures, yielding 1000 cells per treatment.

Analysis of data

ASG analysis. A positive response was indicated by a dose-related reduction in the distance sedimented by the treated DNA relative to the control DNA; this reflects a reduction in the molecular weight of the treated DNA caused by induced single-strand discontinuities. In some cases, the treated DNA peak was only skewed towards the top of the gradient. This was interpreted as a weak positive response. A toxic response was indicated when DNA appeared at the top of the gradient without a dose-related decline in molecular weight. In some cases, there were two

TABLE 1

RESULTS OF CLUSTER DISH ASSAY FOR CYTOTOXICITY IN CHO CELLS

Chemical	S9	Cytotoxicity threshold [a]	
		M (molar)	μg/ml
HMPA	–	1×10^{-5}	1.79
	+	5×10^{-5}	8.95
TOL	–	1×10^{-5}	1.07×10^{-2}
	+	1×10^{-5}	1.07×10^{-2}
BEN	–	1×10^{-5}	7.81×10^{-1}
	+	1×10^{-5}	7.81×10^{-1}
SAF	–	5×10^{-5}	8.11
	+	2.5×10^{-5}	4.05
CAP	–	2×10^{-5}	2.26
	+	5×10^{-5}	5.66
ACN	–	1×10^{-4}	5.31×10^{1}
	+	1×10^{-5}	5.31
DEHP	–	1×10^{-5}	3.91
	+	2×10^{-5}	7.82
ZOIN	–	1×10^{-4}	2.12×10^{1}
	+	1×10^{-4}	2.12×10^{1}
DES	–	1×10^{-4}	2.68×10^{1}
	+	5×10^{-5}	1.34×10^{1}
PB	–	1×10^{-4}	2.32×10^{1}
	+	1×10^{-4}	2.32×10^{1}

[a] See Materials and Methods.

TABLE 2

SUMMARY OF RESULTS OF ALKALINE SUCROSE SEDIMENTATION ANALYSIS IN CHO CELLS [a]

Chemical	+S9	−S9
HMPA	–	–
TOL	± [b]	± [b]
BEN	–	–
SAF	–	–
CAP	–	–
ACN	+	+
DEHP	–	–
ZOIN	–	–
DES	+	+
PB	–	–

[a] See Data Analysis in Materials and Methods.
[b] Weak positive.

DNA peaks from treated cells, representing affected DNA that remained at the top of the gradient and unaffected DNA that sedimented to the same position as the untreated, control, DNA. This phenomenon was also interpreted as a toxic response.

SCE. A dose-related increase in SCE frequency greater than twice the solvent control value was considered a positive response.

In vitro micronucleus. A dose-related increase in micronucleus frequency greater than twice the historical solvent control value for this study was considered a positive response.

Results and discussion

Prior to testing the chemicals for genotoxic effects, a determination of cytotoxicity was made using inhibition of cell growth as the endpoint. Table 1 shows the concentrations at which growth inhibition is evident from visual inspection. These values, ranging from 10^{-5} to 10^{-4} M, were used to assist in the selection of concentration ranges for testing.

In the ASG assay, 2 chemicals, DES and ACN, produced positive dose-related responses that could be attributed to DNA lesions (Table 2). In addition, a third chemical, TOL, caused possible DNA damage. These effects were detected both in the presence and absence of S9 in the treatment mixtures. Table 2 shows the results of the ASG assays expressed as breaks/10^8 dalton. The term 'breaks' is used here to denote all single-strand discontinuities, induced either directly or derived, which are detectable by this assay. Table 3 shows that the

TABLE 3

EFFECT OF CHEMICALS ON ALKALINE SUCROSE-DETECTABLE DNA LESIONS [e]

Chemical	Concentration		Induced breaks/10^8 dalton	
	μg/ml	M (molar)	+S9	−S9
HMPA	1.79×10^{3}	1×10^{-2}	0	0.009
	5.37×10^{3}	3×10^{-2}	0	0
	8.95×10^{3}	5×10^{-2}	0	0
	1.25×10^{4}	7×10^{-2}	0	0
	1.79×10^{4}	1×10^{-1}	0	0.003
TOL	3.21×10^{3}	3×10^{-2}	0	0
	4.28×10^{3}	4×10^{-2}	0.933 [a]	0.085 [a]
	5.35×10^{3}	5×10^{-2}	15.6 [b]	3.68 [b]
	5.89×10^{3}	5.5×10^{-2}	33.9 [b]	–
	6.42×10^{3}	6×10^{-2}	24.6 [b]	23.5 [b]
	7.50×10^{3}	7×10^{-2}	–	29.7 [b]
BEN	3.9×10^{2}	5×10^{-3}	–	–
	7.81×10^{2}	1×10^{-2}	0	0
	2.34×10^{2}	3×10^{-2}	0	0
	4.68×10^{2}	6×10^{-2}	8.68 [b]	7.18 [b]
	7.81×10^{3}	1×10^{-1}	12.3 [b]	6.17 [b]
SAF	8.11	5×10^{-5}	0.033	0.005
	1.62×10^{1}	1×10^{-4}	0.024	0.007
	8.1×10^{1}	5×10^{-4}	0.033	0
	1.62×10^{2}	1×10^{-3}	0	0
	3.24×10^{2}	2×10^{-3}	0.086, 32.6 [bc]	–
CAP	5.66×10^{2}	5×10^{-3}	0	0
	7.92×10^{2}	7×10^{-3}	0	–
	1.13×10^{3}	1×10^{-2}	0	0
	3.39×10^{3}	3×10^{-2}	0	0
	5.66×10^{3}	5×10^{-2}	0	0
	1.13×10^{4}	1×10^{-1}	–	0

TABLE 3 (continued)

Chemical	Concentration		Induced breaks/10^8 dalton	
	μg/ml	M (molar)	+S9	−S9
ACN	5.31×10^{2}	1×10^{-2}	0	0
	1.59×10^{3}	3×10^{-2}	0	0.009
	2.65×10^{3}	5×10^{-2}	0	0
	3.71×10^{3}	7×10^{-2}	0.038 [a]	0.090 [a]
	5.31×10^{4}	1×10^{-1}	0.201 [a]	0.291 [a]
DEHP	3.91×10^{3}	1×10^{-2}	0.019	0
	1.17×10^{4}	3×10^{-2}	0	0
	1.95×10^{4}	5×10^{-2}	–	0
	2.73×10^{4}	7×10^{-2}	0.004	0
	3.91×10^{4}	1×10^{-1}	0	0
ZOIN	2.12×10^{2}	1×10^{-3}	0	0
	1.06×10^{3}	5×10^{-3}	0	0
	1.49×10^{3}	7×10^{-3}	0	0
	2.12×10^{3}	1×10^{-2}	0	0
	6.37×10^{3}	3×10^{-2}	0	0
DES	1.88×10^{1}	7×10^{-5}	–	–
	2.68×10^{1}	1×10^{-4}	0	0.156 [a]
	8.05×10^{1}	3×10^{-4}	0.504 [a]	1.07 [a]
	1.34×10^{2}	5×10^{-4}	1.41 [a]	1.78 [a]
	1.88×10^{2}	7×10^{-4}	2.08 [a]	1.65 [a]
	2.68×10^{2}	1×10^{-3}	2.69 [a]	–
PB	1.16×10^{3}	5×10^{-3}	0	0
	1.63×10^{3}	7×10^{-3}	0	0
	2.32×10^{3}	1×10^{-2}	0	0
	6.97×10^{3}	3×10^{-2}	0.119 [d]	0
	1.16×10^{4}	5×10^{-2}	0.064 [d]	0

[a] Positive effect.
[b] Toxic effect.
[c] Two peaks.
[d] Treated DNA skewed to top of gradient, but no clear-cut effect.
[e] See data analysis in Materials and Methods.

DNA damage produced by DES is greater than that of ACN, the latter being a relatively weak effect. For DES and ACN, the addition of S9 to the treatment mixture produced little change in the level of damage. In the case of TOL, DNA damage was seen only at 4×10^{-2} M. This effect was much more pronounced in the presence of S9 mix. At 5×10^{-2} M, DNA degradation due to cytotoxicity was observed.

Table 4 summarizes the in vitro micronucleus test results. The data are illustrated in Fig. 1. Only ACN caused a dose-related increase in micronuclei. This effect was observed in both the presence and absence of S9 mix. The former effect was confirmed in a subsequent experiment in which the dose–response was more clearly defined (data not shown).

6 of the 10 chemicals were tested for the ability to induce SCE. The results are summarized in Table 5 with the data shown in Table 6. None of the 6 chemicals tested caused an increase in SCE.

The in vitro assays employed in this study detected 3 of the 8 chemicals designated as carcinogens in the IPCS Collaborative Study. Until recently, DES has been regarded as a nonmutagenic carcinogen; however, there is now evidence

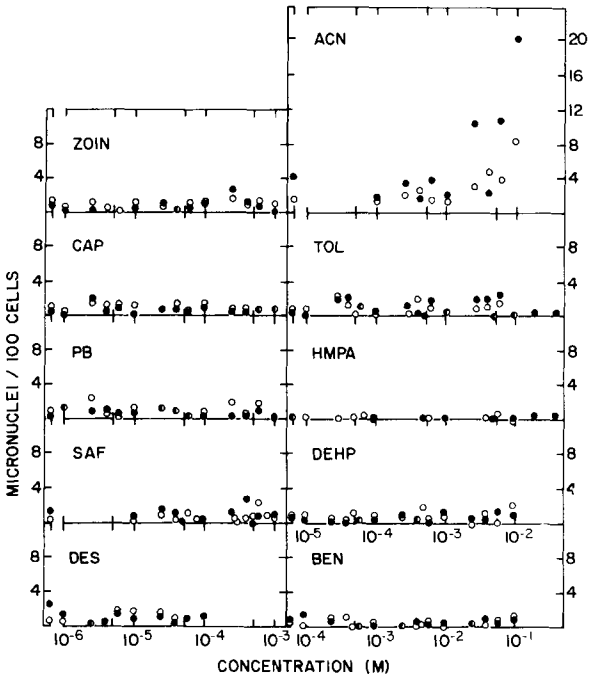

Fig. 1. Frequency of micronuclei in CHO cells. ●, with S9; ○, without S9.

that it causes SCE in the lymphocytes of pregnant women (Hill and Wolff, 1982) and mutation in yeast in the presence of oxidizing agents (Mehta and von Borstel, 1982). Birnboin (personal communication) has also found that DES induced DNA lesions in human white blood cells, as detected using the FADU (McLean et al., 1982) technique. This effect was not inhibitable by superoxide dismutase or catalase and, thus, does not appear to be due to free radicals resulting from auto-oxidation processes.

TABLE 4

SUMMARY OF RESULTS OF IN VITRO MICRONUCLEUS ASSAY [a]

Chemical	+S9	−S9
HMPA	–	–
TOL	–	–
BEN	–	–
SAF	–	–
CAP	–	–
ACN	+	+
DEHP	–	–
ZOIN	–	–
DES	–	–
PB	–	–

[a] See Data Analysis in Materials and Methods.

TABLE 5

SUMMARY OF RESULTS OF SCE ASSAY IN CHO CELLS [a]

Chemical	+S9	−S9
HMPA	–	–
TOL	–	–
BEN	–	–
SAF	–	–
CAP	–	–
DEHP	–	–

[a] See Data Analysis in Materials and Methods.

TOL has been shown previously to be positive in a number of in vitro mammalian assays (de Serres and Ashby, 1981). Accordingly, it is not

TABLE 6

EFFECT OF CHEMICALS ON SCE IN CHO CELLS

Chemical	Concentration		SCE/cell (±SE) [b]	
	μg/ml	M (molar)	+S9	−S9
HMPA	0	0 (DMSO)	6.55 (0.33)	9.63 (0.40)
	1.07×10^{2}	6×10^{-4}	–	9.13 (0.39)
	1.79×10^{2}	1×10^{-3}	6.36 (0.33)	8.63 (0.38)
	7.16×10^{2}	4×10^{-3}	–	8.75 (0.38)
	1.07×10^{3}	6×10^{-3}	7.63 (0.36)	8.33 (0.37)
	1.79×10^{3}	1×10^{-2}	7.07 (0.34)	8.30 (0.37)
	3.58×10^{3}	2×10^{-2}	6.98 (0.38)	–
	7.16×10^{3}	4×10^{-2}	6.56 (0.33)	–

TABLE 6 (continued)

Chemical	Concentration		SCE/cell (±SE) [b]	
	μg/ml	M (molar)	+S9	−S9
TOL	0	0 (DMSO)	6.26 (0.32)	6.27 (0.32)
	1.07	1×10^{-5}	–	7.06 (0.34)
	5.35	5×10^{-5}	–	6.55 (0.33)
	1.07×10^{1}	1×10^{-4}	6.56 (0.38)	6.84) (0.34)
	3.21×10^{1}	3×10^{-4}	–	6.33 (0.32)
	5.35×10^{1}	5×10^{-4}	6.32 (0.37)	–
	6.42×10^{1}	6×10^{-4}	–	6.38 (0.33)
	1.07×10^{2}	1×10^{-3}	7.92 (0.40)	6.96 (0.34)
	4.28×10^{2}	4×10^{-3}	6.12 (0.36)	–
	8.56×10^{2}	8×10^{-3}	7.18 (0.35)	–
	1.07×10^{3}	1×10^{-2}	7.65 (0.36)	–
BEN	0	0	7.05 (0.34)	6.52 (0.33)
	7.81	1×10^{-4}	6.91 (0.33)	6.43 (0.33)
	3.91×10^{1}	5×10^{-4}	6.80 (0.34)	6.97 (0.34)
	7.81×10^{1}	1×10^{-3}	6.05 (0.37)	6.48 (0.33)
	2.34×10^{2}	3×10^{-3}	6.28 (0.32)	–
	3.91×10^{2}	5×10^{-3}	–	6.60 (0.33)
	4.68×10^{2}	6×10^{-3}	7.48 (0.41)	–
	7.81×10^{2}	1×10^{-2}	6.03 (0.32)	6.08 (0.32)
SAF	0	0	7.45 (0.35)	6.08 (0.32)
	8.1	8×10^{-5}	–	5.80 (0.32)
	1.62×10^{1}	1×10^{-4}	6.97 (0.34)	6.11 (0.32)
	4.86×10^{1}	3×10^{-4}	7.23 (0.35)	6.51 (0.33)
	8.10×10^{1}	5×10^{-4}	–	6.82 (0.37)
	9.72×10^{1}	6×10^{-4}	8.25 (0.37)	–
	1.30×10^{2}	8×10^{-4}	8.71 (0.38)	MI [a]
	1.62×10^{2}	1×10^{-3}	MI [a]	MI [a]
	3.24×10^{2}	2×10^{-3}	MI [a]	–
CAP	0	0	5.40 (0.30)	5.91
	5.65	5×10^{-5}	–	6.31 (0.33)
	1.13×10^{1}	1×10^{-4}	6.75 (0.34)	5.98 (0.36)
	5.65×10^{1}	5×10^{-4}	6.00 (0.33)	6.35 (0.35)
	1.13×10^{2}	1×10^{-3}	6.21 (0.37)	6.45 (0.33)
	3.39×10^{2}	3×10^{-3}	6.45 (0.39)	–
	5.65×10^{2}	5×10^{-3}	–	6.05 (0.32)
	6.78×10^{2}	6×10^{-3}	5.40 (0.31)	–
	1.13×10^{3}	1×10^{-2}	6.82 (0.35)	5.83 (0.36)
DEHP	0	0	6.72 (0.33)	6.96 (0.34)
	3.90	1×10^{-5}	–	7.05 (0.34)
	1.95×10^{1}	5×10^{-5}	–	6.75 (0.34)
	3.90×10^{1}	1×10^{-4}	6.53 (0.33)	7.17 (0.35)
	1.95×10^{2}	5×10^{-4}	7.13 (0.34)	7.03 (0.35)
	3.90×10^{2}	1×10^{-3}	7.16 (0.34)	6.80 (0.34)
	1.17×10^{3}	3×10^{-3}	7.57 (0.36)	–
	2.34×10^{3}	6×10^{-3}	6.73 (0.33)	–
	3.90×10^{3}	1×10^{-2}	6.43 (0.32)	–

[a] Mitotic inhibition.

[b] Standard error of mean estimated from poisson statistics.

surprising that evidence of DNA damage was found in this study.

ACN caused DNA damage only at very high concentrations (10^{-1} M) and this effect was evident both with and without S9 in the treatment mix.

Ved Brat and Williams (1982) have shown that ACN requires the metabolism of primary rat hepatocytes to induce SCE in CHO cells. Effects were reported at concentrations of 10^{-4} M and lower. Thus, the finding of effects at high concentrations not requiring metabolic activation suggests these effects may arise through chemical reaction seen only at these higher concentrations.

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Induction of DNA single-strand breaks in CHO cells in culture

Th. Lakhanisky and B. Hendrickx
Unité de Toxicologie Génétique, Brussels (Belgium)

Summary

9 chemicals were tested for their ability to induce DNA single-strand breaks (ssb) in CHO cells. 4 of the compounds tested, *o*-toluidine, benzene, safrole and diethylstilbestrol produced DNA damage detectable by alkaline sucrose sedimention. In the cases of benzene and safrole, presence of S9 mix seemed to have a 'protective' effect in reducing the number of induced ssb. The 5 other chemicals, hexamethylphosphoramide, caprolactam, acrylonitrile, diethylhexylphthalate and benzoin were inactive in our test.

Presence of S9 mix enhanced survival of CHO cells when treated with acrylonitrile, benzoin or diethylstilbestrol; it reduced growth in toluidine-treated cells.

Introduction

Lesions in DNA are indicative of potential mutagenicity and/or carcinogenicity of chemicals.

One of the consequences of DNA damage is the production of single-strand DNA interruptions that can be detected after denaturation of the DNA in alkaline medium. We measured the number of such single-strand breaks by using alkaline sucrose-gradient sedimentation.

Materials and methods

Chemicals

Ham's F10 medium, but without hypoxanthine or thymidine, as well as newborn calf serum, were obtained from Flow Laboratories, U.K; hypoxanthine from Suchardt Ltd., U.K.; sodium penicillin G and streptomycin from Pfizer and R.I.T., Genval, Belgium; [^{3}H]thymidine (^{3}H-TdR) was obtained from the Radiochemical Centre, Amersham, U.K.; EDTA-disodium salt (komplexon III) from Siegfried AG, Switzerland; dimethyl sulfoxide (DMSO), for spectroscopy, from Merck, Germany; Hepes and ethyl methanesulfonate were from Sigma Chemical Co., U.S.A. and dimethylnitrosamine from Merck, Germany.

Cell culture

CHO cells were routinely grown in F10 medium, supplemented with 15% newborn calf serum, 30 μM hypoxanthine, penicillin (100 μ/ml) and streptomycin (100 μl/ml) (complete medium).

Metabolic activation

Metabolic activation was accomplished by adding a 9000 *g* supernatant preparation from Aroclor-1254-induced rat liver (S9) (J. Arany, I.H.E.) and the appropriate cofactors to the treatment mixture according to Maron and Ames (1983).

Cell treatment

Cells were seeded at a density of 10^5/6-cm petri dish in 2 ml medium containing 0.5 μCi/ml ^{3}H-TdR (51 Ci/mM). After 24 h of labelling, the radioactive medium was replaced by fresh nonradioactive medium and the incubation continued for at least 2 h. Cells were washed once with F10 HNS medium (F10 without serum and supplemented with 20 mM Hepes, pH 7.4).

For treatment of washed cells, concentrated solutions of the test compound in DMSO were prepared shortly before use, quickly diluted with warm F10 HNS in presence or absence of S9 mix and used immediately thereafter (final concentra-

TABLE 1
RESULTS OF ALKALINE SUCROSE SEDIMENTATION/CHO CELLS ASSAY AND THEIR CORRELATION WITH GENOTOXICITY

Chemical	Test criterion	Carcino-genicity	Conclu-sions
Hexamethylphosphoramide	–	+	?
o-Toluidine	+	+	+
Benzene	+	+	+
Safrole	+	+	+
Caprolactam	–	–	–
Acrylonitrile	–	+	?
Diethylhexylphtalate	–	+	?
Benzoin	–	–	–
Diethylstilbestrol	+	+	+

tion of DMSO 5%).

Petri dishes with cell culture from which medium was removed were placed in a waterbath at 37 °C and within a few seconds, 2 ml of the mutagen solution was added and incubated for various time intervals. After treatment, the cells were washed twice with ice-cold PBS (8.1 mM Na_2HPO_4, 0.14 M NaCl and 2.6 mM KCl) and after that, kept on crushed ice and sheltered from light.

For survival experiments, 200–400 cells were seeded, allowed to attach for 4 h and treated with the test agent in presence or absence of S9 mix. At the end of treatment, they were washed twice with PBS and incubated in fresh medium for 10 days, when colonies were fixed in 3:1 methanol:glacial acetic acid, stained with Giemsa 10% and counted.

Detection of single-strand DNA breaks by high-salt alkaline sucrose-gradient sedimentation

The ^{3}H-labeled CHO cells on petri dishes were trypsinized at 0 °C (75 μl of 0.25% trypsin + 2 mM EDTa per 6-cm petri dish) and 75 μl of 0.25 M bovine serum albumin in PBS were added. The cells were resuspended by pipetting, 50 μl of cell suspension ($3–6 \cdot 10^5$ cells) were placed between two 75-μl layers of lysis solution (1 M NaOH, 10 mM EDTA) on top of a linear 5–20% sucrose gradient (4.6 ml). The gradient solution contained 0.33 M NaOH, 10 mM EDTA and 2 M NaCl. Cells were allowed to lyse on top of the gradients for 1 h at 20 °C. Then the tubes were centrifuged at the same temperature in a TST 54 rotor in a Kontron preparative ultracentrifuge (17000 rpm, 121 min).

After centrifugation, 28 fractions (8 drops/fraction) were collected from the bottom of the gradients by a peristaltic pump into small vials containing 0.5 ml HCl 0.5 N. After adding 5 ml scintillation liquid (Packard, U.S.A.) the fractions were counted in a Bertold liquid scintillation counter Betaszint 5000/300. The number of ssb per 10^9 dalton DNA were calculated from the sedimentation patterns by means of a computer program as described by van der Schans et al. (1982).

Test criterion

A positive result was indicated by a dose-related increase in the number of ssb, the minimum significant amount of ssb being 5 ssb/10^9 dalton DNA.

Results

A summary of the results obtained for the 9 chemicals is shown in Table 1.

Table 2 shows the response of the test to the 4 compounds that induced single-strand breaks in the DNA of CHO cells.

Hexamethylphosphoramide, acrylonitrile and diethylhexylphtalate registered false negatives when tested with CHO cells by the described treatment protocol. Similarly, caprolactam, and benzoin did not induce ssb, confirming their non-carcinogenicity.

Positive responses were obtained with *o*-toluidine, benzene, safrole, and diethylstilbestrol. The effect observed with *o*-toluidine was dependent on S9 activation. In contrast, the positive response obtained with benzene was annihilated in presence of the activating system. Nevertheless, for both compounds, high concentrations or long exposure times were necessary to induce a significant number of ssb.

Equivocal results were obtained with diethylstilbestrol: at 10^{-4} M, presence of S9 mix enhanced the DNA-strand interruption frequency; but at 10^{-5} M, on the contrary, the number of induced ssb was lower than in the cells treated in absence of metabolization.

This "protective effect" of the activating fraction on the induction of ssb was strongly noticea-

TABLE 2

RESULTS OF SELECTED CHEMICALS EVALUATION

Results of selected chemicals and their evaluation

Dose (M)	Treatment time (min)	S9 mix	ssb/10^9 dalton DNA	Survival (%)	Evaluation
o-Toluidine					
2.0×10^{-2}	60	–	1.3	89.2	–
	60	+	7.2	47.4	+
5.0×10^{-2}	60	–	2.5	84.8	–
	120	–	6.1	ND *	–
Benzene					
1.7×10^{-2}	30	–	9.7	ND	+
	60	–	76.6	20.9	+
	60	+	0.8	33.0	–
	120	–	268.5	ND	+
Safrole					
1.0×10^{-3}	60	–	17.6		+
	60	+	6.4		+
1.3×10^{-3}	60	–	59.9		+
	60	+	7.2		+
1.6×10^{-3}	30	–	8.5		+
	60	–	94.7		+
	60	+	9.0		+
	120	–	182.8		+
3.4×10^{-3}	60	–	108.0		+
	60	+	11.4		+
Diethylstilbestrol					
1.0×10^{-5}	60	–	8.3	70.0	+
	60	+	5.8	89.9	+
1.0×10^{-4}	60	–	9.6	0.0	+
	60	+	12.1	20.6	+
	120	–	28.4	ND	+
Positive controls					
EMS					
5.0×10^{-2}	60	–	13.6	40.0	+
DEN					
1.0×10^{-2}	60	+	12.1	90.0	+
Negative controls					
CHO cells					
–	60	–	2.2	100.0	–

* ND, not determined.

ble in safrole-treated cells. Furthermore, in our test, this carcinogen showed the strongest dose-related response and in a very narrow fork of concentrations: 10^{-3} M to 3.4×10^{-3} M.

Survival fractions determination in case of safrole treated cells was unreliable because of formation of an emulsion-type suspension in the growth medium. But, in general, DNA lesions measured as single-strand breaks did not seem relevant for the cell killing.

Conclusions

From the data obtained in the present study, the gradient sedimentation analysis has shown a sensitivity value of approximately 67%. If com-

bined with the alkaline elution technique, we might expect a significant increase in sensitivity.

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Assays to measure the induction of unscheduled DNA synthesis in cultured hepatocytes

Howard P. Glauert, Wendy S. Kennan, Gerald L. Sattler and Henry C. Pitot

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI (U.S.A)

Summary

8 carcinogens and 2 non carcinogens, as judged by whole animal bioassay, were tested for their ability to induce unscheduled DNA synthesis in primary hepatocyte cultures. The amount of unscheduled DNA synthesis was determined by quantifying the amount of [^{3}H]thymidine incorporated into DNA in the presence of hydroxyurea after isolation of nuclei from hepatocytes treated with the test agent. The carcinogens HMPA, TOL, BEN, SAF, ACN and DEHP, and the noncarcinogen ZOIN induced a significant increase in unscheduled DNA synthesis, whereas the noncarcinogen CAP and the carcinogens DES and PB were negative.

Introduction

Our laboratory participated in the IPCS collaborative study by determining the effect of the 10 test chemicals on unscheduled DNA synthesis in cultured hepatocytes, using the method developed in our laboratory (Althaus et al., 1982). Because hepatocytes are capable of metabolizing a great variety of xenobiotics, the primary hepatocyte culture system provides an excellent procedure for the screening of potential carcinogens. The amount of unscheduled DNA synthesis was measured by [^{3}H]thymidine incorporation into DNA during exposure of the cells to the test chemicals. Hydroxyurea was added to prevent replicative DNA synthesis. Nuclei were isolated from cells and the amount of [^{3}H]thymidine bound to DNA was determined by quantitation using liquid-scintillation spectrometry.

Materials and methods

Culture of adult rat hepatocytes

Hepatocytes were isolated from the livers of adult male albino rats (The Holtzman Co.; 220–280 g, fed ad libitum) by the collagenase perfusion method of Berry and Friend (1969) as modified by Bonney et al. (1974). The hepatocytes were then plated at a cell density of $10–13 \times 10^6$ cells in 10 ml of L-15 medium [supplemented with 18 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, albumin (2 mg/ml), 5% newborn calf serum, penicillin (100 μg/ml), streptomycin (100 μg/ml), glucose (1.5 mg/ml), and insulin (0.5 μg/ml)] onto 100-mm Falcon tissue-culture dishes. A medium change was routinely performed 4 h after plating to remove unattached cells, and the culture was maintained for the times indicated. For treatment of cultures with test agents, stock solutions of chemicals in ethanol or dimethyl sulfoxide were added to the cultures. The concentration of solvent in the medium never exceeded 1%. For treatment with UV, the culture medium was removed, and the monolayers were irradiated with a germicidal lamp (254 nm) at an incident dose rate of 60 J/m^2/sec. Hydroxyurea (10 mM) was added to the medium 1 h before carcinogen treatment, to reduce [*Me*-^{3}H]thymi-

dine incorporation from replicative DNA synthesis. [*Me*-^{3}H]thymidine (10 μCi/plate; 42–48 Ci/mmole) was added to the medium immediately after the agent under study. The hepatocyte cultures were treated with the test chemical 18 h before harvesting, which was done between 45 and 48 h after plating of cells.

Measurement of DNA-repair synthesis

At the end of each experiment, cell monolayers were washed twice with phosphate-buffered saline (136.9 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) containing 2 mM thymidine. Cells were then scraped off the plates with a rubber policeman. All the following procedures were carried out at 4°. Hepatocellular nuclei were isolated as described by Althaus et al. (1982). Hepatocytes were pelleted, and the cells were resuspended in 900 μl of hypotonic buffer (10 mM Tris–HCl, 5 mM $MgCl_2$, pH 8.0) and incubated for 10 min. The cells were then homogenized for 15 sec (Tissuemizer homogenizer, Tekmar, Cincinnati, OH, maximum speed), incubated in 1% Triton X-100 for 5 min, and homogenized again. The homogenate was centrifuged at 600 × *g*, and the pellet was resuspended in 0.25 M buffered sucrose (50 mM Tris–HCl, 25 mM KCl, 15 mM $MgCl_2$, pH 8.0). The suspension was then layered over 0.88 M sucrose–Tris–HCl–KCl–$MgCl_2$ (concentrations same as above) in 15-ml Corex tubes and centrifuged at 2500 rpm for 10 min in a Sorval Model GLC-1 centrifuge. The pellet, which contained the nuclear fraction, was resuspended in 500 μl 10 mM Tris–HCl (pH 8.0) and stored frozen overnight. After thawing, 500 μl of 10 mM Tris–HCl (pH 8.0) and 500 μl of 1 N KOH were added, and the nuclei were incubated at 37° for 45 min. After neutralization with 1 N HCl (400 μl), DNA was precipitated in the presence of 10% trichloroacetic acid (TCA) and 0.5% bovine serum albumin. DNA was hydrolyzed by heating in 5% TCA at 90° for 20 min. The solution was centrifuged and aliquots of the supernatant were used for liquid-scintillatin counting and for measurement of DNA concentration using the diphenylamine method of Burton (1956).

TABLE 1

ABILITY OF 10 CHEMICALS TO INDUCE UNSCHEDULED DNA SYNTHESIS IN CULTURED HEPATOCYTES

Chemical	Test result	Carcinogenicity
Hexamethylphosphoramide	+	+
o-Toluidine	+	+
Benzene	+	+
Safrole	+	+
Caprolactam	−	−
Acrylonitrile	+	+
Diethylhexylphthalate	+	+
Benzoin	+	−
Diethylstilbestrol	−	+
Phenobarbital	−	+

Results and discussion

Each chemical was tested initially at several concentrations to obtain a dose–response curve. The chemicals were then tested at the maximum effective dose, using 4 or 5 plates each for the chemical and for the control. Means were compared using Student's *t*-test (for equal sample variances) or Lohrding's test (for unequal variances but equal coefficients of variation) (Gill, 1978). Values that were greater than control values with greater than 95% confidence were considered 'weakly positive', whereas those that were greater than control values with greater than 99% confidence were classified as 'positive'.

Table 1 summarizes the results from the 10 compounds tested. Table 2 shows the responses and statistics for each compound at the maximum effective dose. The following carcinogens induced a significant increase in unscheduled DNA synthesis: HMPA, TOL, BEN, SAF, ACN and DEHP. The carcinogens DES and PB were negative. The noncarcinogen CAP tested negative whereas the noncarcinogen ZOIN increased unscheduled DNA synthesis significantly.

Neither PB nor DES gave positive results. Although both have induced tumors in experimental animals, it is possible that they were promoting spontaneously initiated cells rather than acting as complete carcinogens. Phenobarbital has been shown to act as a promoter in rat liver (reviewed by Pitot and Sirica, 1980).

The noncarcinogen ZOIN tested strongly positive — more so than any other chemical. ZOIN is

TABLE 2

SUMMARY OF RESULTS USING HEPATOCYTE DNA-REPAIR ASSAY

Chemical	Dose range tested (M)	Dose selected for further testing (M)	UDS induced at optimal concentration [a,b] (% of control)
HMPA	10^{-2}–10^{-6}	10^{-3}	135 ± 6 **
TOL	$2 \cdot 10^{-2}$–10^{-6}	10^{-3}	176 ± 7 **
BEN	$5 \cdot 10^{-2}$–10^{-6}	10^{-3}	117 ± 7 *
SAF	10^{-2}–10^{-6}	$5 \cdot 10^{-4}$	187 ± 6 **
CAP	10^{-2}–10^{-6}	$5 \cdot 10^{-5}$	107 ± 4
ACN	10^{-2}–10^{-6}	10^{-3}	129 ± 4 *
DEHP	10^{-2}–10^{-6}	$5 \cdot 10^{-4}$	165 ± 11 *
ZOIN	10^{-2}–10^{-6}	10^{-3}	249 ± 5 **
DES	$5 \cdot 10^{-3}$–10^{-8}	$5 \cdot 10^{-8}$	111 ± 12
PB	10^{-2}–10^{-6}	$5 \cdot 10^{-3}$	100 ± 7

[a] Mean ± SEM.

[b] Value significantly different from control values are indicated by asteriks; * $P < 0.05$; ** $P < 0.01$.

noncarcinogenic in male or female F344 rats or B6C3F1 mice (DHHS, 1980).

In summary, our testing has shown that 6 of the 8 chemicals that induce tumors in chronic whole animal bioassays also induce unscheduled DNA synthesis in cultured hepatocytes. One of the two established noncarcinogens also induced DNA repair.

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Tests for the induction of unscheduled DNA repair synthesis in HeLa cells

Carl N. Martin and Jean Campbell [1]

Cancer Research Unit, University of York, York and [1] Microtest Research Ltd., University Road, Heslington, York (Great Britain)

Summary

When tested for their ability to induce unscheduled DNA synthesis in HeLa cells 3 compounds, hexamethylphosphoramide, *o*-toluidine, and safrole exhibited a positive response. Hexamethylphosphoramide did not require the addition of an extraneous metabolising preparation, whereas safrole and *o*-toluidine were positive only in the presence of an Aroclor-induced rat-liver preparation plus cofactors. The remaining 7 compounds tested: benzene, diethylhexylphthalate, acrylonitrile, diethylstilboestrol, phenobarbital, caprolactam and benzoin were all negative either in the presence or absence of rat-liver S9.

Measurement of unscheduled DNA synthesis (UDS) in a variety of cell lines in response to treatment with physical and chemical agents has been suggested as a useful short-term in vitro assay method for carcinogens (Martin et al., 1978; Williams, 1976; San and Stich, 1975). Numerous reports have shown a good positive correlation between the ability of a chemical to induce UDS and its carcinogenicity in animals. Sufficient evidence now exists to suggest that UDS, as monitored by uptake of [^{3}H]thymidine into cellular DNA, is a measure of polymerase activity during excision repair consequent upon structural modification of the DNA by chemical or electromagnetic carcinogenic agents.

Uptake of [^{3}H]thymidine has classically been measured in two ways: first, it has been quantified by the counting of silver grains following contact of the treated cell sheet with a radiosensitive photographic emulsion (autoradiography) and secondly by scintillation counting of extracted DNA. The present report describes the use of a scintillometric method using HeLa cells as the biological target (Martin et al. 1977, 1978; Martin and McDermid, 1981).

Compounds in order of testing were: hexamethylphosphoramide (HMPA), safrole, *o*-toluidine hydrochloride, benzene, diethylhexylphthalate (DEHP), acrylonitrile, diethylstilboestrol (DES), phenobarbital, caprolactam, benzoin. HeLa cells, whose normal S-phase DNA synthesis had been inhibited by a combination of growth in arginine-deficient medium and treatment with hydroxyurea, were treated with 8 doses of each agent. Treatments were carried out both in the presence and absence of a rat-liver post-mitochondrial supernatant from Aroclor-1254-induced animals and cofactors.

Materials and methods

Preparation of test chemical solutions

All concentrations were on a weight-per-volume basis and the compound was dissolved in anhydrous dimethyl sulphoxide (DMSO) prepared by drying before use with molecular sieve beads, aluminium sodium silicate, type 4A, 4–8 mesh.

Control compounds

The solvent used for the test compound was used as the negative control (DMSO). The positive control compounds included in this study were: 3,3'-dichlorobenzidine (1.26×10^{-2} mg/ml) +S9 mix and 4-nitroquinoline-1-oxide (1.90×10^{-3} mg/ml) −S9 mix.

Experimental procedures

Metabolic activation The mammalian post-mitochondrial fraction (S9) used for metabolic activation was prepared from male Wistar rats. The liver S9 fraction was prepared in advance and stored at −90 °C until used.

Preparation of liver homogenate (S9) rat-liver enzyme induction was by intraperitoneal injection of Aroclor 1254 (500 mg/kg, 200 mg/ml in corn oil). 5 days after injection, animals were lightly anaesthetised with ether, exsanguinated, killed by cervical dislocation and the livers removed. Livers were then homogenised in 3 vol. 150 mM KCl and the homogenate centrifuged at 9000 *g* for 10 min. The supernatant was dispensed in 4.5-ml aliquots for freezing at −90 °C and thawed just prior to incorporation into S9 mix.

Preparation of S9 mix The S9 mix contained per 0.5 ml:

Glucose 6-phosphate (180 mg/ml)	0.1 ml
NADP (25 mg/ml)	0.1 ml
150 mM KCl	0.1 ml
Rat liver S9 (25% in 150 mM KCl)	0.2 ml

0.5 ml of above was used per flask.

HeLa S3 cells were seeded into 60 disposable tissue-culture flasks (25 cm^2) in 10 ml minimum essential medium with Earle's salts + 15% foetal calf serum. The confluent monolayers obtained after 4 days incubation were then incubated in 10 ml minimum essential medium without arginine + 2.5% dialysed foetal calf serum. After 24 h the medium was replaced with a further 10 ml of arginine-deficient medium and incubation continued for a further 48 h. At the end of this time, a solution of hydroxyurea in water was added to each flask giving a final concentration of 10 mM.

After incubation for 1 h [^{3}H]thymidine was added to give 5 μCi/ml of medium. Alternate 2-fold and 5-fold dilutions at 8 concentrations of each compound were added immediately afterwards in 0.1-ml aliquots. Dilutions were added to triplicate bottles containing S9 and to triplicate bottles containing no metabolising system. Multiple control bottles were included in the experiment. Finally all flasks were incubated at 37 °C for 2.5 h to allow reaction with DNA and uptake of [^{3}H]thymidine. Following incubation, the medium was removed and further enzymic uptake of radioactivity prevented by the addition of 50 ml ice-cold 0.86% NaCl. The cell sheet was then removed as a suspension in 10 ml of warm EDTA solution (NaCl 8.0 g, KCl 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g, glucose 0.2 g, EDTA 0.2 g/l). Cells were pelleted at 1000 rpm in an MSE Mistral centrifuge, and after removal of the supernatant, the pellets were stored frozen at −20 °C overnight.

Cell pellets were thawed and then kept on ice. Precipitation procedures used ice-cold trichloroacetic acid (TCA) throughout. 10% TCA (2 ml) was added to each tube and the precipitate collected after centrifugation at 3000 rpm in an MSE Mistral centrifuge. After removal of the supernatant the cell precipitate was dissolved in 0.3 M KOH (2 ml) and incubated at 37 °C, for 1 h to hydrolyse RNA. Protein and DNA were reprecipitated by addition of 10% TCA (3 ml). Tubes were again centrifuged, the supernatant discarded, and the cell pellet finally suspended in 5% TCA (3.5 ml). Hydrolysis of the DNA was effected by heating at 90 °C for 20 min, leaving protein as a precipitate which was removed by centrifugation. The supernatant was then counted for radioactivity (0.5 ml) in a liquid scintillation counter (Packard Tri-Carb 300C) using 4.5 ml scintillant. The efficiency of counting was calculated by internal computer using automatic external standardisation and a previously constructed quench curve. The DNA concentration was estimated on a further 1 ml of supernatant using the Burton colourimetric assay. The colour was allowed to develop for 18 h in the dark and absorbance measured at 600 nm. Absolute values were determined against a standard graph plotted from solutions of hydrolysed calf thymus DNA of known concentration in 5% TCA set up at the same time and treated in the same manner as the experimental samples.

Results and discussion

The mean dpm/μg DNA and standard deviations of representative doses of each active test compound are compared with negative control values in Table 1. The results of the activity of the 10 tested compounds in the UDS assay in HeLa cells are presented in Table 2.

Incorporation of [^{3}H]thymidine per μg of DNA was calculated for each treatment flask. The mean values ± s.d. for each dose of the test compound

TABLE 1

MAXIMUM RESPONSE OVER BACKGROUND FOR THE 3 COMPOUNDS ACTIVE IN THE UDS ASSAY

Compound	Rat-liver S9	Dose at max. response (μg/ml)	Mean dpm/μg DNA at max. response (±S.D.)	Activity
DMSO	+	–	77.2 ± 3.0	
Hexamethyl-phosphoramide	+	2500	102.4 ± 1.2	+
DMSO	–	–	61.3 ± 1.7	
Hexamethyl-phosphoramide	–	125	91.1 ± 2.9	+
DMSO	+	–	107.3 ± 4.3	
o-toluidine	+	25	152.0 ± 7.2	+
DMSO	–	–	90.0 ± 7.3	
o-toluidine	–	25	96.8 ± 11.0	–
DMSO	+	–	63.9 ± 4.0	
Safrole	+	250	79.4 ± 8.8	+
DMSO	–	–	84.8 ± 9.2	
Safrole	–	25	102.0 ± 29.1	–

TABLE 2

COMPARISON OF ACTIVITY IN UDS ASSAY WITH ANIMAL OR HUMAN CARCINOGENICITY

Compound	Rat-liver S9	p value at max. response [a]	Activity in UDS assay	Carcinogenicity	
				Animal	Human
Hexamethyl-phosphoramide	+	0.001 (4) [b]	+	+	
	–	0.001 (5)			
Safrole	+	0.01 (2)	+	+	
	–	–			
o-Toluidine hydrochloride	+	0.001 (3)	+	+	
	–	–			
Benzene	+	–	–	–	+
	–	–			
Diethylhexyl-phthalate	+	–	–	+	
	–	0.05 (1)			
Acrylonitrile	+	–	–	+	
	–	–			
Diethylstil-boestrol	+	–	–	+	+
	–	–			
Phenobarbital	+	0.05 (1)	–	+	
	–	–			
Caprolactam	+	0.001 (1)	–	–	
	–	0.05 (1)			
Benzoin	+	–	–	–	
	–	–			

[a] p value was calculated using Student's t test for samples with equal variance, with 4 degrees of freedom. See text for explanation of statistical appraisal.

[b] Values in parentheses represent the number of adjacent dose levels at which a statistically significant response ($p < 0.05$) was recorded. Doses comprised of 2.5 mg/ml and 7 alternate 2-fold and 5-fold dilutions thereof.

were calculated and compared with the negative control value using Student's t-test for equal variance. Results which have a p value $\leqslant 0.01$ and have a p value $\leqslant 0.05$ for at least two adjacent concentrations are considered positive. Positive controls are similarly compared with negative controls and must have a p value $\leqslant 0.05$ to validate the test. It can be seen from Table I that of the 10 compounds tested only 3 were active in the UDS assay. All 3 compounds are carcinogenic in animals and an electrophilic species generated prior to covalent DNA binding has been identified for safrole (Phillips et al., 1981). These 3 compounds might be considered as classical initiating carcinogens in that they may alter the structure of the genetic material by covalent interaction which leads to a mutagenic event (Miller and Miller, 1976).

By the nature of the UDS assay it would be expected that any chemical agent acting in this way would be detected as a positive. Hexamethylphosphoramide was active in inducing UDS in the absence of an extraneous metabolising system. The positive result was very striking and suggests that its activation can be mediated by HeLa cell enzymes. Both *o*-toluidine and safrole are known to require metabolic conversion to electrophilic species for biological activity. These two compounds therefore behaved as expected in that they required addition of rat-liver S9 mix for activity in this assay.

None of the remaining compounds tested have been unequivocally shown to involve generation of an electrophilic species and binding to DNA in their induction of cancer although epoxidation of diethylstilboestrol has been suggested as a possible mode of activation (Neumann et al., 1977). In general those compounds that were negative in the UDS assay but are known to be carcinogenic in animals are regarded to act by what are termed epigenetic mechanisms. Benzene has not as yet been shown to be carcinogenic in animals although it is probably implicated in tumours of the haemopoietic system in humans. From the available evidence it appears unlikely that this compound or its metabolites are able to react chemically with DNA in mammalian species. Little evidence exists to suggest that DEHP, acrylonitrile or phenobarbital are carcinogenic due to direct interactions with DNA and for this reason it is perhaps not surprising that an assay designed to detect DNA-damaging agents is refractory to them. Although it has been suggested that DES may be activated via an epoxide intermediate to a positively charged species capable of interaction with negatively charged centres on DNA, it seems more likely that this compound is active primarily at a hormonal level. Again the negative result obtained with this compound is as might be predicted; however, it must be mentioned that when this compound was tested previously a small but significant response was noted on one occasion (Martin et al., 1978) but not when tested as a coded compound (Martin and McDermid, 1981).

In conclusion, therefore, from the data reported above, the unscheduled DNA synthesis assay gave a clear positive result for the compounds safrole, *o*-toluidine and HMPA. For this reason alone it might therefore be strongly argued that measurement of UDS as a short-term assay for chemical carcinogens could usefully be employed in a battery of such test systems. The lack of activity of safrole in the bacterial mutagenicity assay may result from an enzyme defficiency not apparent in the mammalian assay system. In the absence of hard evidence this must remain speculative however.

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Tests for the induction of DNA-repair synthesis in primary cultures of adult rat hepatocytes

G.S. Probst and L.E. Hill

Toxicology Division, Lilly Research Laboratories, Greenfield, IN (U.S.A.)

Summary

10 suspect genotoxins — hexamethylphosphoramide, safrole, *o*-toluidine, benzene, diethylhexylphthalate, acrylonitrile, diethylstilbestrol, phenobarbital, caprolactam and benzoin — were tested for the induction of unscheduled DNA synthesis in cultured hepatocytes. Primary cultures of adult rat hepatocytes were incubated for 20 h with 8 concentrations of each compound. Concentrations ranged from 0.5 to 1000 nmoles/ml, however, several of the compounds were tested using a maximum concentration of 10000 nmoles/ml. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 2-acetylaminofluorene (2AAF) were also tested and served as the positive controls. Unscheduled DNA synthesis was measured by autoradiography, and the study was replicated using 2 independent hepatocyte preparations.

A concentration-dependent induction of DNA-repair synthesis was observed in hepatocytes exposed to the carcinogens MNNG and 2AAF. No induction of DNA-repair synthesis was observed in any of the cultures treated with the test compounds or in the control cultures.

Chemical-induced DNA damage, measured by the induction of DNA-repair synthesis (unscheduled DNA synthesis, UDS) in cultured cells, has been proposed as a short-term in vitro test for the presumptive identification of mutagens and/or carcinogens (Probst et al., 1981; Williams, 1977). Primary cultures of adult rat hepatocytes (Williams, 1978) provide a novel in vitro system for the measurement of UDS in that these cells contain the necessary metabolic enzymes for the activation of many procarcinogens, and thus provide metabolic capability and a molecular target within the same cell. Cultures of adult rat hepatocytes have been shown to be sensitive to both ultimate carcinogens and procarcinogens for the induction of UDS, and selective against non-carcinogens (Probst et al., 1981; Williams 1977, 1978).

This report describes experiments in which primary cultures of adult rat hepatocytes were treated with either hexamethylphosphoramide, safrole, *o*-toluidine, benzene, diethylhexylphthalate, acrylonitrile, diethylstilbestrol, phenobarbital, caprolactam or benzoin to evaluate the induction of UDS.

Materials and methods

Chemicals

All test chemicals were provided by Lancaster Synthesis, Ltd. (East Gate, England) and were tested as supplied without further purification or attempts to identify impurities. Positive control compounds *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and 2-acetylaminofluorene (2AAF) were obtained from Aldrich Chemical Co. (Milwaukee, WI). [*Me*-^{3}H]Thymidine (^{3}H-TdR, spec. act. 25 Ci/mmole) was obtained from Amersham Searle (Arlington Heights, IL). All chemicals were dissolved in reagent-grade DMSO (Fisher Scientific Co., Pittsburgh, PA) and serial dilutions were made in serum-free media to give 8 compound concentrations covering a range of

0.5–1000 mg/ml for each test compound and MNNG, and a range of 0.05–100 nmoles/ml for 2AAF. In several cases where toxicity was not evident at a test compound concentration of 1000 nmoles/ml the compound was retested using a maximum concentration of 10000 nmoles/ml. The highest concentration of DMSO (1%) had no effect on cell morphology, survival or UDS in control cultures (Probst et al., 1981).

Cell cultures

Primary cultures of adult rat hepatocytes used for this study was prepared from the livers of 190 g male Fischer 344 rats (Charles River, Madison, WI) by the method of (Williams et al., 1977). The liver was perfused at 37 °C in situ via the hepatic portal vein for 4–6 min at a rate of 40–50 ml/min with Ca^{2+}–Mg^{2+}-free Hank's balanced salt solution (HBSS) containing 0.5 mM EGTA and buffered with 0.05 M HEPES to pH 7.2. The cells were disaggregated by continuous perfusion at 20–40 ml/min at 37 °C for 10–12 min. The perfusion medium was Williams' medium E buffered to pH 7.2 with 0.05 M HEPES (WEH) (Grand Island Biological Co., Grand Island, NY) that contained 100 units/ml collagenase (Type I, Sigma Chemical Co., St. Louis, MO). Cells were detached by combing in fresh WEH-collagenase medium followed by sequential filtration through 80-mesh nylon. The cells were washed once with WEH containing 10% fetal bovine serum (FBS) and 50 units/ml gentamicin (Reagent Solution, 10 mg/ml, Schering Corp., Port Reading, NJ) and centrifuged at $20 \times g$ for 4 min.

The two hepatocyte preparations used in these replicate studies yielded 3.04×10^8 and 3.28×10^8 hepatocytes with 88.07% and 85.57% viability.

Autoradiographic assay for UDS

Freshly prepared hepatocytes were plated at a density of 3.5×10^4 cells/cm^2 in 26 mm × 33 mm Multiplates containing 10.5 mm × 22 mm plastic coverslips (Thermanox, Lux Scientific Corp., Rockville, D; Williams, 1977) in WEH containing 10% FBS, 50 units/ml gentamicin and 100 units/ml each of penicillin and streptomycin (Grand Island Biological Co., Grand Island, NY). The cultures were incubated at 37 °C in a humidified 95/5% air/CO_2 environment for 2.5 h. After this period for attachment, the cells were washed once with WEH. Then serum-free WEH containing 10 μCi/ml ^{3}H-TdR and the appropriate dilution of the test compound was applied to each culture. After 20 h of incubation the media was removed and the cultures were washed and prepared for fixation. Hepatocytes grown on plastic coverslips were washed and fixed in the Multiplate chambers. The cultures were rinsed once with HBSS, allowed to standard for 10 min in 1% sodium citrate, and fixed by three 1-h washes with ethanol/acetic acid (3 : 1, v/v). All fixing and washing procedures were conducted at 4 °C. Coverslips were air-dried and glued with Permount (Fisher Scientific Co., Pittsburgh, PA) to glass slides, then stained with 1% acetoorcein for 3–5 min. The stained slides were air-dried and individually dipped into undiluted NTB-2 liquid photographic emulsion (Eastman Kodak, Rochester, NY), sealed in light-tight desiccated boxes, incubated for 7 days at 4 °C, developed with Kodak D-19 developer, and fixed with Kodak fixer. Cells were examined by oil-immersion microscopy without coverslips.

Unscheduled DNA synthesis in hepatocytes was quantified as described previously (Probst et al., 1981). Briefly, the number of silver grains over the cell nucleus was counted using a semiautomated Artek Model 880 colony counter (Artek Systems Corp., Farmingdale, NY), adapted for oil-immersion microscopy. Cytoplasmic background counts were determined by counting 3 nuclear-sized areas adjacent to the nucleus. The net nuclear grain count represents the difference between the gross nuclear grain count and the mean cytoplasmic background count. In cases where the cytoplasmic count exceeded the nuclear count, a negative value was recorded for the net nuclear count. This occurred for treatments not inducing UDS.

Nuclei of 20 morphologically unaltered cells, judged to be representative of the UDS responsiveness of the cell population and containing at least 4 grains, were counted for each treatment. Autoradiographic grain counts were conducted for the highest compound concentration that did not produce pronounced cytotoxicity (determined by alterations to cell morphology) and for all lower concentrations of the test compound.

Criteria for a positive response for UDS

A compound was judged to have induced a positive response for UDS when at least 2 successive compound concentrations produced nuclear grain counts which exceeded those of the control by 3 standard deviations of the control value.

Results and discussion

The induction of UDS was measured by autoradiography in primary cultures of adult rat hepatocytes after treatment with each test compound, MNNG or 2AAF. Eight compound concentrations were tested covering a range of 0.5–1000 nmoles/ml with each test compound. In the absence of a cytotoxic effect, several compounds were retested at a maximum concentration of 10000 nmoles/ml. The results of the replicate assays are presented in Table 1.

Control cultures treated with serially diluted DMSO, ranging in concentration from 0.0005 to 1%, showed neither cytotoxicity nor induction of UDS. Cytotoxicity, evidenced by morphological alteration or obliteration of the culture, was noted following treatment with safrole (1000 nmoles/ml), *o*-toluidine (1000 nmoles/ml), benzene (10000 nmoles/ml), diethylstilbestrol (50 nmoles/ml), phenobarbital (5000 nmoles/ml), and benzoin (100 nmoles/ml). At concentrations of up to 10000 nmoles/ml no cytotoxicity was evident from treatment with hexamethylphosphoramide, diethylhexylphthalate, acrylonitrile or caprolactam. For each of these compounds tested, the incidence of net nuclear silver grains was not different from the DMSO-treated control and, therefore, there was no evidence for the induction of UDS by any of these treatments.

A positive autoradiographic response for UDS was noted in cultures treated with either the ultimate carcinogen MNNG or the procarcinogen

TABLE 1

AN EVALUATION OF 10 SUSPECT GENOTOXINS FOR THE INDUCTION OF DNA REPAIR IN PRIMARY CULTURES OF ADULT RAT HEPATOCYTES

Compound	Concentration		Net nuclear silver grains (Mean ± SD) [a]	
	nmoles/ml	μg/ml	Study No. 1	Study No. 2
Hexamethylphosphoramide	10000	1790	NT [b]	−0.33 ± 1.84
	5000	895	NT	−1.62 ± 1.03
	1000	179	−1.52 ± 1.59	−0.56 ± 1.62
	500	89.5	−1.00 ± 2.01	−1.60 ± 1.77
	100	17.9	−1.71 ± 1.08	−1.80 ± 1.21
	50	8.9	−1.56 ± 1.35	−1.47 ± 1.72
	10	1.79	−0.89 ± 1.17	−1.53 ± 1.79
	5	0.89	−1.56 ± 1.57	−0.82 ± 1.59
	1	0.179	−1.36 ± 1.45	−1.47 ± 1.20
	0.5	0.089	−1.23 ± 0.98	−0.91 ± 1.81
Safrole	1000	160	Toxic	Toxic
	500	80	−0.61 ± 2.37	−1.95 ± 3.84
	100	16	−2.11 ± 2.60	−1.23 ± 2.37
	50	8	−1.81 ± 1.58	−1.17 ± 1.75
	10	1.6	−0.85 ± 1.43	−0.60 ± 3.38
	5	0.8	−0.77 ± 1.30	−0.79 ± 3.46
	1	0.16	−0.96 ± 1.94	−2.04 ± 4.14
	0.5	0.08	−0.45 ± 1.70	−1.03 ± 1.56
o-Toluidine	1000	107	Toxic	Toxic
	500	53.5	−1.04 ± 1.40	−0.77 ± 1.62
	100	10.7	−1.00 ± 2.14	−0.65 ± 2.04
	50	5.35	−0.98 ± 0.81	−1.81 ± 1.49
	10	1.07	−0.49 ± 1.49	−1.09 ± 2.12

TABLE 1 (continued)

Compound	Concentration		Net nuclear silver grains (Mean ± SD) [a]	
	nmoles/ml	μg/ml	Study No. 1	Study No. 2
o-Toluidine (continued)	5	0.53	−1.43 ± 1.62	−1.59 ± 1.50
	1	0.10	−1.69 ± 1.31	−1.63 ± 2.36
	0.5	0.05	−1.30 ± 1.12	−1.85 ± 1.79
Benzene	10000	780	NT	Toxic
	5000	390	NT	−0.68 ± 1.29
	1000	78	−1.40 ± 1.20	−1.45 ± 1.23
	500	39	−0.48 ± 1.52	−1.41 ± 1.30
	100	7.8	−0.95 ± 3.68	−0.96 ± 1.69
	50	3.9	−0.83 ± 1.76	−1.71 ± 1.87
	10	0.78	−1.59 ± 1.70	−1.88 ± 2.93
	5	0.39	−1.00 ± 1.52	−1.01 ± 1.85
	1	0.07	−0.77 ± 2.01	−0.61 ± 1.48
	0.5	0.039	−0.05 ± 2.53	−1.62 ± 1.95
Diethylhexylphthalate	10000	3900	NT	−1.00 ± 1.83
	5000	1950	NT	−1.41 ± 1.13
	1000	390	−2.04 ± 1.64	−1.24 ± 2.11
	500	195	−1.60 ± 1.53	−2.28 ± 2.09
	100	39	−1.57 ± 1.77	−1.38 ± 2.19
	50	19.5	−1.34 ± 1.76	−0.70 ± 1.55
	10	3.9	−0.97 ± 1.81	−1.27 ± 1.51
	5	1.95	−1.91 ± 2.35	−1.49 ± 1.59
	1	0.39	−1.19 ± 1.02	−0.72 ± 1.27
	0.5	0.19	−1.36 ± 1.03	−1.39 ± 1.57
Acrylonitrile	10000	530	NT	−1.02 ± 2.08
	5000	265	NT	−0.93 ± 1.48
	1000	53	−1.29 ± 2.27	−1.60 ± 1.31
	500	26.5	−1.67 ± 2.69	−1.02 ± 1.78
	100	5.3	−1.82 ± 1.45	−1.57 ± 2.73
	50	2.65	−1.39 ± 2.05	−1.20 ± 1.97
	10	0.53	−0.73 ± 0.93	−0.96 ± 1.38
	5	0.26	−1.27 ± 1.36	−1.01 ± 1.86
	1	0.05	−0.91 ± 1.36	−0.82 ± 1.28
	0.5	0.026	−0.90 ± 1.50	−0.39 ± 1.48
Diethylstilbestrol	1000	268	Toxic	Toxic
	500	134	Toxic	Toxic
	100	26.8	Toxic	Toxic
	50	13.4	Toxic	Toxic
	10	2.68	−1.01 ± 1.29	−1.20 ± 1.36
	5	1.34	−2.02 ± 2.24	−1.32 ± 1.18
	1	0.268	−0.38 ± 1.74	−0.48 ± 0.93
	0.5	0.134	0.87 ± 2.55	−1.01 ± 1.62
Phenobarbital	10000	2540	NT	Toxic
	5000	1270	NT	Toxic
	1000	254	−1.64 ± 1.53	−0.39 ± 1.59
	500	127	−0.86 ± 2.28	−1.63 ± 1.61
	100	25.4	−1.18 ± 1.76	−1.60 ± 1.87
	50	12.7	−2.17 ± 1.80	−0.91 ± 1.17
	10	2.54	−1.49 ± 1.41	−1.09 ± 1.88

TABLE 1 (continued)

Compound	Concentration		Net nuclear silver grains (Mean ± SD) [a]	
	nmoles/ml	μg/ml	Study No. 1	Study No. 2
Phenobarbital (continued)	5	1.27	−1.33 ± 1.18	−0.50 ± 1.83
	1	0.254	−1.00 ± 1.37	−1.33 ± 1.69
	0.5	0.127	−0.82 ± 1.53	−1.11 ± 1.35
Caprolactam	10000	1130	NT	−0.92 ± 1.05
	5000	565	NT	−0.43 ± 1.59
	1000	113	−0.63 ± 1.89	−1.87 ± 1.37
	500	56.5	−1.24 ± 1.17	−2.75 ± 2.33
	100	11.3	−2.00 ± 2.15	−1.15 ± 1.47
	50	5.65	−1.56 ± 1.11	−0.26 ± 1.70
	10	1.13	−1.01 ± 1.50	−0.97 ± 1.94
	5	0.56	−0.74 ± 2.58	−0.43 ± 1.83
	1	0.113	−2.08 ± 1.93	−0.56 ± 1.46
	0.5	0.056	−0.58 ± 1.46	−0.24 ± 1.35
Benzoin	1000	212	Toxic	Toxic
	500	106	Toxic	Toxic
	100	21.2	−1.42 ± 1.68	Toxic
	50	10.6	−1.08 ± 1.73	−0.32 ± 1.36
	10	2.12	−0.17 ± 1.31	−1.41 ± 1.55
	5	1.06	−0.57 ± 1.20	−1.12 ± 1.32
	1	0.212	−1.00 ± 1.71	−0.86 ± 2.06
	0.5	0.106	−1.01 ± 1.55	−0.69 ± 1.59
2AAF	100	22.6	TNTC [c]	TNTC
	50	11.3	TNTC	TNTC
	10	2.6	TNTC	TNTC
	5	1.3	97.22 ± 16.22	106.74 ± 19.45
	1	0.26	56.71 ± 10.76	75.11 ± 11.61
	0.5	0.13	38.51 ± 8.72	52.05 ± 8.66
	0.1	0.026	8.83 ± 6.22	31.57 ± 10.91
	0.05	0.013	−0.35 ± 1.44	11.03 ± 5.93
MNNG	1000	147	Toxic	Toxic
	500	73.5	Toxic	Toxic
	100	14.7	56.82 ± 9.02	101.70 ± 18.38
	50	7.35	28.10 ± 7.65	49.37 ± 17.04
	10	1.47	5.19 ± 7.66	24.66 ± 10.14
	5	0.735	−1.04 ± 1.38	10.23 ± 5.89
	1	0.147	−0.64 ± 2.03	2.34 ± 4.21
	0.5	0.073	−1.58 ± 1.36	−1.03 ± 2.91
DMSO	1.0%		−1.09 ± 2.47	−1.49 ± 1.36
	0.5		−1.04 ± 1.31	−1.36 ± 1.62
	0.1		−2.47 ± 1.53	−0.78 ± 1.44
	0.05		−1.26 ± 1.30	−1.03 ± 1.29
	0.01		−0.60 ± 2.11	−1.26 ± 1.40
	0.005		−1.24 ± 1.40	−1.19 ± 1.71
	0.001		−0.70 ± 1.08	−0.95 ± 1.53
	0.0005		−0.87 ± 2.10	−1.04 ± 1.74

[a] Represents counts of nuclei from 20 morphologically unaltered cells from each treatment.
[b] NT, not tested.
[c] TNTC, silver grains too numerous for automated counting.

2AAF. In these cultures, the highest levels of UDS were noted at the highest noncytotoxic concentrations of compound, and UDS decreased in a dose-related fashion with lower concentrations. Cytotoxicity resulted from treatment with 500 and 1000 nmoles/ml of MNNG in each of the replicate assays. Treatment with 10, 50 or 100 nmoles/ml of 2AAF resulted in a positive response for UDS; however, the silver grain density was excessive for automated counting.

As a result of this study, it is concluded that the cultured adult rat hepatocytes were sensitive to the induction of UDS both by the ultimate carcinogen MNNG and the procarcinogen 2AAF and that hexamethylphosphoramide, safrole, *o*-toluidine, benzene, diethylhexylphthalate, acrylonitrile, diethylstilbestrol, phenobarbital, caprolactam or benzoin were inactive for the induction of UDS.

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Tests for the induction of chromosome aberrations, polyploidy and sister-chromatid exchanges in rat liver (RL_4) cells

Robert A.J. Priston and Brian J. Dean

Shell Toxicology Laboratory, Sittingbourne Research Centre, Sittingbourne, Kent (Great Britain)

Summary

The 10 compounds were tested in the rat liver (RL_4) assay for their ability to induce structural chromosome aberrations, polyploidy and sister-chromatid exchanges (SCE).

Of the 8 established carcinogens, diethylstilboestrol (DES) induced an increase in the number of polyploid cells, and hexamethylphosphoramide (HMPA) and *o*-toluidine (TOL) induced increased frequencies of SCE. TOL also induced a small increase in chromatid damage. The remaining five carcinogens and both noncarcinogens (caprolactam and benzoin) were negative in all assays under the experimental conditions described.

Epithelial-like cells isolated from rat liver have formed the basis of a number of assays for genotoxic chemicals using endpoints such as gene mutation (Tong and Williams, 1980), neoplastic transformation (San et al., 1979), unscheduled DNA synthesis (Williams, 1980), structural chromosome aberrations (Dean and Hodson-Walker, 1979), and sister-chromatid exchanges (Meyer and Dean, 1981; Cunningham and Ringrose, 1983). Two factors recommend their use in short-term tests for mutagens and carcinogens: (a) they retain substantial residual metabolising enzyme activity enabling them to activate a range of carcinogenic chemicals to reactive molecules; and (b) their epithelial nature makes them more relevant than fibroblasts to the target cells of most chemical carcinogens. The RL_4 line has a spectrum of responses to the clastogenic effects of chemicals comparative to the RL_1 line used in earlier studies (Dean, 1981).

This paper describes the results of assays for structural chromosome aberrations and SCE in RL_4 cells after treatment with a series of eight established carcinogens and two noncarcinogens selected for the CSSTT Collaborative Study.

Materials and methods

Chemicals

The chemicals included in this study were hexamethylphosphoramide (HMPA), *o*-toluidine (TOL), benzene (BEN), safrole (SAF), caprolactam (CAP), acrylonitrile (ACN), diethylhexylphthalate (DEHP), benzoin (ZOIN), diethylstilboestrol (DES) and phenobarbital (PB). They are described in detail elsewhere in this volume.

Cell line

The RL_4 cell line was derived in this laboratory from a 10-day-old male Wistar rat. The cells have a cycle time of 13 h and are maintained in monolayer culture by regular subculturing in Dulbecco's Minimal Essential Medium containing 10% foetal bovine serum and 1% nonessential amino acids (Dean and Hodson-Walker, 1979). This medium was also used in the subsequent assays.

Test concentrations

An approximation of the cytotoxicity of each compound (with one exception, see below) was

obtained by estimating growth inhibition using monolayer cultures in multiwell tissue culture trays. The precise range of doses to be used in subsequent cytogenetic and SCE assays was determined by measuring the effect of the chemicals on cloning efficiency in polystyrene dish cultures. The cloning efficiency of 3 compounds was estimated from cultures in 15-ml glass screw-capped Leighton tubes; ACN was tested in this way because of its volatility, and both SAF and BEN were found to attack the plastic of disposable ware.

Cytotoxicity

For each test substance (except ACN) each well of two disposable 24-well tissue culture trays was seeded with 5×10^4 cells in 2 ml growth medium. After 24 h incubation at 37 °C in a humidified 5% CO_2 atmosphere, the growth medium was replaced with 2 ml of fresh growth medium containing the test substance. Each dilution of the test compound was added to 3 replicate wells and the trays were reincubated for a further 24 h. The medium was then discarded from each tray and the cell sheets were rinsed in Hanks BSS, fixed in methanol and stained with 10% Giemsa stain. An assessment of the concentration that inhibited growth by approximately 50% was made by visual examination. The cytotoxicity of ACN was assessed using 15-ml glass screw-capped Leighton tubes seeded with 1×10^5 cells per tube. Three tubes were used for each concentration and the procedures for assessing growth inhibition was similar to that described above.

Cloning efficiency

The cloning efficiency was determined on cultures treated with a narrow range of concentrations of the test compound estimated from the cytotoxicity assay. Culture dishes (9 cm) were seeded with 400–500 cells in 10 ml growth medium and incubated at 37 °C in a humidified, 5% CO_2 atmosphere. The cloning efficiency of ACN, SAF and BEN was determined using 100-ml Leighton tubes seeded with 200 cells in 10 ml medium. Triplicate cultures were used at each dose level. After a further 24 h incubation, the medium containing the test compound was replaced with fresh medium and incubation was continued for a further 6–8 days to allow the development of visible colonies. After fixing the colonies with methanol and staining with 10% Giemsa, the colonies were counted and the concentration of the compound that reduced the cloning efficiency to 50% of the control levels was determined.

Cytogenetic assay

Usually, 3 dose levels representing $1\times$, $0.5\times$ and $0.25\times$ the concentration producing a 50% reduction in plating efficiency were selected for the cytogenetic assays. When appropriate, additional concentrations above or below those selected were included. Triplicate cultures on glass microscope slides were used at each concentration plus a solvent control group and a positive control group (1 μg/ml 7,12-dimethylbenzanthracene).

Labelled and sterilised 3 in. $\times$ 1 in. glass microscope slides were placed in individual 9-cm polystyrene culture dishes and seeded with 2.5×10^4 cells in 0.5 ml growth medium. These were incubated for 2 h at 37 °C in a humidified, 5% CO_2 incubator for the cells to attach. Then, 10 ml of growth medium was added to each dish and incubation was continued. For experiments with ACN, SAF and BEN, slides were placed individually in 100-ml Leighton tubes and seeded with 10 ml of growth medium containing 1×10^5 cells. After 24 h the medium was removed from each culture and replaced with appropriate dilutions of test and control materials in fresh medium. After 22 h, colcemid solution was added to a final concentration of 0.3 μg per ml, and after an additional 2 h incubation the slides were removed and processed for the demonstration of metaphase chromosomes (Dean and Hodson-Walker, 1979).

The stained slides were randomised for blind scoring and 100 metaphase cells were analysed for structural aberrations. Mitotic indices were calculated from the examination of 500 cells from top dose and solvent control cultures.

SCE assay

The selection of concentrations of test chemicals for use in the SCE assays and the initiation of slide cultures was identical to that described above. 24 h after initiation of the cultures, culture medium was replaced with 10 ml medium containing appropriate dilutions of test or control substances. Bromodeoxyuridine (BrdUrd) solution was added

to a final concentration of 15 μM and the cultures were returned to the incubator. After 22 or 30 h incubation (the optimum period was estimated from a preliminary study of the proportion of second division metaphases) colcemid was added as above. This and all subsequent manipulations were carried out in subdued lighting. After a further 2 h incubation, the slides were removed and processed for the demonstration of SCE (Perry and Wolff, 1974). The processed slides were randomised for blind scoring and about 30 cells with differentially stained chromatids were examined for SCE. The proportion of cells in their first metaphase (M1), second metaphase (M2) and subsequent metaphases (M2 +) was also recorded.

Results

The results of the analysis of structural chromosome aberrations are shown in Table 1 and of SCE assays in Table 2. The findings are summarised in Table 3.

The highest concentration of each compound used was determined by an initial cytotoxicity assay followed by measurement of the plating efficiency (PE). The mitotic index (MI) was determined during analysis (Table 1) to confirm the appropriateness of the selected dose levels. With the exception of HMPA, BEN and SAF, the highest concentration used in the assays corresponded to the dose that reduced the PE by about 50%. Concentrations of HMPA, BEN and SAF only marginally reduced the PE; but, in subsequent assays, these concentrations significantly reduced the MI. Thus there was evidence of cytotoxicity, growth inhibition, reduction in MI or cell cycle delay at the highest dose of all the compounds tested.

Judgement on the activity of the compounds was made on the results of the single assays, except with DES, when a second assay, using a longer treatment time was conducted.

HMPA

A concentration of 2000 μg/ml was selected for the top dose. This reduced the MI to 58% of that of the control cultures in addition to producing significant cell-cycle delays. There was no significant increase in the frequency of polyploidy or structural aberrations in cells exposed to 500, 1000 or 2000 μg/ml for 24 h. There was a significant increase in the frequency of SCE at the highest dose level ($p \leq 0.05$) and it is concluded that HMPA was positive in this assay, but with only a weak response.

TOL

The concentration of TOL that reduced the PE by 50% was between 500 and 750 μg/ml and 700 μg/ml was used as the highest dose level. This concentration reduced the MI to 31% that of the controls. Over the dose range 87.5–700 μg/ml, TOL induced a dose related increase in chromatid gaps that was significant at 700 μg/ml ($p \leq 0.01$). In the absence of a significant increase in the incidence of other chromatid or chromosome aberrations the increase in gaps alone is regarded as indicative of weak clastogenic activity. In the SCE assay, cultures exposed to 175, 350 or 700 μg/ml TOL did not produce harlequin-stained chromosomes of suitable quality. In a second experiment, in which cells were exposed to 21.8, 43.75 and 87.5 μg/ml, the frequency of SCE/cell was increased at each dose level when compared with the control cultures. The lack of a dose–response curve may be explained by saturation of the residual metabolising enzymes by the lowest dose used.

SAF

A concentration of 100 μg/ml SAF was judged to reduce the PE of RL_4 cells by about 50% and this was used as the top dose in subsequent chromosome assays. The MI was reduced from 7.6% in the controls to 1% in the 100 μg/ml SAF cultures. Concentrations ranging from 12.5 to 100 μg/ml did not influence the frequency of structural chromosome aberrations or polyploidy. In the SCE assay there was considerable mitotic delay in the 100 μg/ml cultures and insufficient cells were available for analysis. The frequency of SCE per cell was increased over control values at each remaining dose level, but the increase was not statistically significant.

BEN

In preliminary cytotoxicity studies, significant growth inhibition was observed in multiwell dish cultures at 2000 and 4000 μg/ml BEN. However, concentrations up to 2000 μg/ml did not signifi-

TABLE 1

STRUCTURAL CHROMOSOME ABERRATIONS IN RL_4 CELLS

Compound	Conc. (μg/ml)	Number of cultures	Number of cells analysed	Percent cells showing							Frequency per cell of		Mitotic index (%)
				Poly-ploidy	Chromatid gaps	Chromatid aberrations	Chromosome aberrations	Multiple aberrations	All aberra-tions excl. gaps	All aberra-tions incl. gaps	All aberra-tions excl. gaps	All aberra-tions incl. gaps	
HMPA	0	3	300	2.7	1.0	0.7	0.3	0	1.0	2.0	0.010	0.020	9.1
HMPA	500	3	300	2.7	2.3	0.3	0	0	0.3	2.7	0.003	0.027	–
HMPA	1000	3	300	1.7	2.7	1.0	1.0	0	2.0	4.7	0.020	0.047	–
HMPA	2000	3	278	1.8	1.1	0	0	0	0	1.0	0	0.010	5.3
DMBA	1.0	2	200	2.5	12.0	8.5	0.5	2.0	9.0	21.0	0.115	0.390	–
TOL	0	3	300	2.7	0.7	0	0	0	0	0.7	0	0.007	10.6
TOL	87.5	3	300	2.7	1.3	0.3	0.7	0	1.0	2.3	0.010	0.023	–
TOL	175	3	300	2.0	2.3	0.7	0.3	0	1.0	3.3	0.010	0.037	–
TOL	350	3	300	0.7	3.0	0.3	0	0	0.3	3.3	0.003	0.037	–
TOL	700	3	300	2.7	6.3	0.3	0	0	0.3	6.7	0.003	0.093	3.4
DMBA	1.0	2	200	1.0	14.5	5.0	0.5	0	5.5	20.0	0.065	0.390	–
BEN	0	3	300	2.0	1.3	1.0	0	0	1.0	2.3	0.010	0.027	6.9
BEN	250	3	300	1.7	1.7	0.3	0.3	0	0.7	2.3	0.006	0.023	–
BEN	500	3	300	2.3	1.7	0	0.3	0	0.3	2.0	0.003	0.023	–
BEN	1000	3	300	1.7	1.3	1.0	0	0	1.0	2.3	0.010	0.023	3.8
DMBA	1.0	2	200	1.0	22.5	14.5	1.0	3.5	21.0	34.5	0.315	0.770	–
SAF	0	3	300	1.0	0.7	0.3	0	0	0.3	1.0	0.003	0.010	7.6
SAF	12.5	3	300	1.7	2.0	0.7	0.7	0.3	1.0	3.0	0.024	0.044	–
SAF	25	3	300	1.0	2.0	0.3	0	0	0.3	2.3	0.003	0.023	–
SAF	50	3	300	0.7	1.3	0	0	0	0	1.3	0	0.017	–
SAF	100	3	252	1.2	1.2	0.4	0	0	0.4	1.6	0.003	0.022	1.0
DMBA	1.0	3	293	2.1	8.9	10.2	0.7	4.1	19.3	20.1	0.284	0.421	–
CAP	0	3	300	2.3	1.0	0.3	0	0	0.3	1.3	0.003	0.013	5.5
CAP	250	3	300	2.7	2.3	1.0	0	0	1.0	2.7	0.010	0.037	–
CAP	500	3	300	2.0	2.0	1.0	0.3	0	1.3	3.3	0.013	0.033	–
CAP	1000	2	200	1.0	6.0	0	1.5	0	1.5	7.5	0.115	0.075	2.5
DMBA	1.0	2	200	2.0	1.3	9.0	0	2.0	11.0	23.0	0.165	0.350	–
ACN	0	3	300	0.7	1.3	0	0	0	0	1.3	0	0.013	2.3
ACN	1.25	3	228	0	1.3	0.9	0	0	0.9	2.2	0.009	0.022	–

ACN	2.5	3	235	0.4	0	0	0	0	0	0	0	0	–
ACN	5.0	3	300	0.3	1.3	0	0.3	0	0.3	1.7	0.003	0.020	–
ACN	10.0	3	300	0	2.7	0.7	0	0	0.7	3.3	0.007	0.043	1.2
DMBA	1.0	2	56	0	1.8	12.5	3.6	1.8	17.9	19.6	0.215	0.233	–
DEHP	0	3	300	1.0	0	0	0	0	0	0	0	0	8.4
DEHP	250	3	300	1.3	0	0.7	0	0	0.7	0.7	0.007	0.007	–
DEHP	500	3	300	1.3	0	0	0	0	0	0	0	0	–
DEHP	1000	3	279	1.4	1.4	0.4	0	0	0.4	1.8	0.004	0.017	1.1
DMBA	1.0	2	200	1.0	7.0	4.0	1.5	1.0	6.5	12.5	0.110	0.190	–
ZOIN	0	3	300	0.3	0	0.3	0.3	0	0.7	0.7	0.007	0.007	4.3
ZOIN	50	3	300	0.3	0.7	0.3	0.3	0	0.7	1.3	0.007	0.014	–
ZOIN	100	3	279	1.1	1.1	0	0	0	0	1.1	0	0.011	–
ZOIN	200	3	292	1.4	1.0	1.0	0.7	0	1.7	2.7	0.017	0.027	–
ZOIN	400	3	200	0	1.0	1.5	0	0	1.5	2.5	0.015	0.025	0.6
DMBA	1.0	2	184	1.6	8.2	7.1	1.6	1.1	9.8	17.9	0.119	0.228	–
DES [a]	0	3	225	4.0	2.2	0	0	0	0	2.2	0	0.022	3.0
DES	1.25	3	300	8.0	2.0	1.0	0	0	1.0	3.0	0.010	0.033	–
DES	2.5	3	253	4.7	0	1.2	0	0	1.2	1.2	0.012	0.012	–
DES	5.0	3	282	1.1	3.2	1.4	0	0	1.4	4.6	0.014	0.053	–
DES	10.0	3	123	4.1	2.4	0	0	0	0	2.4	0	0.024	1.5
DMBA	1.0	3	209	2.4	9.1	5.7	1.0	1.4	8.1	17.2	0.091	0.268	–
DES [b]	0	3	300	7.3	1.0	0.7	0	0	0.7	1.7	0.007	0.017	6.4
DES	1.25	3	300	8.0	0.7	0	0.3	0	0.3	1.0	0.003	0.010	–
DES	2.5	3	300	7.3	0.3	0	0	0	0	0.3	0	0.003	–
DES	5.0	3	300	11.0	0.7	0.3	0.3	0	0.7	1.3	0.006	0.013	–
DES	10.0	3	261	33.0	2.7	1.2	0.4	0	1.6	4.2	0.016	0.043	2.0
DMBA	1.0	3	106	0	20.8	15.1	0.9	4.7	16.5	37.3	0.368	0.896	–
PB	0	3	300	0.7	0.7	0.3	0	0	0.3	1.0	0.003	0.010	1.1
PB	250	3	250	3.2	1.2	0	0.4	0	0.4	1.6	0.004	0.016	–
PB	500	3	268	0.7	0.4	0.4	0	0	0.4	0.7	0.004	0.008	–
PB	750	3	269	2.2	0.4	0.7	0.4	0	1.1	1.5	0.012	0.016	–
PB	1000	3	151	2.0	0.7	0.7	0.7	0	1.3	2.0	0.012	0.019	0.7
DMBA	1.0	3	250	0.4	0.8	4.4	0.8	1.2	6.4	7.2	0.096	0.112	–

[a] DES, after 24 h exposure.
[b] DES, after 42 h exposure.

TABLE 2

SISTER-CHROMATID EXCHANGES IN RL_4 CELLS

Compound	Conc. (μg/ml)	Time in BrdUrd (h)	Number of cultures	Total cells scored	Percent of cells in cell cycle stage			Total number of chromosomes	Total number of SCE	Mean number of SCE per chromosome	Mean number of SCE per cell	± SE
					M1	M2	M2+					
HMPA	0	24	3	90	41	59	0	3856	974	0.253	10.82	0.36
HMPA	500	24	3	90	36	64	0	3770	1049	0.279	11.66	0.45
HMPA	1000	24	3	90	28	72	0	3862	984	0.255	10.93	0.34
HMPA	2000	24	3	90	51	49	0	3814	1148	0.301	12.76 *	0.40
DMBA	0.1	24	2	50	61	38	1	2116	1344	0.636	26.88	0.91
TOL	0	24	3	90	9	91	0	3982	806	0.203	8.96	0.40
TOL	21.8	24	3	90	4	96	0	3978	1152	0.290	12.80 **	0.43
TOL	43.75	24	3	90	10	90	0	3974	1080	0.272	12.00 **	0.40
TOL	87.5	24	3	90	12	88	0	3920	1184	0.302	13.16 **	0.47
DMBA	0.1	24	3	90	26	74	0	4012	2158	0.538	23.98	0.60
BEN	0	24	2	60	11	89	0	2570	374	0.146	6.24	0.35
BEN	250	24	3	90	12	88	0	3791	531	0.140	5.90	0.24
BEN	500	24	3	90	6	94	0	3812	523	0.138	5.82	0.26
BEN	1000	24	3	85	31	69	0	3575	421	0.118	4.96	0.26
DMBA	0.1	24	2	60	24	76	0	2452	856	0.350	14.27	0.57
SAF	0	24	3	90	6	94	0	3934	961	0.245	10.68	0.33
SAF	12.5	24	3	90	4	96	0	3947	1174	0.298	13.05	0.40
SAF	25	24	2	60	5	95	0	2657	719	0.271	11.99	0.40
SAF	50	24	3	90	29	71	0	3987	1135	0.285	12.62	0.42
SAF	100	24	3	4	88	12	0	–	–	–	–	–
DMBA	0.1	24	2	60	3	97	0	2656	1811	0.682	30.19	0.85
CAP	0	24	3	90	32	66	2	3888	803	0.207	8.93	0.36
CAP	250	24	2	60	38	62	0	2569	563	0.220	9.39	0.65
CAP	500	24	3	90	43	57	0	3930	883	0.225	9.82	0.37
CAPA	1000	24	3	90	52	48	0	3956	915	0.232	10.17	0.35
DMBA	0.1	24	2	55	77	22	1	2345	1024	0.437	18.62	0.93
ACN	0	24	2	60	0	100	0	2230	565	0.254	9.42	0.52
ACN	1.25	24	2	60	3	93	0	2308	515	0.224	8.58	0.37

ACN	2.5	24	3	90	9	91	0	3589	658	0.184	7.31	0.30
ACN	5.0	24	3	90	24	76	0	3632	858	0.237	9.53	0.46
ACN	10.0	24	3	0	95	5	0	–	–	–	–	–
DMBA	0.1	24	3	90	27	73	0	3665	1607	0.439	17.86	0.85
DEHP	0	24	2	60	6	94	0	2508	557	0.222	9.29	0.37
DEHP	125	24	2	60	5	95	0	2564	592	0.231	9.87	0.43
DEHP	250	24	2	60	13	87	0	2569	631	0.246	10.52	0.45
DEHP	500	24	3	90	14	86	0	3650	792	0.217	8.80	0.33
DEHP	1000	24	3	88	30	70	0	3782	700	0.185	7.96	0.26
DMBA	0.1	24	2	47	16	84	0	1909	710	0.372	15.11	0.69
ZOIN	0	24	2	60	8	92	0	2634	369	0.140	6.15	0.40
ZOIN	50	24	3	90	9	91	0	3942	591	0.150	6.57	0.39
ZOIN	100	24	3	90	15	85	0	3943	609	0.155	6.77	0.33
ZOIN	200	24	3	90	13	87	0	3945	629	0.160	6.99	0.30
ZOIN	400	24	3	90	17	83	0	3897	518	0.133	5.76	0.30
DMBA	0.1	24	2	60	24	76	0	2655	1166	0.440	19.44	0.88
DES	0	24	2	60	12	87	1	2627	499	0.190	8.32	0.47
DES	1.25	24	3	90	13	87	0	3792	641	0.169	7.13	0.29
DES	2.5	24	3	90	14	86	0	3883	805	0.208	8.95	0.43
DES	5.0	24	3	0	97	3	0	–	–	–	–	–
DMBA	0.1	24	2	60	27	73	0	2628	1077	0.410	17.95	0.79
DES	0	32	2	60	34	56	10	2528	439	0.174	7.32	0.39
DES	1.25	32	2	60	15	82	3	2505	467	0.187	7.79	0.30
DES	2.5	32	2	60	24	74	2	2503	497	0.199	8.29	0.45
DES	5.0	32	2	60	40	60	0	2475	424	0.172	7.07	0.39
DES	10.0	32	2	0	99	1	0	–	–	–	–	–
DMBA	0.1	32	2	42	61	38	1	1811	689	0.381	16.41	1.04
PB	0	24	2	60	4	96	0	2516	721	0.287	12.02	0.70
PB	125	24	3	90	3	95	2	3888	1220	0.314	13.56	0.42
PB	250	24	3	90	9	90	1	3793	1082	0.286	12.02	0.47
PB	500	24	3	72	32	68	0	2997	917	0.306	12.74	0.59
PB	1000	24	2	0	92	8	0	–	–	–	–	–
DMBA	0.1	24	2	60	25	75	0	2539	1407	0.555	23.45	0.98

* Significantly greater than control $p \leq 0.05$.

** Significantly greater than control $p \leq 0.01$.

TABLE 3

INDUCTION OF STRUCTURAL ABERRATIONS, POLYPLOIDY AND SCE IN RL_4 CELLS

Compound	Aberrations	polyploidy	SCE
HMPA	–	–	+w
TOL	+w	–	+
SAF	–	–	–
BEN	–	–	–
CAP	–	–	–
ACN	–	–	–
DEHP	–	–	–
ZOIN	–	–	–
DES	–	+	–
PB	–	–	–

cantly affect the PE, though colony size was reduced above 1250 μg/ml. In the subsequent chromosome assay, exposure of cells to 1000 μg/ml reduced the MI to 55% that of control cultures. There was no evidence of increases in structural aberrations, polyploidy or SCE in cultures grown for 24 h in the presence of BEN up to 1000 μg/ml.

CAP

A concentration of 1000 μg/ml inhibited growth of monolayer cultures by about 50% but only marginally reduced the PE. MI was reduced from 5.5% in control cultures to 2.5% in cultures exposed to 1000 μg/ml CAP. Apparent increases in the frequencies of both aberrations and SCE were observed over the range 250–1000 μg/ml but these were not statistically significant.

ACN

A 50% reduction in PE was achieved at between 5 and 10 μg/ml ACN and the chromosome assay was conducted on cultures exposed to 1.25, 2.5, 5.0 or 10.0 μg/ml. The MI was reduced by about 50% at 10 μg/ml. There was no indication of induction of aberrations at any dose level. Considerable mitotic delay reduced the number of M2 cells in the SCE assay at 10 μg/ml. SCEs were not increased at the remaining dose levels.

DEHP

Because of precipitation of DEHP in the growth medium, the highest dose used in the chromosome assay was 1000 μg/ml. Although this dose level had negligible effects in cytotoxicity and PE assays, the MI was reduced from 8.4% in the control cultures to 1.1% in the 1000 μg/ml DEHP cultures. There was no increase in the frequencies of chromosome aberrations or SCE at dose levels ranging from 125 to 1000 μg/ml DEHP.

ZOIN

Based on growth inhibition and PE observations, doses of 50, 100, 200 and 400 μg/ml ZOIN were chosen for the chromosome assays. At 400 μg/ml the MI was 13.5% that of the control cultures. Assays for aberrations, polyploidy, and SCE were negative.

DES

A concentration of 10 μg/ml DES reduced the PE and MI by approximately 50%. In the chromosome assay, in which cells were harvested after 24 h treatment, polyploidy was increased in cultures exposed to 1.25 μg/ml. The increase in the incidence of polyploid cells was confirmed in a second assay in which cells were harvested after 42 h. Structural aberrations were not significantly increased.

Two SCE assays were conducted. In the first assay using a 24-h exposure period, only cultures in the 1.25 and 2.5 μg/ml groups produced sufficient M2 divisions for analysis. Increasing the exposure time to 32 h enabled cells exposed to 5 μg/ml DES to be analysed. There was no indication of an increase in SCE frequency in either experiment.

PB

A dose level of 100 μg/ml was chosen as the highest concentration to be tested and reduced the MI to about 17% that of control cultures. No evidence of increases in the frequencies of chromosome damage or SCE was obtained at dose levels of 250, 500, 750 or 1000 μg/ml PB.

DMBA

7,12-Dimethylbenzanthracene (DMBA) was used as the positive control material in all the studies i.e. 1.0 μg/ml in cytogenetic assays and 0.1 μg/ml in the SCE assays. The compound gave consistent positive results with the percentage cells containing aberrations ranging from 7.2 to 34.5%

of cells (mean 19.5%) after 24 h exposure. The mean number of SCE per cell after 24 h exposure ranged from 14.3 to 30.2, with a mean of 20.8.

Discussion

Only 1 of the 10 compounds induced an increase in the frequency of structural chromosome aberrations in RL_4 cells under the test conditions described. The study with *o*-toluidine (TOL) resulted in a significant increase in chromatid gaps, although this was not accompanied by an increase in any other type of aberration. TOL was classified as a weak clastogen. Diethylstilboestrol (DES) induced an increase in polyploidy in cultures exposed to 1.25 μg/ml for 24 h. When the exposure time was increased to 42 h, cultures exposed to 5.0 and 10.0 μg/ml DES also showed a high frequency of polyploid cells, presumably because the longer exposure time allowed a greater proportion of the cells to proceed to the second mitosis after treatment.

Two compounds, HMPA and TOL, were considered positive in the SCE assay. SCE frequencies in cultures exposed to SAF were increased over the control values, but analysis of the data using a Student's *t*-test and Dunnett's modified *t*-test (Dunnett, 1964), failed to demonstrate a statistical difference. SAF was, therefore, classified as negative.

A number of parameters were measured to ensure that appropriate concentrations of the test materials were applied. Preliminary cytotoxicity and plating efficiency assays indicated suitable dose ranges and these were confirmed by determining the mitotic index and by recording the percentages of cells at M1, M2 and M2 + in the SCE assay. These latter data provided an insight into the effects of the compounds on cell division. For example, with the exception of HMPA and TOL, all compounds reduced the number of cells in their second mitosis after 24 h in a dose-related manner suggesting that they caused significant delays to the cell cycle.

Of the 8 established carcinogens, 3 induced either chromosome damage, polyploidy or SCE, and the 2 alleged noncarcinogens were negative in all assays.

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Tests for chromosome aberrations and aneuploidy in the Chinese hamster fibroblast cell line CH1-L

Natalie Danford

Genetic Toxicology Research Group, Department of Genetics, University College of Swansea, Singleton Park, Swansea (Great Britain)

Summary

10 compounds were examined for their ability to induce chromosome aberrations and aneuploidy in a low passage number fibroblast cell line derived from Chinese hamster liver. HMPA, TOL and DES were positive for both endpoints. ACN and PB induced chromosome aberrations, and BEN and DEHP induced aneuploidy only. SAF, CAP and ZOIN did not induce either chromosome aberrations or aneuploidy.

Investigations on the induction of chromosome aberrations in cultured mammalian cells have tended to concentrate on two cell types, CHO Chinese hamster ovary cells and human peripheral lymphocytes. They have been widely validated (Preston et al., 1981), but have two main disadvantages. First, the metabolic capacities of both are fairly low, usually necessitating the use of an external metabolising system (Madle and Obe, 1980). Second, the karyotype of CHO is not sufficiently stable to recommend it as a monitor of aneuploidy. Human lymphocytes should have a very stable karyotype, but the normal method of preparation makes it extremely difficult to score hypodiploidy reliably.

This paper describes results obtained with a low passage number Chinese hamster fibroblast cell line, derived from liver. This was used to measure simultaneously chromosome aberrations and levels of mitotic aneuploidy, following exposure to the 10 compounds selected for the IPCS Collaborative Study/CSSTT. The compounds in this study are difficult to detect in standard short-term tests, and aneuploidy was therefore used as an additional endpoint. The efficiency of the assay for aberrations was slightly reduced by the requirement of a longer exposure time, as the cells had to reach their second metaphase post-treatment for induced aneuploidy to be detectable. Both hypodiploidy and hyperdiploidy were measured; the use of a very short hypotonic treatment retained the chromosomes within the cell membrane, preventing accidental chromosome loss during slide preparation.

The cell line had already been studied for its ability to detect a range of compounds requiring metabolic activation. In addition to the 4 used as positive controls in this paper 7,12-dimethylbenzanthracene and 1,6-dinitropyrene were inducers of chromosome aberrations without any external metabolising system (Danford et al., in preparation). The 10 compounds were therefore tested in these cells without the addition of S9 microsomal extract or other extrinsic metabolising agent.

Materials and methods

Cell line

The cell line, designated CH1-L, was derived from the liver of a 3-week-old male Chinese hamster. A fibroblast culture was isolated by G. Hodson-Walker, Shell Research Laboratories, Sittingbourne, Kent, UK, using the technique of trypsinisation of minced tissue (Williams et al., 1971).

The cells were routinely maintained in

Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% foetal calf serum and penicillin/streptomycin (50 i.u./50 μg/ml) (Gibco Europe Ltd.). They were grown as a monolayer, and once confluent, they were split 1:4 or 1:9 using 0.25% trypsin (Flow Laboratories Ltd.) to detach them. They were incubated at 37°C in a 95% air, 5% CO_2 atmosphere. The cell cycle time was estimated to be 18–19 h, using cell counts to measure the doubling time.

Chemicals

The chemicals used in the experiments, their molecular weights and the concentrations tested are given in Table 1. All chemicals were dissolved in dimethyl sulphoxide (DMSO) except for diethylhexylphthalate, which was dissolved in DMSO + 1% Tween 80 (BDH Ltd., Poole, UK). Benzoin and diethylhexylphthalate were handled in glass containers until added to the culture medium.

Cytotoxicity assay

Cells were seeded into 24-well Linbro plates (Flow Laboratories Ltd.) at 25000 cells/ml, 2 ml/well, to determine the doses at which toxicity occurred (Dean and Hodson-Walker, 1979). After 24 h incubation, the medium was removed, and replaced in each of 2 wells by medium plus test agent at one dose. 2 compounds at 5 concentrations (and a DMSO negative control) were tested on each plate. With some compounds, it was necessary to first test a wide range (e.g. 0.1–2000 μg/ml) and subsequently use a narrow range (e.g. 10–100 μg/ml). With others, previously studied in a number of cultured mammalian cell lines, only one (narrow) range was used, based on dose levels tested in these other studies.

The toxicity plates were harvested after 36 h culture in the presence of the test chemicals. The medium was removed, and the cells rinsed with Hank's balanced salt solution (BSS), fixed with methanol for 10 min, and air-dried. The cells were stained for 5 min with 10% Giemsa stain, diluted with 0.01 M phosphate buffer, pH 6.8. Examination of the plates on an inverted microscope, and intensity of staining allowed an assessment of toxicity of the chemicals. Subsequent chromosome assays were carried out up to doses showing some degree of toxicity (preferably up to about 50%).

Cytogenetic assay

Slide cultures were prepared using confluent or near-confluent flasks. Single-cell suspensions (75000 cells/ml) were seeded onto individual sterile glass slides in 90-mm petri dishes at 1 ml/slide. After 2 h to allow the cells to attach, a

TABLE 1

MOLECULAR WEIGHTS, CONCENTRATIONS TESTED, AND PASSAGE NUMBERS OF THE CH1-L CELLS WHEN TESTED

Compound		M.W. [a]	Concentration [b]		Passage No. CH1-L cells	Set [c]
			μg/ml	mM		
Hexamethylphosphoramide	(HMPA)	179.20	20	0.112	14	1
o-Toluidine	(TOL)	107.15	120	1.120	15	2
Benzene	(BEN)	78.11	250	3.201	10	4
Safrole	(SAF)	162.18	60	0.370	14	1
Caprolactam	(CAP)	113.16	2000	17.674	11	5
Acrylonitrile	(ACN)	53.06	25	0.471	9	3
Diethylhexylphthalate	(DEHP)	390.54	50	0.128	11	5
Benzoin	(ZOIN)	212.22	200	0.942	9	3
Diethylstilboestrol	(DES)	268.34	30	0.112	15	2
Phenobarbital	(PB)	232.23	1000	4.306	10	4

[a] Molecular weight (from: Windholz et al. (1976). Merck Index, 9th edn.).

[b] Maximum concentration tested; Other concentrations: ×0.5, 0.25, 0.1.

[c] Compounds tested in 5 sets of pairs.

further 11 ml of medium was added to each dish. 30 slides were set up at one time, so that 4 concentrations of 2 chemicals, plus negative and positive controls were subsequently prepared in triplicate. An additional negative control, DMSO + 1% Tween 80, was included when diethylhexylphthalate was examined.

The test agent was added 24 h after the slide cultures were initiated. Serial dilutions were prepared so that identical volumes of DMSO plus test chemical were added to each dish. Stock solutions in culture medium were prepared at 5 times the required concentration. and then 3 ml added to the 12 ml already in the dishes. The final volume of DMSO was 4 μl/ml culture medium in each case except with caprolactam, when it was 16 μl/ml, including in the negative and positive (cyclophosphamide) controls. In one instance, the cells had not grown sufficiently after 1 day, so they were treated after 48 h. These cultures were subsequently treated with benzoin and acrylonitrile. The cultures were incubated for 36 h after the addition of the test agent. After 33 h 0.3 μg/ml Colcemid (Gibco Bio-cult Ltd.) was added as a mitotic arrester.

Slides were harvested using standard techniques for metaphase preparations, with slight modifications to prevent cells bursting open and scattering the chromosomes (Danford, 1984). Following a rinse with Hank's BSS, just 4 min hypotonic (0.056% KCl) exposure was used. The cells were then fixed with 3 : 1 methanol : acetic acid for 0.5 min (twice) and 30 min, and then air dried. Harvesting was carried out in situ in the dishes or in vertical staining tanks, with as little movement as possible prior to completion of fixation. The slides were stained for 5 min with 10% Giemsa solution diluted with 0.01 M phosphate buffer pH 6.8, rinsed, dried and mounted with DePeX (BDH Ltd.). All the slides were prepared within an 11-day period. Passage numbers of the cultures used for each chemical are given in Table 1. Slide cultures for each chemical were also set up simultaneously to examine effects on the mitotic spindle and stages of mitosis; the results of these experiments are described elsewhere in this volume by E.M. Parry.

2 replicates were taken from each dose and the positive control, and from all 3 negative controls. In the case of benzene, the data from 2 slides was not sufficiently conclusive, and the third replicate was subsequently also examined. All slides were independently coded at random. From each slide, 100 metaphases were scored, unless toxicity had greatly reduced the mitotic index; they were selected by finding cells in which a circle of cytoplasm was visible around the chromosomes, which had to be sufficiently clear to allow assessment of structural aberrations and accurate counting of the number of chromosomes. If cells were found in which the amount of damage prevented determination of the chromosome number, extra cells were scored to increase the total to 100, before decoding of the slides. As these additional cells were not selected strictly at random (i.e. cells with damage were not assessed) they were not included in the structural aberration data. Cells with more than 10 aberrations were classified as having multiple aberrations or severe damage.

Analysis of data

Chromosome aberrations. The data were examined by comparing the control values, excluding and including gaps, with each dose by means of Fisher's Exact test (Sokal and Rohlf, 1969). Regression analysis was also carried out, using both the percentage of cells with aberrations and the frequency of aberrations/cell against dose. The frequency of aberrations/cell was calculated using a value of 10 for each cell with multiple aberrations, although this would usually result in an underestimate of the total (Kilian et al., 1977; Whorton et al., 1979).

Chromosome numbers. These data were analysed using χ^2 2×2 test, except where any value was less than 10, in which case Fisher's Exact test was applied. The control values were compared with each dose as follows: (probability, p, indicated in the tables under columns)

(i) Aneuploidy/diploidy + polyploidy (p: final column)

(ii) Hypodiploidy/diploidy + polyploidy (p: "20–21" column)

(iii) Hyperdiploidy/diploidy + polyploidy (p: "23" column).

With DES, however, the increase in polyploidy at some doses made it more suitable to take diploid numbers only throughout (i.e. (i) aneuploidy + polyploidy/diploidy, etc).

Percentage values were used to calculate the regression on dose of: (i) Diploidy, (ii) Diploidy + polyploidy, (iii) Hypodiploidy, (iv) Hyperdiploidy.

Levels of significance are indicated as follows: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

Results

HMPA (Tables 2 and 3)

The percentage of cells with aberrations and frequencies of aberrations, excluding and including gaps, were significantly increased in the treated samples at almost all doses. There was a significant dose–response ($F_{1,3} = 14.04$ *) when the total number of aberrations including gaps was regressed on dose.

The number of aneuploid cells increased with dose, with the percent of diploid cells ($F_{1,3} = 19.851$ **) and diploid + polyploid ($F_{1,3} = 16.107$ *) decreasing significantly with increasing

TABLE 2

ABERRATION LEVELS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO HMPA

Concentration (μg/ml)	Number of cells analysed	Chromatid gaps	Chromatid aberrations	Chromosome aberrations	Multiple aberrations	All aberrations excluding gaps	All aberrations including gaps
(*a*) *Percentage of cells with aberrations*							
0	300	3.0	0	0	0	0	3.0
2	200	7.0	3.0	0.5	0	3.5 ***	9.0 ***
5	200	3.5	1.5	0.5	0	2.0 **	5.5
10	200	9.5	1.5	0	0	1.5	11.0 †
20	200	10.5	5.0	0.5	0	5.5 †	13.5 †
2AAF	162	50.0	42.6	14.8	29.6	80.2 †	92.6 †
		Chromatid gaps	Chromatid breaks	Chromatid exchanges	Chromosome aberrations	All aberrations excluding gaps	All aberrations including gaps
(*b*) *Frequency per cell*							
0	300	0.030	0	0	0	0	0.030
2	200	0.075	0.020	0.010	0.005	0.035 ***	0.110 ***
5	200	0.040	0.010	0.005	0.010	0.025 **	0.065
10	200	0.095	0.005	0.015	0	0.020 *	0.115 †
20	200	0.115	0.070	0.015	0.005	0.090 †	0.205 †
2AAF	162	1.191	0.438	0.296	0.172	3.870 †	5.062 †

2AAF, +ve control; 2-acetylaminofluorene (100 μg/ml).
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

TABLE 3

CHROMOSOME NUMBERS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO HMPA

Concentration (μg/ml)	Number of analysed	Number of cells with chromosome (centromere) No.					Percent euploid	*p*
		20–21	22	23	⩾ 24	Polyploid		
0	300	16	253	25	5	1	84.7 ± 3.51	
2	200	15	159	20	2	4	81.5 ± 3.54	n.s.
5	200	9	155	24	6	6	80.5 ± 7.78	n.s.
10	200	21 **	144	26 *	5	4	74.0 ± 0	***
20	200	22 **	139	24 *	8	7	73.0 ± 5.66	***
2AAF	75	13 †	39	11 †	9	3	56.0	†

2AAF, +ve control; 2-acetylaminofluorene (100 μg/ml).
−ve control vs. HMPA (total) = ***.
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

concentrations of HMPA. This was due to an increase in both hypo- and hyper-diploid cells, which were significantly greater than the control at 10 and 20 μg/ml, although the regression was not statistically significant in either case.

HMPA thus induced chromosome aberrations and aneuploidy in this assay system.

TOL (Tables 4 and 5)

All concentrations tested, both excluding and including gaps, caused a significant increase in the level of chromosome aberrations. Although dose–response was observed, this was not statistically significant.

A similar situation was found with regard to

TABLE 4

ABERRATION LEVELS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO TOL

Concentration (μg/ml)	Number of cells analysed	Chromatid gaps	Chromatid aberrations	Chromosome aberrations	Multiple aberrations	All aberrations excluding gaps	All aberrations including gaps
(*a*) *Percentage of cells with aberrations*							
0	300	3.7	0.7	0	0	0.7	4.0
12	200	10.5	4.0	0.5	0	4.5 ***	14.0 †
30	200	10.5	3.5	0.5	0	3.5 *	12.5 †
60	200	11.0	5.5	0	0	5.5 ***	15.5 †
120	200	11.5	4.5	1.0	0	5.5 ***	16.5 †
BP	200	13.0	12.0	1.5	1.5	14.5 †	25.0 †
		Chromatid gaps	Chromatid breaks	Chromatid exchanges	Chromosome aberrations	All aberrations excluding gaps	All aberrations including gaps
(*b*) *Frequency per cell*							
0	300	0.043	0.003	0.003	0	0.007	0.050
12	200	0.135	0.015	0.025	0.005	0.045 ***	0.180 †
30	200	0.110	0.055	0.005	0.005	0.065 †	0.175 †
60	200	0.140	0.080	0.020	0	0.100 †	0.240 †
120	200	0.145	0.060	0.020	0.010	0.090 †	0.235 †
BP	200	0.160	0.100	0.090	0.015	0.355 †	0.515 †

BP, + ve control; benzo[*a*]pyrene (1 μg/ml).
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

TABLE 5

CHROMOSOME NUMBERS IN CHINESEHAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO TOL

Concentration (μg/ml)	Number of analysed						Percent euploid	*p*
		Number of cells with chromosome (centromere) No.						
		20–21	22	23	⩾ 24	Polyploid		
0	300	21	227	40	8	4	77.0 ± 5.29	
12	200	11	145	31	8	5	75.0 ± 0	n.s.
30	200	8	151	30	2	9	80.0 ± 0	n.s.
60	200	15	131	41 *	6	7	69.0 ± 0	*
120	200	16	133	39 **	9	3	68.0 ± 2.83	*
BP	200	22	129	37	7	5	67.0 ± 7.07	**

BP, + ve control; benzo[*a*]pyrene (1 μg/ml).
− ve control vs. TOL (total) = n.s.
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

aneuploidy levels. The higher 2 doses (60 and 120 μg/ml) had significantly greater levels of hyperdiploidy, but dose–response was nonsignificant. The increases in frequency of chromosome aberrations and hyperdiploidy were more step-wise than gradual.

TOL was thus positive for both endpoints.

BEN (Tables 6 and 7)

An extra replicate (3 in all) was scored at each dose, after analysis of the data from 2 slides had been inconclusive. For chromosome aberrations, no significant regression was observed, although several individual results were greater than the controls if gaps were included; and at 125 μg/ml, there was a significant increase excluding gaps. This, however, was at a low level, and statistically no greater than some historical controls.

The analysis of the data on chromosome numbers also showed no significant regression. How-

TABLE 6

ABERRATION LEVELS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO BEN

Concentration (μg/ml)	Number of cells analysed	Chromatid gaps	Chromatid aberrations	Chromosome aberrations	Multiple aberrations	All aberrations excluding gaps	All aberrations including gaps
(*a*) *Percentage of cells with aberrations*							
0	300	2.3	0	0	0	0	2.3
25.0	300	6.0	0.7	0.3	0	1.0	7.0 **
62.5	300	3.0	0.7	0	0	0.7	3.3
125.0	300	5.7	2.3	0	0	2.3 **	7.3 ***
250.0	300	4.7	1.0	0.3	0	1.3	5.7
EMS	200	10.5	10.0	3.5	0	12.0 †	21.0 †
		Chromatid gaps	Chromatid breaks	Chromatid exchanges	Chromosome aberrations	All aberrations excluding gaps	All aberrations including gaps
(*b*) *Frequency per cell*							
0	300	0.023	0	0	0	0	0.023
25.0	300	0.070	0.003	0.003	0.003	0.010	0.080 ***
62.5	300	0.033	0	0.007	0	0.007	0.040
125.0	300	0.063	0.023	0.007	0	0.030 ***	0.093 †
250.0	300	0.057	0.007	0.003	0.003	0.013	0.070 **
EMS	200	0.115	0.080	0.035	0.035	0.150 †	0.265 †

EMS, +ve control; ethyl methanesulphonate (200 μg/ml).
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

TABLE 7

CHROMOSOME NUMBERS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO BEN

Concentration (μg/ml)	Number of cells analysed	Number of cells with chromosome (centromere) No.					Percent euploid	*p*
		20–21	22	23	⩾ 24	Polypoid		
0	300	14	263	16	1	6	89.7 ± 1.53	
25.0	300	13	254	24	4	5	86.3 ± 5.13	n.s.
62.5	300	25	236	24	4	11	82.3 ± 5.77	**
125.0	300	19	248	22	5	6	84.7 ± 4.16	n.s.
250.0	300	20	234	35 ***	3	8	80.7 ± 5.13	***
EMS	200	15	164	15	1	5	84.5 ± 3.54	n.s.

EMS, +ve control; ethyl methanesulphonate (200 μg/ml).
−ve control vs. BEN (total) = ***.
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

ever, aneuploidy was increased at 62.5 and 250 μg/ml, in the former due to both hypo- and hyper-diploidy (though neither was significant on its own), and in the latter due to hyperdiploidy only. Using pooled data for all doses, both aneuploidy and hyperdiploidy were highly significantly increased.

Benzene was negative for chromosome aberrations and positive for aneuploidy.

SAF (Tables 8 and 9)

The aberration levels excluding gaps that were observed following exposure to SAF were significantly greater than the controls at 15 and 60 μg/ml. However, no dose–response was observed and compared with historial controls, including those in this study, the maximum levels of 2.5% and 0.025/cell are not significant. The percentage and frequency of gaps were significantly increased

TABLE 8

ABERRATION LEVELS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO SAF

Concentration (μg/ml)	Number of cells analysed	Chromatid gaps	Chromatid aberrations	Chromosome aberrations	Multiple aberrations	All aberrations excluding gaps	All aberrations including gaps
(*a*) *Percentage of cells with aberrations*							
0	300	3.0	0	0	0	0	3.0
6	200	8.5	0.5	0	0	0.5	9.0 ***
15	200	6.0	2.5	0	0	2.5 **	7.5 *
30	200	6.0	1.0	0.5	0	1.5	7.0 *
60	200	6.5	2.0	0	0	2.0 *	8.0 **
2AAF	162	50.0	42.6	14.8	29.6	80.2 †	92.6 †
		Chromatid gaps	Chromatid breaks	Chromatid exchanges	Chromosome aberrations	All aberrations excluding gaps	All aberrations including gaps
(*b*) *Frequency per cell*							
0	300	0.030	0	0	0	0	0.030
6	200	0.095	0.005	0	0	0.005	0.100 ***
15	200	0.060	0.015	0.010	0	0.025 **	0.085 **
30	200	0.060	0.010	0	0.005	0.015	0.075 *
60	200	0.085	0.015	0.005	0	0.020 *	0.105 ***
2AAF	162	1.191	0.438	0.296	0.172	3.870 †	5.062 †

2AAF, +ve control; 2-acetylaminofluorene (100 μg/ml).
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

TABLE 9

CHROMOSOME NUMBERS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO SAF

Concentration (μg/ml)	Number of cells analysed	Number of cells with chromosome (centromere) No.					Percent euploid	*p*
		20–21	22	23	⩾ 24	Polyploid		
0	300	16	253	25	5	1	84.7 ± 3.51	
6	200	10	170	15	3	2	86.0 ± 1.41	n.s.
15	200	16	157	25	2	0	78.5 ± 2.12	n.s.
30	200	12	164	16	3	5	84.5 ± 2.12	n.s.
60	200	14	160	20	4	2	81.0 ± 4.24	n.s.
2AAF	75	13 †	39	11 †	9	3	56.0	†

2AAF, +ve control; 2-acetylaminofluorene (100 μg/ml).
−ve control vs. SAF (total) = n.s.
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

at all doses, but this is not generally accepted as being sufficient to classify a compound as positive.

The number of aneuploid cells did not differ from the control. SAF was therefore negative for both chromosome aberrations and aneuploidy.

CAP (Tables 10 and 11)

No significant increase in either aberrations or aneuploidy was observed, with the exception of hypodiploidy, where the regression analysis was marginally significant ($F_{1,3} = 10.745$ *). However, an increase from 5.7 to 8.5% is slight, and no individual doses differed significantly from the control.

CAP was negative in this system.

ACN (Tables 12 and 13)

Acrylonitrile was the most potent inducer of

TABLE 10

ABERRATION LEVELS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO CAP

Concentration (μg/ml)	Number of cells analysed	Chromatid gaps	Chromatid aberrations	Chromosome aberrations	Multiple aberrations	All aberrations excluding gaps	All aberrations including gaps
(*a*) *Percentage of cells with aberrations*							
0	300	4.0	0	0	0	0	4.0
200	200	2.5	0.5	0	0	0.5	2.5
500	200	3.5	0	0	0	0	3.5
1000	200	4.5	0	0	0	0	4.5
2000	200	2.5	0	0	0	0	2.5
CP	200	13.5	7.5	2.0	0	9.0 †	20.0 †
		Chromatid gaps	Chromatid breaks	Chromatid exchanges	Chromosome aberrations	All aberrations excluding gaps	All aberrations including gaps
(*b*) *Frequency per cell*							
0	300	0.040	0	0	0	0	0.040
200	200	0.025	0	0.005	0	0.005	0.030
500	200	0.035	0	0	0	0	0.035
1000	200	0.045	0	0	0	0	0.045
2000	200	0.025	0	0	0	0	0.025
CP	200	0.145	0.065	0.060	0.020	0.145 †	0.290 †

CP, + ve control; cyclophosphamide (2000 μg/ml).
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

TABLE 11

CHROMOSOME NUMBERS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO CAP

Concentration (μg/ml)	Number of cells analysed	Number of cells with chromosome (centromere) No.					Percent euploid	*p*
		20–21	22	23	⩾ 24	Polyploid		
0	300	17	225	48	3	7	77.3 ± 2.08	
200	200	13	150	23	2	12	81.0 ± 2.83	n.s.
500	200	11	159	24	2	4	81.5 ± 4.95	n.s.
1000	200	13	160	19	4	4	82.0 ± 0	n.s.
2000	200	17	144	27	5	7	75.5 ± 0.71	n.s.
CP	200	9	130	56 ***	3	2	66.0 ± 5.66	***

CP, + ve control; cyclophosphamide (2000 μg/ml).
− ve control vs. CAP (total) = n.s.
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

structural aberrations tested in this study (excluding positive controls). Significant dose–response was observed for the percent of cells with aberrations excluding gaps ($F_{1,3} = 50.15$ ***), including gaps ($F_{1,3} = 64.69$ ***), and for frequency of aberrations excluding gaps ($F_{1,3} = 26.62$ **) and including gaps ($F_{1,3} = 44.64$ ***). Most individual results were also significant (Table 12).

With regard to chromosome numbers, there was a significant decrease in cells with diploid + polyploid numbers of chromosomes ($F_{1,3} = 11.535$ *) and a significant increase in hypodiploidy ($F_{1,3} = 13.416$ *). At the highest dose, the hypodiploidy level was increased. If the levels of chromosome aberrations are high, however, an increase in hypodiploidy may be due to loss of damaged chromosomes. For this reason, ACN is not considered to have induced aneuploidy in this test.

ACN was therefore positive for structural aber-

TABLE 12

ABERRATION LEVELS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO ACN

Concentration (μg/ml)	Number of cells analysed	Chromatid gaps	Chromatid aberrations	Chromosome aberrations	Multiple aberrations	All aberrations excluding gaps	All aberrations including gaps
(a) Percentage of cells with aberrations							
0	300	4.0	1.0	0	0	1.0	4.7
2.5	200	6.5	4.0	0	0	4.0 *	10.5 †
6.25	200	6.5	2.5	0	0	2.5	9.0 ***
12.5	200	11.0	7.0	0	0	7.0 †	15.5 †
25.0	200	19.5	12.5	2.0	1.0	14.5 †	30.0 †
DMN	200	8.5	4.5	0.5	0.5	5.0 ***	11.0 †
		Chromatid gaps	Chromatid breaks	Chromatid exchanges	Chromosome aberrations	All aberrations excluding gaps	All aberrations including gaps
(b) Frequency per cell							
0	300	0.040	0.007	0.003	0	0.010	0.050
2.5	200	0.065	0.025	0.015	0	0.040	0.105 †
6.25	200	0.065	0.015	0.010	0	0.025	0.090 **
12.5	200	0.130	0.085	0	0	0.085 †	0.215 †
25.0	200	0.250	0.190	0.030	0.025	0.345 †	0.595 †
DMN	200	0.090	0.030	0.020	0.005	0.105 †	0.195 †

DMN, +ve control; dimethylnitrosamine (2000 μg/ml).
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

TABLE 13

CHROMOSOME NUMBERS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO ACN

Concentration (μg/ml)	Number of cells analysed	Number of cells with chromosome (centromere) No.					Percent euploid	p
		20–21	22	23	⩾ 24	Polyploid		
0	300	22	209	57	2	10	73.0 ± 3.0	
2.5	200	11	140	40	2	7	73.5 ± 0.71	n.s.
6.25	200	21	140	32	2	5	72.5 ± 2.12	n.s.
12.5	200	21	142	31	2	4	73.0 ± 4.24	n.s.
25.0	200	28 **	137	31	3	1	69.0 ± 5.66	n.s.
DMN	200	13	141	38	2	6	73.5 ± 4.95	n.s.

DMN, +ve control; dimethylnitrosamine (2000 μg/ml).
−ve control vs. ACN (total) = n.s.
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

rations and negative for numerical chromosome aberrations.

DEHP (Tables 14 and 15)

There was no significant increase in chromosome aberrations at any dose.

The levels of aneuploidy were not significantly different from the controls, and no significant dose–response was observed for diploidy, diploidy + polyploidy or hypodiploidy. The hyperdiploidy level, however, increased highly significantly with dose ($F_{1,3} = 167.09$ †).

DEHP was negative for chromosome aberrations and positive for numerical aberrations.

ZOIN (Tables 16 and 17)

No increase in chromosome aberrations was seen following exposure to ZOIN. The level of

TABLE 14

ABERRATION LEVELS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO DEHP

Concentration (μg/ml)	Number of cells analysed	Chromatid gaps	Chromatid aberrations	Chromosome aberrations	Multiple aberrations	All aberrations excluding gaps	All aberrations including gaps
(*a*) *Percentage of cells with aberrations*							
0	300	2.7	0.3	0	0	0.3	3.0
5.0	200	4.0	0.5	0	0	0.5	4.0
12.5	200	5.0	0	0	0	0	5.0
25.0	200	4.0	1.0	0	0	1.0	5.0
50.0	200	4.0	1.0	0	0	1.0	5.0
CP	200	13.5	7.5	2.0	0	9.0 †	20.0 †
		Chromatid gaps	Chromatid breaks	Chromatid exchanges	Chromosome aberrations	All aberrations excluding gaps	All aberrations including gaps
(*b*) *Frequency per cell*							
0	300	0.027	0.003	0	0	0.003	0.030
5.0	200	0.040	0.005	0	0	0.005	0.045
12.5	200	0.055	0	0	0	0	0.055
25.0	200	0.040	0	0.010	0	0.010	0.050
50.0	200	0.040	0.010	0	0	0.010	0.050
CP	200	0.145	0.065	0.060	0.020	0.145 †	0.290 †

CP, +ve control; cyclophosphamide (2000 μg/ml).
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

TABLE 15

CHROMOSOME NUMBERS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO DEHP

Concentration (μg/ml)	Number of analysed	Number of cells with chromosome (centromere) No.					Percent euploid	*p*
		20–21	22	23	⩾ 24	Polyploid		
0	300	24	220	44	4	8	76.0 ± 3.46	
5.0	200	18	140	33	3	6	73.0 ± 4.24	n.s.
12.5	200	25	133	33	4	5	69.0 ± 1.41	n.s.
25.0	200	17	138	34	7	4	71.0 ± 2.83	n.s.
50.0	200	10	137	43 *	6	4	70.5 ± 2.12	n.s.
CP	200	9	130	56 ***	3	2	66.0 ± 5.66	***

CP, +ve control; cyclophosphamide (2000 μg/ml).
−ve control vs. DEHP (total) = n.s.
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

TABLE 16

ABERRATION LEVELS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO ZOIN

Concentration (μg/ml)	Number of cells analysed	Chromatid gaps	Chromatid aberrations	Chromosome aberrations	Multiple aberrations	All aberrations excluding gaps	All aberrations including gaps
(a) Percentage of cells with aberrations							
0	300	4.0	1.0	0	0	1.0	4.7
20	200	5.0	1.0	0	0	1.0	5.5
50	200	5.5	0	0	0	0	5.5
100	200	6.0	0	0	0	0	6.0
200	200	4.5	1.5	0	0	1.5	5.5
DMN	200	8.5	4.5	0.5	0.5	5.0 ***	11.0 †
		Chromatid gaps	Chromatid breaks	Chromatid exchanges	Chromosome aberrations	All aberrations excluding gaps	All aberrations including gaps
(b) frequency per cell							
0	300	0.040	0.007	0.003	0	0.010	0.050
20	200	0.055	0.005	0.005	0	0.010	0.065
50	200	0.055	0	0	0	0	0.055
100	200	0.065	0	0	0	0	0.065
200	200	0.050	0.005	0.010	0	0.015	0.065
DMN	200	0.090	0.030	0.020	0.005	0.105 †	0.195 †

DMN, +ve control; dimethylnitrosamine (2000 μg/ml).
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

TABLE 17

CHROMOSOME NUMBERS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO ZOIN

Concentration (μg/ml)	Number of cells analysed	Number of cells with chromosome (centromere) No.					Percent euploid	p
		20–21	22	23	⩾ 24	Polyploid		
0	300	22	209	57	2	10	73.0 ± 3.0	
20	200	13	143	39	3	2	72.5 ± 3.54	n.s.
50	200	16	143	33	4	4	73.5 ± 2.12	n.s.
100	200	14	136	40	5	5	70.5 ± 2.12	n.s.
200	200	15	133	43	4	5	69.0 ± 1.41	n.s.
DMN	200	13	141	38	2	6	73.5 ± 4.95	n.s.

DMN, +ve control; dimethylnitrosamine (2000 μg/ml).
−ve control vs. ZOIN (total) = n.s.

aneuploidy increased significantly with dose ($F_{1,3} = 15.869$ *), but the reduction in diploid + polyploid cells was only from 73 to 69%, and neither hypo- nor hyper-diploidy, nor any individual results, were significant.

ZOIN was thus negative for both numerical and structural aberrations.

DES (Tables 18 and 19)

Observations on chromosome aberrations at 15 μg/ml were possibly underestimated, as the quality of metaphases was greatly reduced. It was more marked at this dose than at 30 μg/ml, where the mitotic index was very low, but those metaphases present were reasonably sharp.

There was an increase in chromosome aberrations at 3 and 30 μg/ml; in the latter, the levels were very high and cells with multiple aberrations were seen. The regression of frequency of aberrations excluding gaps on dose was statistically significant ($F_{\widehat{1,3}} = 10.55$ *).

TABLE 18

ABERRATION LEVELS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO DES

Concentration (μg/ml)	Number of cells analysed	Chromatid gaps	Chromatids aberrations	Chromosome aberrations	Multiple aberrations	All aberrations exluding gaps	All aberrations including gaps
(*a*) *Percentage of cells with aberrations*							
0	300	3.7	0.7	0	0	0.7	4.0
3.0	200	11.0	3.5	0.5	0	4.0 **	14.0 †
7.5	200	5.5	0.5	0.5	0	1.0	6.5
15.0	200	4.5	2.5	0.5	0	2.5	6.0
30.0	171	10.5	10.5	1.2	4.1	15.8 †	22.8 †
BP	200	13.0	12.0	1.5	1.5	14.5 †	25.0 †
		Chromatid gaps	Chromatid breaks	Chromatid exchanges	Chromosome aberrations	All aberrations excluding gaps	All aberations including gaps
(*b*) *Frequency per cell*							
0	300	0.043	0.003	0.003	0	0.007	0.050
3.0	200	0.135	0.035	0.015	0.005	0.055 ***	0.190 †
7.5	200	0.055	0	0.005	0.005	0.010	0.065
15.0	200	0.060	0.010	0.015	0.005	0.030	0.090
30.0	171	0.140	0.076	0.035	0.012	0.532 †	0.672 †
BP	200	0.160	0.100	0.090	0.015	0.355 †	0.515 †

BP, +ve control; benzo[*a*]pyrene (1.0 μg/ml).
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

TABLE 19

CHROMOSOME NUMBERS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO DES

Concentration (μg/ml)	Number of cells analysed	Number of cells with chromosome (centromere) No.					Percent diploid	*p*
		20–21	22	23	≥ 24	Polyploid		
0	300	21	227	40	8	4	75.7 ± 5.51	
3.0	200	13	130	44 ***	11	2	65.0 ± 0	**
7.5	200	23	130	26	12	9	65.0 ± 8.49	**
15.0	200	2 †	18	3 †	7	170	9.0 ± 0	†
30.0	168	12	92	21	3	40	54.8	†
BP	200	22	129	37	7	5	64.5 ± 4.95	**

BP, +ve control; benzo[*a*]pyrene (1 μg/ml).
−ve control vs. DES (total) = †.
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

DES had a particularly interesting effect on chromosome numbers. At 15 μg/ml, the two replicates had 83 and 87% polyploid cells. The level was only 23.8% (24 and 23.53%) at 30 μg/ml, probably mainly due to long cell cycle delay at the highest dose; that is, many cells observed were in the first division post-treatment, and thus there was not sufficient time for polyploidy to be induced. Thre was also an increase in hyperdiploidy at 7.5 μg/ml. No dose–response was observed with chromosome numbers, which were distorted by the levels of polyploidy.

DES induced both structural and numerical chromosome aberrations.

PB (Tables 20 and 21)

The numbers of chromosome aberrations observed following treatment with PB was significantly greater than the controls at all doses, excluding and including gaps. No dose–response was observed, and some of the values were not greater compared with historial controls, but others (percentage of cells, 100 and 250 μg/ml excluding gaps; frequencies/cell, 100 and 500 μg/ml excluding gaps) were substantially higher and not due to single replicates. Although the increase was slight compared with, for example, ACN, they cannot be discounted.

There was no effect on chromosome numbers except at 100 μg/ml, where hypodiploidy was significantly increased. This was due mainly to one replicate, which was also the one in which the greatest amount of chromosome damage was observed.

TABLE 20

ABERRATION LEVELS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO PB

Concentration (μg/ml)	Number of cells analysed	Chromatid gaps	Chromatid aberrations	Chromosome aberrations	Multiple aberrations	All aberrations excluding gaps	All aberrations including gaps
(a) Percentage of cells with aberrations							
0	300	2.3	0	0	0	0	2.3
100	200	6.0	4.5	0	0	4.5 †	8.5 ***
250	200	5.0	3.0	1.0	0	4.0 ***	9.0 ***
500	200	6.0	2.5	0	0	2.5 ***	8.0 ***
1000	200	6.0	2.5	0	0	2.5 ***	7.5 ***
EMS	200	10.5	10.0	3.5	0	12.0 †	21.0 †
		Chromatid gaps	Chromatid breaks	Chromatid exchanges	Chromosome aberrations	All aberrations excluding gaps	All aberrations including gaps
(b) Frequency per cell							
0	300	0.023	0	0	0	0	0.023
100	200	0.070	0.030	0.040	0	0.070 †	0.140 †
250	200	0.050	0.020	0.010	0.010	0.040 ***	0.090 ***
500	200	0.075	0.035	0.035	0	0.070 †	0.145 †
1000	200	0.065	0.015	0.010	0	0.025 **	0.090 ***
EMS	200	0.115	0.080	0.035	0.035	0.150 †	0.265 †

EMS, +ve control; ethyl methanesulphonate (200 μg/ml).
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

TABLE 21

CHROMOSOME NUMBERS IN CHINESE HAMSTER LIVER FIBROBLASTS FOLLOWING EXPOSURE TO PB

Concentration (μg/ml)	Number of cells analysed	Number of cells with chromosome (centromere) No.					Percent euploid	*p*
		20–21	22	23	⩾ 24	Polyploid		
0	300	14	263	16	1	6	89.7 ± 1.53	
100	200	18 *	156	17	3	6	81.0 ± 5.66	***
250	200	17	167	10	0	6	86.5 ± 3.54	n.s.
500	200	15	165	15	2	3	84.0 ± 4.24	n.s.
1000	200	18	169	11	1	1	85.0 ± 2.83	n.s.
EMS	200	15	164	15	1	5	84.5 ± 3.54	n.s.

EMS, +ve control; ethyl methanesulphonate (200 μg/ml).
−ve control vs. PB (total) = **.
Levels of significance: * $p = 0.025$–0.05; ** $p = 0.01$–0.025; *** $p = 0.001$–0.01; † $p < 0.001$.

PB induced chromosome aberrations but not aneuploidy.

Positive controls

All the positive controls induced chromosome aberrations, DMN being the least and 2AAF by far the most potent.

DMN and EMS were negative for induction of aneuploidy, hypodiploidy and hyperdiploidy. The data for 2AAF was highly significant for all 3 parameters, although the sample size was small (75 cells). BP was significant overall for aneuploidy but nonsignificant for hypo- or hyper-diploidy. CP showed highly significantly greater levels of aneuploidy, due almost entirely to an increase in hyperdiploidy.

Discussion

Although these results will not be discussed in great detail, a few aspects will be expanded.

Hypodiploidy and structural chromosome aberrations

It was observed with HMPA, ACN and PB that when levels of chromosome aberrations were high, there was an increase in hypodiploidy. If these two events are observed together, hypodiploidy may well be due to excessive chromosome breakage and not to chromosome lagging or other effects on chromosome separation or movement. For this reason, ACN and PB were classified as negative for aneuploidy, while HMPA, which also showed hyperdiploidy, was considered to have induced aneuploidy.

Negative controls

The levels of structural chromosome aberrations varied slightly from set to set, but the aneuploidy levels showed highly significant heterogeneity (analysis of variance: $F_{4,13}^{\sim} = 12.45$ †).

Variation between replicates was in most instances quite low, but between samples the percentage of aneuploid cells ranged from 10.3 to 27.0%. This did not appear to be correlated with passage number, although different split ratios at earlier passages may account in part for variation between the different stocks used here. Set 3, which had to be grown for an extra day before treatment, had the highest spontaneous levels of both structural chromosome aberrations and aneuploidy. It is therefore also possible that cells that have not grown sufficiently when they would normally be ready for treatment may be unhealthy and not merely a little sluggish to start (Hsu, 1979).

Conclusions

Table 22 summarises the results and assigns a + or − to each compound. Since two endpoints were being assessed and there are considerable

TABLE 22

SUMMARY OF RESULTS

Compound	Structural aberrations		Aneuploidy		Conclusion
	Increase	Dose–response	Increase	Dose–response	
HMPA	+	+	+	+	+
TOL	+	+	+(w)	−	+
BEN	−	−	+(w)	−	+(w)
SAF	−	−	−	−	−
CAP	−	−	−	−	−
ACN	+	+	−	−	+
DEHP	−	−	+(w)	+	+(w)
ZOIN	−	−	−	−	−
DES	+	+	+ [a]	−	+
PB	+(w)	−	−	−	+(w)

−, negative; +(w), weak positive; +, strong positive.

[a] Also significant increase in polyploidy.

differences between positive compounds, a weighted value is given.

Clearly, the two parameters assessed are not correlated, since 3 were positive for both, 3 for neither and 4 positive for one and negative for the other. Both can thus provide information on the effects of chemical agents on cultured mammalian cells.

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Tests for the induction of chromosomal aberrations and sister chromatid exchanges in cultured Chinese hamster ovary (CHO) cells

D.K. Gulati, P.S. Sabharwal and M.D. Shelby [1]

Environmental Health Research and Testing, Inc., Lexington, KY (U.S.A.) and [1] National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC (U.S.A.)

Summary

8 carcinogens and 2 noncarcinogens were tested for their ability to induce chromosomal aberrations and sister-chromatid exchanges (SCE) in cultured Chinese hamster ovary cells. Chemicals were tested up to a maximum concentration of 5 mg/ml unless limited by solubility or toxicity. Hexamethylphosphoramide, *o*-toluidine, acrylonitrile, and safrole were positive in the SCE assays. *o*-Toluidine, acrylonitrile, phenobarbital and diethylstilbestrol were positive in the chromosomal aberration assay.

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8 rodent carcinogens (HMPA, SAF, TOL, BEN, DEHP, ACN, PB and DES) and 2 rodent noncarcinogens (CAP, ZOIN) were selected by the International Program for Chemical Safety as test chemicals in the Collaborative Study on Short-Term Tests for Carcinogens and Mutagens (Ashby et al., 1983). The carcinogens have proven difficult or impossible to detect in bacterial mutagenicity tests and were selected as a group of carcinogens that might provide a basis for identifying assays complementary to the Salmonella/microsome assay. Reported here are the test results for these 8 carcinogens and 2 noncarcinogens for the induction of chromosomal aberrations and sister-chromatid exchanges in cultured Chinese hamster ovary cells. The protocol used in this study has been developed through projects supported by the Cellular and Genetic Toxicology Branch of the National Institute of Environmental Health Sciences. A detailed description of the assay is in preparation (Galloway et al., 1985).

Materials and methods

Cell culture

All cytogenetic testing was performed in Chinese hamster ovary (CHO) cells, a permanent cell line with an average generation time of 12–14 h. The cells were provided by A. Bloom of Columbia University Medical Center, New York.

CHO cells were maintained in modified McCoy's 5A Medium (Gibco) supplemented with glutamine (Gibco) and 10% fetal bovine serum (FBS). Glutamine and FBS were added immediately prior to use. Cells were grown in plastic T-75 flasks (Corning) in an atmosphere of 5% CO_2 at 37 °C and approximately 90% humidity.

Test chemicals

All test chemicals were provided by the CSSTT program in uncoded 2-g lots and stored at 4 °C. Chemicals were handled under safe yellow lights. Immediately before each use, the test article was weighed (both liquids and solids) and appropriately dissolved in DMSO. Serial dilutions were prepared from the main stock in the same solvent to achieve the desired final concentrations by additions of 100 μl of the test solution in the culture flasks.

Metabolic activation system

Rat-liver S9 fraction was purchased from Litton

Bionetics, Kensington, (MD) and was prepared from 8–10-week-old Sprague–Dawley, male rats obtained from Charles River Breeders. Animals were induced with Aroclor 1254 suspended in corn oil administered in one intraperitoneal (i.p.) injection at 500 mg/kg for 5 days prior to sacrifice and starved 12 h prior to sacrifice. The S9 fraction was thawed immediately before use and added to a "core" reaction mixture. The final concentration of NADP, isocritric acid, and S9 fraction in the culture medium was 2.4 mg/ml, 4.5 mg/ml, and 15 μl/ml, respectively.

Experimental controls

3 different controls were used with each experiment: (a) negative control, cells received no treatment; (b) solvent control, cells were treated with 10 μl/ml DMSO; and (c) positive control, cells were treated with cyclophosphamide (+S9) or mitomycin C (−S9).

Selection of dose

10 or 11 dose levels in descending half-log series were used for the first SCE tests beginning with 5 mg/ml (highest dose). Cells were harvested by shake-off. The 3–5 highest doses yielding sufficient mitotic cells were scored. Results from these initial SCE tests were used to determine dose ranges for all subsequent tests.

Protocol for sister chromatid exchange assay

Cells were seeded at a density of 8×10^5/flask. In assays without metabolic activation, one day after culture initiation the medium was replaced with fresh medium and cells were treated with test or control article for 2 h to allow some interaction with cells before addition of bromodeoxyuridine (BrdUrd). BrdUrd was added (final concentration 10 μM) and incubation was continued for an additional 24 h. Medium was then removed to allow an opportunity to wash off any test compound precipitate that might interfere with cell fixation and minimize the chance of exposure of personnel to cultures containing test compounds. Fresh medium containing 10 μM BrdUrd and colcemid was added, and incubation continued for 2–3 h. The total incubation time with both the test article and BrdUrd was about 26 h. In some cases, cytostatic effects led to delayed harvests and hence extended exposure times.

In assays with the metabolic activation, medium was removed one day after culture initiation, the cells were washed twice with phosphate-buffered saline (PBS), and fresh medium without serum was added. Cells were incubated for 2 h in the presence of the test or control compound and the S9 reaction mixture. Serum was omitted to avoid the possible binding of short-lived, highly reactive intermediates produced by S9 enzymes to serum proteins. After the 2-h exposure, cells were washed at least twice with PBS, and complete medium containing 10% serum and 10 μM BrdUrd was added. Cells were incubated for an additional 26 h with colcemid present for the final 2–3 h. Cells were collected by mitotic shake-off and treated for about 3 min at room temperature with hypotonic KCl (75 mM). Cells were then washed twice with fixative (3 : 1 methanol : glacial acetic acid, v/v), dropped on to slides and air-dried.

Test for cell cycle delay and repeated harvests

In the event a test article caused significant cell-cycle delay, a technique was used for assessing this and, if necessary, performing later harvests on the same culture. After 2–3 h incubation with colcemid, cells were harvested by mitotic shake-off and collected by centrifugation. The supernatant medium was then returned to appropriate flasks which were reincubated at 37 °C. After fixation of harvested cells, test slides were made and stained for 10 min in Hoechst 33258, rinsed in water, and mounted. These slides were examined by fluorescence microscopy to assess the frequency of cells that had completed two cell cycles in BrdUrd. If there was a significant delay, the same culture was harvested repeatedly. Continued colchicine treatment causes contraction of chromosomes. Duplicate cultures were set up, when necessary, to allow a delayed harvest without prolonged colchicine treatment.

Staining and scoring of SCE slides

Staining for detection of SCE was accomplished by a modified fluorescence plus Giemsa (FRG) technique (Goto et al., 1978). Slides were stained for 10 min with Hoechst 33258 (50 μg/ml in phosphate buffer, pH 6.8), mounted in McIlvaine's Buffer and exposed at 55–65 °C to "black-light". Slides were then stained with 5% Giemsa for 5–20

min and air dried. M_2 cells were scored for the frequency of SCE per cell. In addition, the relative frequency of M_1 (first metaphase), M_{1+}, M_2, and M_2+ was estimated for each dose level by scoring 50 cells.

Chromosomal aberration assay

CHO cells were seeded in T-75 cm^2 flasks at a cell density of 1.2×10^6. In assays without the metabolic activation system, the medium was replaced one day after culture initiation and cells treated with test or control compound for 8–10 h. The medium was then removed, cultures washed with PBS, and medium replaced with fresh medium containing colcemid (0.1 μg/ml). After a further 2–3 h of incubation, cells were harvested by mitotic shake-off and fixed.

In tests that included metabolic activation, the medium was replaced one day after culture initiation with fresh medium without serum. Cells were incubated for 2 h in the presence of the test or control compounds and the S9 mix. Cultures were then washed at least twice with PBS and incubation continued for 8–10 h. Colcemid was present for the last 2–3 h of incubation. Cells were harvested and fixed as described for SCE studies.

Slides were stained in 5% Giemsa for 5–10 min. 100 cells were scored per dose. Only metaphases with chromosome number ranges between 19 and 23 were counted. The chromosome number and the incidence of chromatid- or chromosome-type aberrations were recorded. The categories of aberrations scored were: chromatid gaps, chromatid breaks, chromatid fragments, chromosome gaps, triaradials, quadriradials, complex arrangements, acentric fragments, minutes, double minutes, dicentrics, rings, pulverized chromosome(s), chromosome breaks, chromosome intrachanges, translocations, interstitial deletions and uncoiled chromosomes.

Analysis of results

The mean SCE frequency per cell and per chromosome was computed for each dose level. Group means were statistically analyzed by a one-way analysis of variance F-Test. A value was considered significant when $p < 0.01$. For chromosomal aberration assays, a dose-related increase was regarded as positive response. Chromatid/chromosome gaps were excluded when computing dose–response.

Improved methods of data analysis for both SCE and aberrations are being developed at NIEHS/NTP, and a manuscript describing these methods is in preparation (Galloway et al., 1985). Briefly, a decision scheme is used that considers both a trend analysis of the dose–response curve and the magnitude of the response at each dose level. A repeat of each test is required, and the results of two tests are then used to assign a negative, questionable, or positive conclusion.

Results and discussion

The effects of test chemicals on SCE frequencies in CHO cells are presented in Tables 1–10 and are summarized in Table 11. HMPA, TOL and ACN treatments caused approximately 2-fold increases in SCE frequencies over corresponding solvent controls. TOL and ACN treatment also significantly delayed mitotic cycle. For example, in control cultures, about 95% of the cells reached M_2 after 26 h of growth in BrdUrd-containing medium. In flasks treated with 500 μg/ml TOL, cells reached M_2 only after 43 h.

SAF treatment caused a significant increase in SCE frequency at some dose levels and was regarded positive. Results with the remaining test articles, CAP, ZOIN, BEN, DEHP, PB and DES were considered negative. ZOIN and PB were also cytostatic but did not enhance SCE frequency even following delayed harvests.

Subsequent to the meeting of investigators, the new method of SCE analysis was applied to test data from all 10 chemicals. In only 3 cases would the conclusions presented in Table 11 have changed. Based on those analyses, BEN (−S9) and ZOIN (+S9) would be questionable (?) and DEHP (−S9) would be positive (+).

The effects of test chemicals on the incidence of chromosomal aberrations are also presented in Tables 1–10. HMPA, CAP, BEN, ZOIN, SAF and DEHP did not significantly increase the frequency of chromosomal damage. ACN and PB gave weak positive responses. The initial test of SAF for aberrations gave negative results, but in the repeat test, there appeared to be an effect in the presence of S9 at 50 μg/ml. However, a third test (data is

TABLE 1

CYTOGENETIC EFFECTS OF HEXAMETHYLPHOSPHORAMIDE (680-31-9) IN CHO CELLS

Sister-chromatid exchanges				Chromosome aberrations			
− S9		+ S9		− S9		+ S9	
Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)
Solvent [a]	7.8 ± 0.4	Solvent	8.0 ± 0.3	Solvent	0	Solvent	1 (1)
PC [a] (0.01)	36.7 ± 1.03	PC (1.5)	37.1 ± 1.1	PC (0.5)	58 (36)	PC (25)	45 (37)
				16	2 (2)		
50	9.1 ± 0.5	50	8.7 ± 0.4	50	3 (3)	50	3 (3)
160	9.0 ± 0.4	160	9.1 ± 0.4	160	2 (2)	160	2 (2)
500	9.4 ± 0.4	500	8.6 ± 0.4	500	4 (3)	500	1 (1)
1600	9.9 ± 0.5	1600	9.1 ± 0.4	1600	4 (3)	1600	3 (3)
5000	14.1 ± 0.5	5000	10.1 ± 0.4	5000	3 (3)	5000	1 (1)
Solvent	8.0 ± 0.3	Solvent	8.4 ± 0.3	Solvent	1 (1)	Solvent	0 (0)
PC (0.01)	40.4 ± 1.4	PC (1.5)	31.1 ± 0.8	PC (0.5)	54 (39)	PC (50)	79 (40)
1000	9.4 ± 0.4	1000	8.5 ± 0.4	1000	0 (0)	1000	2 (2)
2000	10.2 ± 0.4	2000	8.9 ± 0.4	2000	1 (1)	2000	3 (3)
3000	11.0 ± 0.4	3000	9.0 ± 0.4	3000	1 (1)	3000	1 (1)
4000	12.8 ± 0.6	4000	9.7 ± 0.5	4000	3 (3)	4000	1 (1)
5000	16.4 ± 0.6	5000	9.6 ± 0.3	5000	2 (2)	5000	1 (1)

[a] See Materials and Methods for details on controls.

Conclusions:	− S9	+ S9
SCE	+	−
CA	−	−

not presented) in this dose range revealed no effect. On the basis of nonreproducibility, SAF was considered negative for aberrations.

Results with TOL and DES were particularly interesting. The incidence of chromosomal aberrations in CHO cells exposed to TOL at 50, 160, 500 and 1600 μg/ml for 2 h in the presence of S9 was essentially the same as that of control cells. Since SCE tests showed TOL to be strongly cytostatic, CHO cells were exposed to 1000 and 2000 μg/ml TOL, and cell fixation was delayed by 6 h. The number of aberrations per cell in cultures exposed to 0, 1000 and 2000 mg/ml TOL was 0, 0.5 and 0.75 respectively. These data suggest that TOL is strongly positive, but due to its cytostatic effect, a positive response is obtained only when the cell fixation is delayed.

DES treatment in the presence of S9 at up to 7.5 μg/ml caused no significant increase in aberrations when the interval between the beginning of exposure and fixation was 12.5 h. However, DES treatment at 7.5 μg/ml caused chromosomal damage in 77% of the cells when cell fixation was delayed. To confirm these data, DES was retested at 2.5, 5, 7.5 and 10 μg/ml. Duplicate cultures were set up at the top 3 dose levels. The first set of cultures were harvested twice, 12 and 15 h after the beginning of the treatment. Cells in the second set of cultures were fixed 18 h after the beginning of treatment. Data from this experiment confirmed our earlier finding that DES induces significant chromosomal damage. DES is cytostatic and the interval between the beginning of treatment and fixation may be critical when studying its cytogenetic effects.

TABLE 2

CYTOGENETIC EFFECTS OF *o*-TOLUIDINE (95-53-4) IN CHO CELLS

Sister chromatid exchanges				Chromosome aberrations			
−S9		+S9		−S9		+S9	
Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)
Solvent [a]	9.2 ± 0.5	Solvent	9.3 ± 0.5	Solvent	3 (3)	Solvent	2 (2)
PC [a] (0.1)	39.9 ± 1.4	PC (1.5)	32.6 ± 0.7	PC (0.25)	29 (20)	PC (25)	43 (27)
1.6	10.2 ± 0.5	16	8.7 ± 0.5	16	0 (0)	50	3 (3)
5	10.7 ± 0.5	50	8.9 ± 0.4	50	3 (3)	160	2 (2)
16	10.9 ± 0.5	160	9.4 ± 0.5	160	2 (2)	500	1 (1)
50	12.1 ± 0.6	500	9.3 ± 0.4	500	13 (7)	1600	1 (1)
160	13.8 ± 0.5	1600	11.2 ± 0.5	1600	11 (10)	5000	toxic
500	toxic	5000	toxic	5000	toxic		
Solvent	9.3 ± 0.4	Solvent	8.8 ± 0.5	Solvent	0 (0)	Solvent	0 (0)
PC (0.01)	53.9 ± 1.5	PC (2)	37.4 ± 0.9	PC (0.5)	138 (67)	PC (50)	121 (68)
25	11.0 ± 0.4	500	9.6 ± 0.4	250	0 (0)	250	5 (5)
				500	0 (0)		
50	11.2 ± 0.6	700	10.4 ± 0.5	1000 [b]	5 (4)	500	6 (5)
100	12.5 ± 0.5	1000	10.6 ± 0.4	1500 [b]	10 (10)	1000 [b]	6 (5)
250	15.0 ± 0.6	1500	10.5 ± 0.4	2000 [b]	toxic	2000 [c]	75 (38)
500	17.4 ± 0.8	2000	toxic	1000 [c]	2 (2)	2500 [c]	toxic
				1500 [c]	12 (10)		

[a] See Materials and Methods for details on control.
[b] Harvest delayed 4 h.
[c] Harvest delayed 8 h (−S9) or 6 h (+S9).

Conclusions:	−S9	+S9
SCE	+	+
CA	+	+

TABLE 3

CYTOGENETIC EFFECTS OF BENZENE (71-43-2) IN CHO CELLS

Sister chromatid exchanges				Chromosome aberrations			
− S9		+ S9		− S9		+ S9	
Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)
Solvent [a]	9.0 ± 0.4	Solvent	8.0 ± 0.4	Solvent	2 (2)	Solvent	2 (2)
PC [a] (0.01)	36.5 ± 1.4	PC (1.5)	31.2 ± 0.8	PC (0.25)	22 (20)	PC (25)	33 (25)
16	8.9 ± 0.5	16	8.2 ± 0.4	16	3 (3)	16	1 (1)
50	8.5 ± 0.4	50	8.7 ± 0.4	50	0 (0)	50	1 (1)
160	9.2 ± 0.4	160	8.4 ± 0.4	160	2 (2)	160	1 (1)
500	8.6 ± 0.4	500	8.8 ± 0.4	500	0 (0)	500	1 (1)
1600	toxic	1600	9.2 ± 0.4	1600	2 (2)	1600	3 (3)
		5000	8.9 ± 0.5	5000	toxic	5000	1 (1)
Solvent	8.0 ± 0.3			Solvent	1 (1)	Solvent	0 (0)
PC (0.01)	40.4 ± 1.4			PC (0.5)	54 (39)	PC (50)	79 (40)
100	8.3 ± 0.4			100	1 (1)	500	0 (0)
250	8.4 ± 0.5			500	1 (1)	1000	1 (1)
500	9.3 ± 0.5			1000	2 (2)	2000	4 (4)
750	10.0 ± 0.4			2000	toxic	3000	1 (1)
1000	10.7 ± 0.5					4000	2 (2)
						5000	0 (0)

[a] See Materials and Methods for details on controls.

Conclusions:	− S9	+ S9
SCE	−	−
CA	−	−

TABLE 4

CYTOGENETIC EFFECTS OF SAFROLE (94-59-7) IN CHO CELLS

Sister-chromatid exchanges				Chromosome aberrations			
− S9		+ S9		− S9		+ S9	
Treatment (μg/ml)	SCE/cell ± S.E.	Treatment μg/ml	SCE/cell ± S.E.	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)
Solvent [a]	11.0 ± 0.5	Solvent	9.2 ± 0.5	Solvent	0 (0)	Solvent	3 (3)
PC [a] (0.01)	45.6 ± 1.5	PC (2)	41.6 ± 1.3	PC (0.5)	53 (36)	PC (50)	97 (52)
1.6	11.5 ± 0.6	0.5	11.1 ± 0.5	0.16	1 (1)	0.5	0 (0)
5	12.6 ± 0.5	1.6	11.6 ± 0.5	0.5	0 (0)	1.6	0 (0)
16	11.8 ± 0.5	5	11.5 ± 0.5	1.6	0 (0)	5	0 (0)
25	12.0 ± 0.5	16	10.7 ± 0.5	5	3 (3)	16	3 (3)
50	toxic	25	12.5 ± 0.6	16	3 (3)	50	5 (4)
		50	toxic	50	2 (2)	160	toxic
				160	toxic		
Solvent	7.6 ± 0.3	Solvent	8.4 ± 0.5	Solvent	1 (1)	Solvent	2 (2)
PC (0.01)	41.2 ± 1.4	PC (2)	38.0 ± 1.0	PC (0.5)	154 (73)	PC (50)	54 (39)
10	8.4 ± 0.4	10	8.9 ± 0.3	40	2 (2)	30	2 (2)
30	10.5 ± 0.4	30	9.4 ± 0.5	50	7 (7)	40	4 (4)
50	11.1 ± 0.4	50	9.9 ± 0.4	75	3 (3)	50	12 (8)
		75	10.3 ± 0.4	100	6 (5)	75	toxic

[a] See Materials and Methods for details on controls.

Conclusions:	− S9	+ S9
SCE	+	+
CA	−	−

TABLE 5

CYTOGENETIC EFFECTS OF CAPROLACTAM (105-60-2) IN CHO CELLS

Sister-chromatid exchanges				Chromosome aberrations			
− S9		+ S9		− S9		+ S9	
Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)
Solvent [a]	7.6 ± 0.4	Solvent	8.4 ± 0.4	Solvent	3 (3)	Solvent	0 (0)
PC [a] (0.01)	51.1 ± 1.5	PC (1.5)	24.6 ± 0.7	PC (0.5)	54 (38)	PC (50)	106 (61)
16	8.3 ± 0.4	50	8.1 ± 0.4	50	2 (2)	16	1 (1)
50	8.6 ± 0.4	160	8.7 ± 0.4	160	3 (3)	50	1 (1)
160	8.5 ± 0.4	500	8.4 ± 0.4	500	0 (0)	160	0 (0)
500	8.4 ± 0.4	1600	8.5 ± 0.5	1600	2 (2)	500	0 (0)
1600	8.3 ± 0.4	5000	8.4 ± 0.3	5000	2 (2)	1600	0 (0)
5000	toxic					5000	1 (1)
Solvent	8.2 ± 0.5	Solvent	8.1 ± 0.4	Solvent	0 (0)	Solvent	2 (2)
PC (0.01)	47.3 ± 1.4	PC (2)	38.6 ± 1.0	PC (0.29)	53 (38)	PC (25)	49 (32)
1000	8.5 ± 0.4	2000	8.5 ± 0.4	2000	4 (4)	2000	0 (0)
2000	9.7 ± 0.5	3000	8.7 ± 0.5	3000	4 (4)	3000	0 (0)
3000 [b]	9.3 ± 0.4	4000	8.3 ± 0.5	4000	1 (1)	4000	1 (1)
3000 [c]	8.3 ± 0.4	5000	8.6 ± 0.3	5000	1 (1)	5000	3 (3)

[a] See Materials and Methods for details on controls.
[b] Harvest delayed 8 h.
[c] Harvest delayed 24 h.

Conclusions:	− S9	+ S9
SCE	−	−
CA	−	−

TABLE 6

CYTOGENETIC EFFECTS OF ACRYLONITRILE (107-13-1) IN CHO CELLS

Sister-chromatid exchanges				Chromosome aberrations			
− S9		+ S9		− S9		+ S9	
Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)
Solvent [a]	8.3 ± 0.4	Solvent	8.4 ± 0.4	Solvent	4 (4)	Solvent	(0)
PC [a] (0.01)	46.6 ± 1.2	PC (1.5)	27.2 ± 0.8	PC (0.5)	99 (63)	PC (50)	74 (51)
0.16	7.9 ± 0.5	1.6	8.5 ± 0.5	5	1 (1)	1	0 (0)
0.5	8.2 ± 0.4	5	8.3 ± 0.5	10	1 (1)	5	0 (0)
1.6	8.4 ± 0.4	16	8.2 ± 0.4	25	3 (2)	10	3 (3)
5.0	9.0 ± 0.5	50	10.9 ± 0.4	50	2 (2)	50	2 (2)
16.0	10.1 ± 0.5	160	toxic	100	toxic	100	6 (6)
50	toxic						
Solvent	8.2 ± 0.5	Solvent	8.1 ± 0.4	Solvent	0 (0)	Solvent	2 (2)
PC (0.01)	47.3 ± 1.4	PC (2)	38.6 ± 1.0	PC (0.25)	53 (38)	PC (25)	49 (32)
10 [b]	10.7 ± 0.6	10 [b]	8.8 ± 0.5	5	2 (2)	25	4 (4)
20 [b]	12.4 ± 0.7	25 [b]	10.3 ± 0.4	10	2 (2)	50	5 (4)
20 [c]	13.7 ± 0.6	50 [b]	12.4 ± 0.5	25	1 (1)	75	5 (5)
30 [c]	19.6 ± 0.8	75 [b]	17.0 ± 0.7	50	1 (1)	100	13 (11)
				75	toxic	150	toxic

[a] See Materials and Methods for details on controls.
[b] Harvest delayed by 2 h.
[c] Harvest delayed by 12 h.

Conclusions:	− S9	+ S9
SCE	+	+
CA	−	wk +

TABLE 7

CYTOGENETIC EFFECTS OF DIETHYLHEXYLPHTHALATE (117-81-7) IN CHO CELLS

Sister-chromatid exchanges				Chromosome aberrations			
− S9		+ S9		− S9		+ S9	
Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)
Solvent [a]	7.7 ± 0.5	Solvent	7.2 ± 0.4	Solvent	0 (0)	Solvent	0 (0)
PC [a] (0.01)	45.3 ± 1.5	PC (1.5)	26.0 ± 0.9	PC (0.5)	99 (63)	PC (50)	74 (51)
5	7.9 ± 0.4	160	8.2 ± 0.4	50	0 (0)	50	0 (0)
16 [b]	7.7 ± 0.4	500	7.8 ± 0.4	160	0 (0)	160	0 (0)
50 [b]	8.6 ± 0.5	1600	8.2 ± 0.4	500	0 (0)	500	0 (0)
160 [b]	9.1 ± 0.4	5000	8.0 ± 0.4	1600	0 (0)	1600	0 (0)
500 [b]	9.2 ± 0.4			5000	0 (0)	5000	0 (0)
1600	Cytostatic						
Solvent	7.9 ± 0.4	Solvent	7.2 ± 0.4	Solvent	2 (2)	Solvent	3 (3)
PC (0.005)	35.7 ± 0.6	PC (2)	36.7 ± 1.2	PC (0.5)	76 (48)	PC (50)	74 (52)
3000 [c]	9.2 ± 0.4	3000	8.2 ± 0.4	2000	3 (3)	2000	4 (4)
4000 [c]	9.6 ± 0.4	4000	8.2 ± 0.4	3000	1 (1)	3000	4 (3)
5000 [c]	10.5 ± 0.6	5000	8.5 ± 0.5	4000	1 (1)	4000	2 (2)
				5000	1 (1)	5000	2 (2)

[a] See materials and Methods for details on controls.
[b] Harvest delayed by 8 h.
[c] Harvest delayed by 14 h.
[d] Positive by subsequent statistical analysis.

Conclusions:	− S9	+ S9
SCE	− [d]	−
CA	−	−

TABLE 8

CYTOGENETIC EFFECTS OF BENZOIN (119-53-9) IN CHO CELLS

Sister-chromatid exchanges				Chromosome aberrations			
−S9		+S9		−S9		+S9	
Treatment (μg/ml)	SCE/cell ±S.E.	Treatment (μg/ml)	SCE/cell ±S.E.	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)
Solvent [a]	8.1 ± 0.5	Solvent	8.3 ± 0.4	Solvent	0 (0)	Solvent	0 (0)
PC [a] (0.01)	46.0 ± 1.4	PC (1.5)	22.4 ± 0.7	PC (0.5)	62 (37)	PC (25)	47 (31)
5	7.8 ± 0.4	160	7.9 ± 0.4	50	3 (3)	100	1 (1)
16	8.8 ± 0.5	500	8.8 ± 0.4	160	3 (3)	500	0 (0)
50	7.6 ± 0.3	1600	9.4 ± 0.4	300	2 (2)	1000	2 (2)
160	8.7 ± 0.4	2000	8.7 ± 0.4	400	1 (1)	1500	0 (0)
500	Cytostatic			500	7 (5)	2000	2 (2)
Solvent	7.9 ± 0.4	Solvent	7.6 ± 0.4	Solvent	1 (1)	Solvent	0 (0)
PC (0.005)	35.7 ± 0.6	PC (1.5)	26.1 ± 0.9	PC (0.25)	35 (32)	PC (50)	63 (41)
100 [b]	9.7 ± 0.5	200	8.2 ± 0.5	100	2 (2)	500	1 (1)
200 [b]	9.3 ± 0.4	500	9.2 ± 0.4	200	2 (2)	1000	1 (1)
300 [b]	10.7 ± 0.4	1000	10.4 ± 0.5	300	0 (0)	1500	2 (2)
400 [b]	10.8 ± 0.5	1500	10.3 ± 0.4	400	3 (3)	2000	1 (1)
500 [b]	10.4 ± 0.4	2000	Toxic	500	5 (5)		

[a] See Materials and Methods for details on controls.

[b] Harvest delayed 20 h.

Conclusions:	−S9	+S9
SCE	−	−
CA	−	−

TABLE 9

CYTOGENETIC EFFECTS OF DIETHYLSTILBESTROL (56-53-1) IN CHO CELLS

Sister-chromatid exchanges				Chromosome aberrations			
−S9		+S9		−S9		+S9	
Treatment (μg/ml)	SCE/cell ±S.E.	Treatment (μg/ml)	SCE/cell ±S.E.	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)
Solvent [a]	7.8 ± 0.4	Solvent	8.7 ± 0.4	Solvent	0 (0)	Solvent	0 (0)
PC [a] (0.01)	45.1 ± 1.3	PC (1.5)	27.5 ± 0.9	PC (0.5)	62 (37)	PC (25)	47 (31)
0.16	8.3 ± 0.4	0.16	7.5 ± 0.3	1.0	4 (4)	0.5	2 (1)
0.5	8.4 ± 0.4	0.5	7.8 ± 0.4	2.5	3 (2)	1.0	0 (0)
1.6	7.7 ± 0.4	1.6	8.6 ± 0.5	5.0	8 (7)	2.5	0 (0)
5	Cytostatic	5	8.4 ± 0.5	7.5	8 (6)	5.0	2 (2)
				10	2 (2)	7.5	85 (77) [b]
				15	0 (0)	10.0	53 (48) [b]
Solvent	8.6 ± 0.5	Solvent	8.1 ± 0.4	Solvent	1 (1)	Solvent	0 (0)
PC (0.01)	38.6 ± 1.1	PC (1.5)	22.7 ± 0.9	PC (0.25)	35 (32)	PC (50)	39 (30)
1	8.5 ± 0.4	0.5	7.8 ± 0.5	5	1 (1)	2.5	0 (0)
2.5	8.8 ± 0.5	1.0	7.1 ± 0.4	7.5	2 (2)	5	4 (4)
5	8.6 ± 0.6	2.5	7.7 ± 0.3	10	1 (1)	5 [c]	2 (1)
10	No M_2 cells	5	8.7 ± 0.4	15	2 (2)	7.5 [d]	30 (18)
	even after			20	4 (4)	7.5 [c]	148 (49)
	16 h delay					10 [d]	toxic

[a] See Materials and Methods for details on controls.
[b] Most aberrations on X-chromosomes.
[c] Harvest delayed by 6 h.
[d] Harvest delayed by 3 h.

Conclusions:	−S9	+S9
SCE	−	−
CA	−	+

TABLE 10

CYTOGENETIC EFFECTS OF PHENOBARBITAL (50-06-6) IN CHO CELLS

Sister-chromatid exchanges				Chromosome aberrations			
− S9		+ S9		− S9		+ S9	
Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	SCE/cell ± S.E.	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)	Treatment (μg/ml)	Abs./100 cells (% cells w/abs.)
Solvent [a]	8.3 ± 0.4	Solvent	7.7 ± 0.4	Solvent	0 (0)	Solvent	0 (0)
PC [a] (0.01)	38.7 ± 1.2	PC (1.5)	21.9 ± 0.7	PC (0.5)	62 (37)	PC (25)	47 (31)
16	8.2 ± 0.5	16	7.6 ± 0.4	160	1 (1)	50	3 (3)
50	9.4 ± 0.5	50	7.3 ± 0.3	500	1 (1)	160	3 (3)
160	8.6 ± 0.4	160	7.8 ± 0.3	1600	4 (3)	500	7 (7)
500	10.0 ± 0.5	500	8.3 ± 0.4	2000	5 (4)	1600	5 (5)
1600	Cytostatic	1600	9.0 ± 0.4			2000	5 (5)
		5000	toxic				
Solvent	7.9 ± 0.4	Solvent	8.1 ± 0.4	Solvent	1 (1)	Solvent	2 (2)
PC (0.005)	35.7 ± 0.6	PC (1.5)	24.9 ± 0.9	PC (0.5)	81 (49)	PC (50)	58 (39)
250	9.9 ± 0.5	1000	8.1 ± 0.4	250	3 (2)	500	2 (2)
500	8.7 ± 0.4	2000	8.4 ± 0.4	500	2 (2)	1000	1 (1)
1000	Cytostatic	3000	9.0 ± 0.4	1000	5 (5)	2000	4 (4)
		4000	8.6 ± 0.4	2000	0 (0)	3000	7 (5)
				3000	0 (0)	4000	toxic

[a] See Materials and Methods for details on controls.

Conclusions:	− S9	+ S9
SCE	−	−
CA	wk +	wk +

TABLE 11

SUMMARY OF CHO CYTOGENETIC RESULTS

Chemical	SCE		CA	
	− S9	+ S9	− S9	+ S9
HMPA	+	−	−	−
TOL	+	+	+	+
SAF	+	+	−	−
BEN	−	−	−	−
CAP	−	−	−	−
ACN	+	+	−	+
DEHP	− [a]	−	−	−
ZOIN	−	−	−	−
DES	−	−	−	+
PB	−	−	+	+

[a] Positive by subsequent statistical analysis.

The overall conclusions for each chemical are illustrated at the bottom of each table. In summary, both noncarcinogens were negative in SCE as well as chromosomal aberration assays. HMPA, TOL, ACN and SAF were positive in the SCE studies and TOL, DES and PB in chromosomal aberration assays. Considering the goals of the IPCS/CSSTT program 6 of the 8 carcinogens were detected in one or both endpoints when tested according to the protocol described in this article. The present results suggest that the test system and protocol performed well and may prove valuable as a complement to the Salmonella/microsome assay.

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The in vitro chromosomal aberration test using Chinese hamster lung (CHL) fibroblast cells in culture

Motoi Ishidate Jr. and T. Sofuni

Division of Mutagenesis, Biological Safety Research Centre, National Institute of Hygienic Sciences, Tokyo (Japan)

Summary

In the present chromosomal aberration test using Chinese hamster cells in culture, 6 carcinogens (hexamethylphosphoramide, *o*-toluidine, benzene, safrole, acrylonitrile and diethylstilbestrol) and 2 non-carcinogens (caprolactam and benzoin) induced structural or numerical chromosome aberrations with or without metabolic activation, while 2 carcinogens (diethylhexylphthalate and phenobarbital) did not significantly induce any chromosome aberrations.

As a primary screening tool for environmental mutagens and/or carcinogens, chromosomal aberration tests in vitro have been carried out on more than 500 compounds at our laboratory for the past 10 years, and the results on these chemicals were recently summarized (Ishidate, 1983). The cell line used was a clonal subline of Chinese hamster fibroblasts, CHL, which was originally established from the lung of a new-born female by Koyama et al. (1970). In the present experiment, the chromosomal aberration tests have been carried out, using this cell line, on 10 chemicals supplied for the CSSTT, in the system with or without a metabolic activation by rat-liver S9.

Materials and methods

A Chinese hamster lung fibroblast cell line (CHL) (Koyama et al., 1970) was used for this study. The cells were cultured with Eagle's MEM (Gibco) supplemented with 10% heat-inactivated calf serum in a CO_2 incubator at 37°C. The doubling time was estimated to be about 15 h, and the modal chromosome number was 25. This cell line is commercially available in Japan.

As a preliminary test, the cells were exposed to the test chemical at different doses for 48 h, fixed with formaldehyde, and stained with crystal violet. The dose of the test chemical giving approximately 50% cell growth inhibition was estimated by measurement of relative cell density of each plate using a photodensitometer desinged for measurement of color absorption (Olympus, Monocellater).

For the chromosomal aberration test, the cells were treaated for 24 and 48 h with the test chemical at a minimum of 3 doses, including the 50% cell growth inhibition dose (Ishidate and Odashima, 1977). The cells were harvested after 2 h of treatment with colcemid (0.2 μg/ml), and chromosome preparations were made by an air-drying method. For the system of metabolic activation of the test chemicals, the cells were exposed to the test chemical together with the S9 mix prepared from the liver of Fischer rats pretreated with 500 mg/kg of PCB (KC-400). The S9 mix consisted of 3 ml of the S9 fraction, 2 ml of 20 mM Hepes buffer (pH 7.2), 1 ml of 50 mM $MgCl_2$, 1 ml of 330 mM KCl, 1 ml of 50 mM G-6-P, 1 ml of 40 mM NADP and 1 ml of distilled water (Matsuoka et al., 1979). The final concentration of the S9 fraction was adjusted at 5% in each plate. After 6 h of treatment with S9 mix, the reaction mixture was replaced by a fresh medium and the cells were cultured for 18 h. Colcemid treatment and chro-

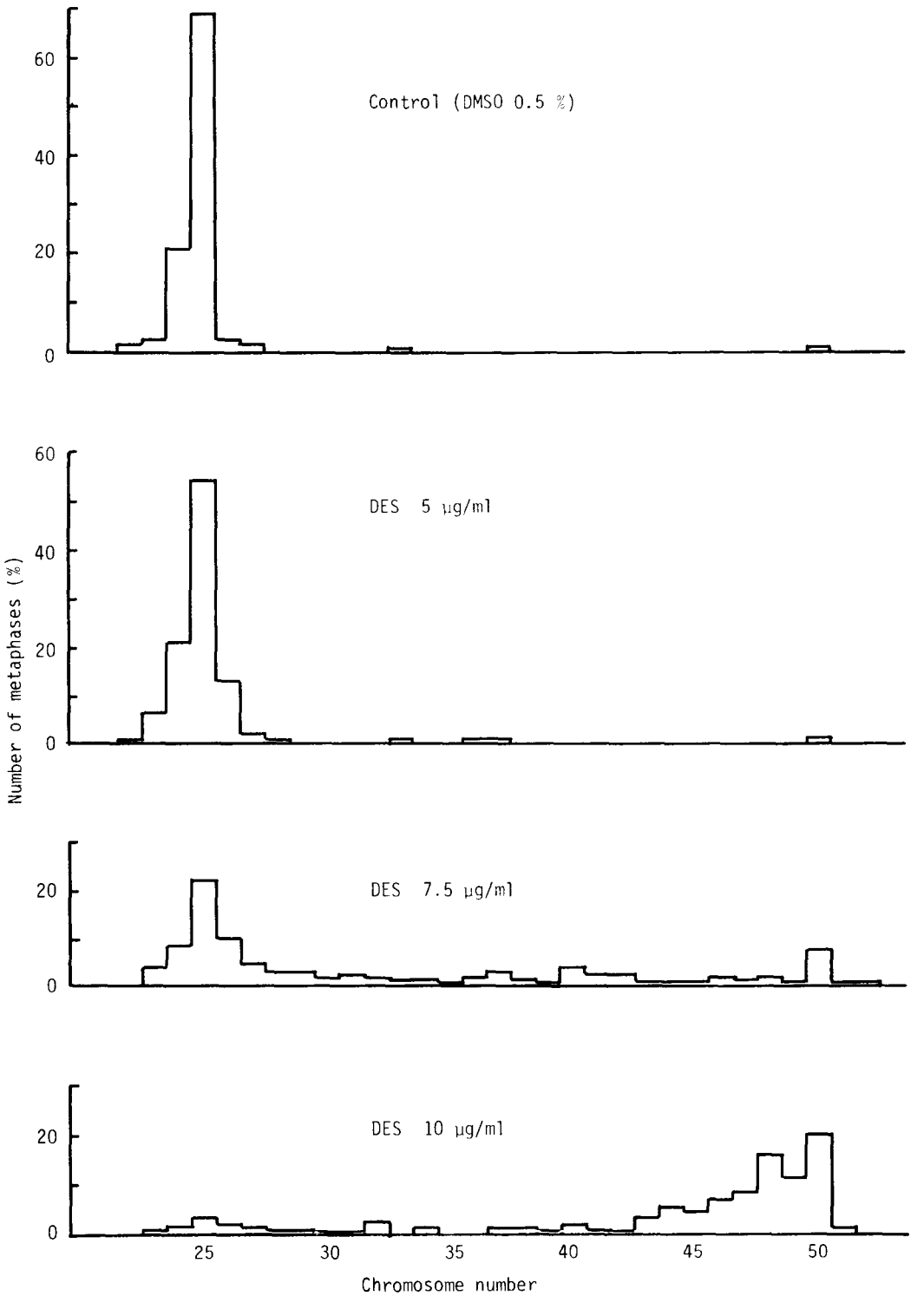

Fig. 1. Diagram of chromosome number distribution in cultured Chinese hamster lung (CHL) cells treated with diethylstilbestrol (DES) for 48 h.

mosome preparation were made as in the conventional method (without metabolic activation).

As positive controls, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or mitomycin C (MMC), and benzo[*a*]pyrene (BP) or dimethylnitrosamine (DMN) were used for the conventional and metabolic activation methods, respectively. Physiological saline and DMSO were used as solvent, and solvent-treated cells were used as negative controls in both methods.

Two plates were used at each dose level, and 100 well-spread metaphases were examined in each plate. Frequencies of cells with structural chromosome aberrations, aberrations per cell, and polyploid cells were recorded. A response was considered positive when more than 10% of cells had structural aberrations (including gaps) or more than 0.1 aberrations per cell. If the frequency of polyploid cells exceeded 10%, positive result was also given.

Results and discussion

Of 10 test compounds under study, hexamethylphosphoramide (HMPA), acrylonitrile (ACN), diethylstilbestrol (DES) and phenobarbital (PB), were examined on their clastogenic potential without metabolic activation; the results for these compounds are summarized in Table 1. HMPA and ACN induced structural chromosome aberrations, while DES and PB did not show any significant increase of structural chromosome aberrations. Minimum effective doses were remarkably different between two positive compounds, that is, 12.5 μg/ml in ACN and 6000 μg/ml in HMPA. Frequencies of aberrations induced by HMPA were

TABLE 1

FREQUENCIES OF STRUCTURAL CHROMOSOME ABERRATIONS IN A CHINESE HAMSTER CELL LINE (CHL) TREATED WITH 4 TEST CHEMICALS WITHOUT METABOLIC ACTIVATION

Compound	Solvent	Dose (μg/ml)	Percent of cells with aberrations				Aberrations per cell				Overall result
			24 h		48 h		24 h		48 h		
			−gaps	+gaps	−gaps	+gaps	−gaps	+gaps	−gaps	+gaps	
Hexamethyl-phosphoramide	Saline	2000	2.5	6.0	0.5	2.5	0.025	0.07	0.01	0.03	±
		4000	2.0	4.0	3.0	7.0	0.02	0.05	0.03	0.075	±
		6000	4.5	11.5	5.5	10.5	0.045	0.135	0.08	0.155	+
		8000	32.4	42.0	35.5	42.5	0.625	0.915	0.67	0.855	+
Acrylonitrile	Saline	3.13	0.5	0.5	0.5	0.5	0.005	0.005	0.005	0.005	−
		6.25	2.0	3.0	2.5	5.5	0.02	0.035	0.025	0.055	±
		12.5	19.0	26.5	30.5	36.5	0.245	0.375	0.6	0.76	+
		25.0	---------- Tox ----------				---------- Tox ----------				
Diethylstil-bestrol	DMSO	2.5	1.5	2.0	0.0	0.0	0.035	0.04	0.0	0.0	−
		5.0	0.0	1.0	1.0	1.5	0.0	0.01	0.01	0.02	−
		7.5	1.5	2.5	0.5	0.5	0.015	0.025	0.005	0.005	−
		10.0	2.0	4.0	3.5	4.0	0.025	0.045	0.035	0.04	−
Phenobarbital	DMSO	250	2.0	2.5	0.5	0.5	0.02	0.025	0.005	0.005	−
		500	0.5	1.0	0.5	1.0	0.005	0.01	0.005	0.01	−
		1000	1.0	2.0	0.0	0.5	0.01	0.02	0.0	0.005	−
		2000	0.5	2.5	2.5	4.0	0.005	0.03	0.04	0.07	±
MNNG [a]	Saline	2.5	40.5	46.8	39.5	43.0	0.747	0.842	0.585	0.66	+
Mitomycin C	Saline	0.05	25.5	26.5	56.9	60.4	0.35	0.41	1.168	1.294	+
–	Saline	–	0.5	1.3	0.3	1.5	0.005	0.013	0.003	0.015	
–	DMSO	–	0.5	1.0	0.5	0.5	0.005	0.01	0.01	0.01	

[a] MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

almost identical in both 24- and 48-h treatments. In ACN, on the other hand, the frequency of aberrations was higher in the 48-h treatment than in the 24-h treatment.

Conspicuous induction of polyploid cells was recognized in the 48-h treatment of DES (Table 3), although the other 3 compounds did not significantly induce polyploid cells. The distribution of chromosome number in cells treated by DES, presented in Fig. 1, indicates a dose-dependent increase of polyploid cells. This finding supports our previous study (Sawada and Ishidate, 1978).

Results on 6 test compounds examined by the metabolic activation method are shown in Table 2. All 5 compounds, excluding diethylhexylphthalate (DEHP), induced structural chromosome aberrations in the presence of S9 mix. Minimum effective doses were different among the compounds tested: 20 μg/ml in benzoin (ZOIN), 175 μg/ml in safrole (SAF), 550 μg/ml in benzene (BEN), 1000 μg/ml in *o*-toluidine (TOL) and 12000 μg/ml in caprolactam (CAP). TOL and CAP also induced polyploid cells with relatively low frequencies. The former was positive only in the absence of S9 mix

TABLE 2

FREQUENCIES OF STRUCTURAL CHROMOSOME ABERRATIONS IN A CHINESE HAMSTER CELL LINE (CHL) TREATED WITH 6 TEST CHEMICALS WITH METABOLIC ACTIVATION

Compound	Solvent	Dose (μg/ml)	Percent of cells with aberrations				Aberrations per cell				Overall result
			−S9		+S9		−S9		+S9		
			−gaps	+gaps	−gaps	+gaps	−gaps	+gaps	−gaps	+gaps	
o-Toluidine	DMSO	1000	0.5	2.0	6.0	8.5	0.005	0.02	0.095	0.125	+
		1250	3.0	3.5	15.5	17.5	0.03	0.04	0.17	0.215	+
		1500	1.5	1.5	26.5	35.5	0.015	0.015	0.35	0.525	+
Benzene	DMSO	550	0.5	2.0	7.0	8.5	0.005	0.02	0.09	0.11	+
		1100	1.0	3.5	14.0	17.5	0.01	0.035	0.25	0.31	+
		2200	1.5	2.5	15.0	17.5	0.015	0.025	0.305	0.355	+
		4400	1.5	2.0	21.5	22.5	0.015	0.02	0.465	0.525	+
Safrole	DMSO	75	1.5	4.5			0.02	0.05			±
		100	2.5	4.5			0.025	0.06			±
		125	2.5	4.0	4.0	6.5	0.025	0.04	0.04	0.065	±
		150	2.0	4.0	5.5	8.5	0.02	0.04	0.06	0.095	±
		175	− − Tox − −		12.5	16.0	− − Tox − −		0.21	0.265	+
		200	− − Tox		31.5	36.0	− − Tox − −		0.685	0.805	+
Caprolactam	Saline	6000	2.0	3.5	1.0	2.0	0.02	0.04	0.01	0.02	−
		8000	1.0	3.5	2.0	4.0	0.02	0.05	0.035	0.06	±
		10000	5.0	7.5	4.0	4.5	0.07	0.095	0.07	0.085	±
		12000	− − Tox − −		12.3	15.1	− − Tox − −		0.253	0.329	+
Diethylhexyl-phthalate	DMSO	1375	0.0	1.0	0.0	1.0	0.0	0.01	0.0	0.01	−
		2750	1.0	1.5	1.0	1.0	0.01	0.015	0.01	0.01	−
		4130	1.5	2.5	1.5	2.5	0.015	0.025	0.015	0.025	−
Benzoin	DMSO	10	0.5	3.0	0.0	2.5	0.005	0.035	0.0	0.025	−
		20	1.0	4.5	12.5	14.0	0.01	0.045	0.285	0.335	+
		40	0.0	4.0	28.0	28.5	0.0	0.04	1.04	1.12	+
Benzo[*a*]pyrene	DMSO	20			49.0	50.0			1.078	1.15	+
DMN [a]	Saline	500			57.5	61.5			1.085	1.21	+
−	Saline	−	1.0	1.0	1.0	1.5	0.01	0.01	0.02	0.025	
−	DMSO	−	0.8	1.9	1.2	2.9	0.009	0.02	0.013	0.032	

[a] DMN, dimethylnitrosamine.

TABLE 3

FREQUENCIES (PERCENT) OF POLYPLOID CELLS IN A CHINESE HAMSTER CELL LINE (CHL) TREATED WITH 10 TEST CHEMICALS WITH AND WITHOUT METABOLIC ACTIVATION

Compound	Solvent	Dose (μg/ml)	Without metabolic activation		With metabolic activation		Overall result
			24 h	48 h	−S9	+S9	
Hexamethyl-phosphoramide	Saline	2000	0.5	0.5			−
		4000	0.0	1.0			−
		6000	0.5	0.5			−
		8000	1.7	0.0			−
o-Toluidine	DMSO	1000			10.5	0.5	+
		1250			20.5	0.0	+
		1500			9.0	0.5	±
Benzene	DMSO	550			1.0	0.5	−
		1100			4.0	1.0	−
		2200			3.0	0.0	−
		4400			4.5	1.5	−
Safrole	DMSO	75			1.0		−
		100			0.5		−
		125			2.5	2.0	−
		150			3.0	1.5	−
		175				1.0	−
		200				0.0	−
Caprolactam	Saline	6000			13.5	4.0	+
		8000			10.5	3.5	+
		10000			7.5	2.5	±
		12000				11.0	+
Acrylonitrile	Saline	3.13	0.0	0.0			−
		6.25	1.0	0.5			−
		12.5	0.5	3.5			−
Diethylhexyl-phthalate	DMSO	1375			0.0	0.0	−
		2750			1.0	0.0	−
		4130			0.5	0.0	−
Benzoin	DMSO	10			0.5	2.0	−
		20			0.5	5.5	±
		40			1.0	3.0	−
Diethylstil-bestrol	DMSO	2.5	0.0	1.5			−
		5.0	0.0	1.0			−
		7.5	2.0	33.0			+
		10.0	4.5	84.5			+
Phenobarbital	DMSO	250	0.0	4.0			−
		500	0.0	2.0			−
		1000	0.5	4.5			−
		2000	0.0	0.0			−
MNNG [a]	Saline	2.5	2.5	0.5			−
Mitomycin C	Saline	0.05	0.0	0.5			−
Benzo[*a*]pyrene	DMSO	20				2.0	−
DMN [b]	Saline	500				0.5	−
–	Saline	–	1.3	0.3	0.5	1.0	
–	DMSO	–	0.5	1.0	0.8	0.9	

[a] MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

[b] DMN, dimethylnitrosamine.

and the latter was positive both with and without S9 mix, although there was no dose–response relationship (Table 3). DEHP, on the other hand, showed negative results on structural and numerical chromosome aberrations.

The majority of chromosome aberrations induced by the 7 test compounds studied here, were chromatid-type aberrations including gaps, breaks, and intra- and inter-exchanges. There was no significant increase of chromosome-type aberrations, such as dicentric and ring chromosomes. Among chromatid-type aberrations, chromatid breaks and exchanges were preponderant, while chromatid gaps were less frequent. Further, there was tendency for the frequency of chromatid exchanges to be higher than that of chromatid breaks, with two exceptions. In HMPA and ZOIN, chromatid breaks were more predominant than chromatid exchanges, and this tendency was extremely remarkable in ZOIN.

Of 8 carcinogens studied here, 5 (HMPA, TOL, BEN, SAF and ACN) induced structural aberrations with or without metabolic activation; 1 (DES) induced polyploid cells without metabolic activation. The remaining 2 carcinogens (DEHP and PB) were negative in the present study. DEHP did not show any cytotoxicity at even the maximum dose used here, and this finding may relate to the insolubility of this chemical. The previous report from our laboratory (Ishidate et al., 1981) indicated that PB induced polyploid cells with a relatively low frequency (13% at 2000 μg/ml). However, such a tendency was not noted in the present specimen.

2 noncarcinogens, CAP and ZOIN, were positive in the present study. Clastogenic potential of ZOIN was relatively strong, since the minimum effective dose was 20 μg/ml. However, the majority of aberrations induced by ZOIN were chromatid breaks, and chromatid exchanges were less frequent. CAP showed only one effective dose that was extremely high, that is, 12000 μg/ml, without a dose–response relationship. Therefore, clastogenic potential of CAP may be extremely low.

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Tests for chromosome aberrations and sister-chromatid exchanges in Chinese hamster ovary (CHO) cells in culture

A.T. Natarajan [1,2], C.J.M. Bussmann [1], A.C. van Kesteren-van Leeuwen [1], M. Meijers [1,2] and J.L.S. van Rijn [1]

[1] *Department of Radiation Genetics and Chemical Mutagenesis, State University of Leiden, Sylvius Laboratories, Leiden (The Netherlands) and* [2] *J.A. Cohen Institute, Interuniversity Institute of Radiopathology and Radiation Protection, Leiden (The Netherlands)*

Summary

o-Toluidine, benzene, caprolactam, acrylonitrile (ACN) and phenobarbitone (PHE) were tested for their ability to induce chromosomal aberrations and sister-chromatid exchanges (SCEs), with and without metabolic activation. ACN and PHE were positive in inducing chromosomal aberrations and SCEs. The other 3 chemicals were negative.

Under the collaborative study sponsored by the World Health Organization, we tested 10 established or suspected human carcinogens for their ability to induce chromosomal aberrations and sister-chromatid exchanges (SCEs) in Chinese hamster ovary cells (CHO). In the first series of experiments, a routine protocol used, namely, treatment of cells following one cycle in a medium containing 5-bromodeoxyuridine (BrdUrd); subsequent analysis after the second cell cycle for both

TABLE 1

CHROMOSOMAL ABERRATIONS INDUCED BY *o*-TOLUIDINE

Test compound	Concentration (mM)	Cells scored	Percent of normal cells	Aberrations/100 cells						
				Chromatid aberrations			Chromosome aberrations		Total	Multiple aberrations
				Gaps	Breaks	Exch.	Breaks	Exch.		
TOL +S9	5	300	90.7	8	2	0	0.33	0	10.33	0
	10	300	89.3	6.33	4.67	0	1.33	0.33	12.67	0
	20	300	87.7	11.67	1	1	0.67	0.33	14.67	0
CP+S9	3	300	35.0	40.0	111.33	33.67	38.0	1	224.33	0.33
Control +S9	–	300	91.0	7.67	1.33	0.33	0.33	–	9.67	0
TOL −S9	5	300	89.0	9.0	3.67	0	1.0	0	13.67	0
	10	300	90.7	7.33	3.67	0.67	0.33	0	12.0	0
	20	300	89.7	7.67	1.67	1.67	1.0	0	12.0	0
MMS	1.8	300	60.3	18.33	15.67	30.67	4.0	0.33	70.33	1.33
Control	–	300	91.7	8.0	1.67	0	0.33	0	10.0	0

TABLE 2

CHROMOSOMAL ABERRATIONS INDUCED BY BENZENE

Test compound	Concentration (mM)	Cells scored	Percent of normal cells	Aberrations/100 cells						
				Chromatid aberrations			Chromosome Aberrations		Total	Multiple aberrations
				Gaps	Breaks	Exch.	Breaks	Exch.		
BE +S9	3	300	84.3	8	13.3	0	2.33	0.33	26.0	0
	6	300	86.7	7.7	8.0	0.33	0.67	0	16.7	0
	12	300	87.3	6.7	8.0	0.33	0.67	0.33	16.03	0
Control +S9	–	300	89.3	7.0	5.3	0	0.33	0	12.63	0
BE −S9	3	300	85.7	9.0	2.3	0.33	2.33	0.67	16.3	0
	6	300	87.3	7.0	6.3	0	2.0	0	15.3	
	12	300	89.3	7.3	5.7	0.67	1.0	0	14.7	0
Control		300	86.0	6.7	8.3	0.33	1.33	0.33	17.0	0

Positive controls are the same as *o*-toluidine.

chromosomal aberrations and SCEs was employed. Based on these preliminary results, 5 chemicals were chosen for testing using conventional protocol, that is, treating exponentially-growing cells and scoring for aberrations after one cell cycle and scoring for SCEs after two cycles in BrdUrd. Carcinogens such as safrole, hexamethylphosphoramide were not included in this group, because extensive data had already been accumulated in our laboratory using these two compounds. The compounds included in this study are: *o*-toluidine (TOL), benzene (BEN), caprolactam (CAP), acrylonitrile (ACN) and phenobarbital (PB).

Material and methods

CHO cells were grown in Ham's F10 medium supplemented with 15% newborn calf serum and antibiotics. The test chemicals were dissolved in

TABLE 3

CHROMOSOMAL ABERRATIONS INDUCED BY ACRYLONITRILE

Test compound	Concentration (mM)	Cells scored	Percent of normal cells	Aberrations/100 cells						
				Chromatid aberrations			Chromosome aberrations		Total	Multiple aberrations
				Gaps	Breaks	Exch.	Breaks	Exch.		
ACN +S9	1	300	81.3	9.67	19.0	3.0	10.0	1.33	43.3	0.33
	2	300	85.3	10.67	7.0	1.0	3.0	2.0	24.0	0.33
	4	600	36.2	35.83	120.67	24.67	15.67	2.33	206.5	7.33
Control +S9	–	600	87.3	11.5	5.17	0	1.17	0.17	19.67	0
ACN −S9	1	400	85.0	12.5	10.25	2.25	4.5	0	29.5	0
	2	400	78.8	11.5	20.25	0.75	4.25	1.25	38.0	0
	4	600	51.7	27.17	84.33	10.17	12.33	0.83	137.8	3.0
Control		600	89.2	7.5	3.33	0	0.67	0	11.5	0

Positive controls are the same as *o*-toluidine.

TABLE 4

CHROMOSOMAL ABERRATIONS INDUCED BY CAPROLACTAM

Test compound	Concentration (mM)	Cells scored	Percent of normal cells	Aberrations/100 cells						
				Chromatid aberrations			Chromosome aberrations		Total	Multiple aberrations
				Gaps	Breaks	Exch.	Breaks	Exch.		
CL +S9	37.5	300	89.0	6.67	3.33	0.67	3	0	13.67	0
	75.0	300	88.0	5.33	5.33	0	0.67	0	11.33	0
	150	300	85.7	4.67	6.33	0.33	1.67	1	14.0	0
Control +S9		300	91.7	6.33	4.0	0	0.33	0.33	11.0	0
CL −S9	37.5	400	85.5	6.5	4.75	0.25	2	0.25	13.75	0
	75	300	90.7	2.67	2	0.33	4	0.33	9.33	0
	150	400	86.5	6.25	5.75	0	1.75	0.5	14.25	0
Control		300	90.3	7.67	2.67	0.33	0	0.33	11.0	0

Positive controls are the same as *o*-toluidine.

DMSO for treatment. Exponentially-growing cells were treated for 1 h at 37 °C with different concentrations of the test chemical in the presence or absence of S9 mixture (for details see Natarajan and van Kesteren-van Leeuwen, 1981). For studying chromosomal aberrations, cells were fixed at 3 different times following treatment from 13 to 19 h. For scoring of SCEs, the treated cells were grown in a medium containing 5 μM BrdUrd for two cycles and fixed between 25 and 36 h following treatment. From each fixation time 100 cells were scored for chromosomal aberrations and 25 cells were scored for determination of frequencies of SCEs. Cyclophosphamide (CP) was used as positive control as a chemical needing metabolic activation; methyl methanesulfonate (MMS) was used as a positive control for directly acting chemicals.

TABLE 5

CHROMOSOMAL ABERRATIONS INDUCED BY PHENOBARBITAL

Test compound	Concentration (mM)	Cells scored	Percent of normal cells	Aberrations/100 cells						
				Chromatid aberrations			Chromosome aberrations		Total	Multiple aberrations
				Gaps	Breaks	Exch.	Breaks	Exch.		
PB +S9	3.75	300	91.3	3.7	1.3	0	5.3	0	10.3	0
	7.5	300	86.3	6.7	2.7	1.3	4.0	0	14.7	0
	15.0	300	63.7	7.0	28.0	28.0	6.7	0	75.0	5.3
Control +S9		300	90.3	7.0	3.0	0	2.0	0	12.0	0
PB −S9	3.75	300	91.7	4.3	1.7	0	3.3	0	9.3	0
	7.5	300	91.0	7.7	1.7	1.0	1.7	0.33	12.43	0
	15.0	300	87.3	5.3	2.7	2.0	3.0	0	13.0	0
Control		300	92.3	4.7	1.0	0	3.0	0	8.7	0

Positive controls are the same as *o*-toluidine.

TABLE 6

FREQUENCIES OF SCEs FOLLOWING *o*-TOLUIDINE TREATMENT

Test compound	Concentration (mM)	Cells scored	SCEs/cell
TOL	5.0	25	9.2
	10.0	25	8.8
	20.0	25	11.4
MMS	0.6	30	61.8
Control	–	25	8.2
TOL + S9	5.0	25	8.6
	10.0	25	10.1
	20.0	25	10.0
CP	0.03	25	17.4
Control + S9	–	25	8.3

TABLE 8

FREQUENCIES OF SCEs FOLLOWING ACRYLONITRILE TREATMENT

Test compound	Concentration (mM)	Cells scored	SCEs/cell
ACN	1.0	25	12.0
	2.0	25	12.5
	4.0	no II division	
MMS	0.6	30	61.8
Control	–	25	10.3
ACN + S9	1.0	25	13.2
	2.0	25	19.3
	4.0	no II division	
CP	0.03	25	27.8
Control + S9	–	25	11.9

Results and discussion

Pooled data from 3 fixation times, pertaining to the frequencies of chromosomal aberrations are presented in Tables 1–5. The data indicate that acrylonitrile is positive for induction of chromosomal aberrations, with and without metabolic activation. It was more effective after activation. Phenobarbital was positive only after metabolic activation.

Data pertaining to induction of SCEs are presented in Tables 6–10. Acrylonitrile was found positive when metabolically activated. Phenobarbital was weakly active in inducing SCEs, with or without metabolic activation.

o-Toluidine, caprolactam and benzene were

TABLE 7

FREQUENCIES OF SCEs FOLLOWING BENZENE TREATMENT

Test compound	Concentration (mM)	Cells scored	SCEs/cell
BEN	3.0	25	14.1
	6.0	25	10.5
	12.0	25	12.2
MMS	0.6	30	61.8
Control	–	25	12.3
BEN + S9	3.0	25	13.9
	6.0	25	11.4
	12.0	25	13.3
CP	0.03	25	25.2
Control + S9	–	25	11.5

TABLE 9

FREQUENCIES OF SCEs FOLLOWING CAPROLACTAM TREATMENT

Test compound	Concentration (mM)	Cells scored	SCEs/cell
CAP	37.75	25	6.8
	75.0	25	9.3
	150.0	25	9.3
MMS	0.6	30	61.8
Control		25	6.2
CAP + S9	37.75	25	8.3
	75.0	25	9.6
	150.0	25	9.0
CP	0.03	25	19.3
Control + S9	–	25	7.8

TABLE 10

FREQUENCIES OF SCEs FOLLOWING PHENOBARBITONE TREATMENT

Test compound	Concentration (mM)	Cells scored	SCEs/cell
PB	3.75	25	12.6
	7.5	25	14.4
	15.0	25	16.0
MMS	0.6	30	61.8
Control	–	25	8.8
PB + S9	3.75	25	15.4
	7.5	25	13.8
	15.0	no II divisions	
CP	0.03	20	24.4
Control + S9	–	25	9.1

negative in both tests. In experiments in which prolonged treatment time was employed, weak activity of safrole and hexamethylphosphoramide was detected, but the results are not included in this report as the data collected did not conform to the guidelines of this collaborative study. It can be concluded that, by modifying the protocol, one may be able to improve the sensitivity of the assay systems used in this study.

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Tests for the induction of sister-chromatid exchanges in human peripheral lymphocytes in culture

G. Obe, A. Hille, R. Jonas, S. Schmidt and U. Thenhaus

Institut für Genetik, Freie Universität Berlin, Arnimallee 5-7, D-1000 Berlin 33 (F.R.G.)

Summary

Acrylonitrile, benzene, benzoin, caprolactam, diethylhexylphthalate, hexamethylphosphoramide, phenobarbitone, safrole, *o*-toluidine and diethylstilboestrol were tested in respect to their possible SCE inducing capacities in human peripheral lymphocytes in vitro with and without metabolic activation. All compounds gave negative results.

All compounds were tested with respect to their ability to induce SCE in human peripheral lymphocytes in vitro with and without metabolic activation.

(1) Without metabolic activation

Each culture contained 5.0 ml fluid of the following composition: 4.0 ml McCoy's 5A medium, 0.5 ml fetal bovine serum and 0.12 ml PHA M (all Biochrom, Berlin), 0.1 ml BrdUrd (final concentration, 2×10^{-5} M; Serva, Heidelberg), 0.4 ml whole blood, 400 IE penicillin (Hoechst, Frankfurt) and 0.4 mg dihydrostreptomycin sulfate (Heyl, Berlin). The test compounds were dissolved in DMSO (Merck, Darmstadt) and were added to the cultures 24 h after they were started. The final DMSO concentration was 1% (v/v). Positive controls received diepoxybutane (DEB; Merck, Darmstadt), dissolved in medium to give a final concentration of 0.28 μg/ml. In some experiments DMSO was tested alone in a final concentration of 1% (v/v). The treatment conditions in the experimental controls were the same as described for the test compounds. Metaphase preparations were made 48 h later, including a colcemide treatment of 4.5 h (0.08 μg/ml). Preparations were made following a routine protocol and the differential staining was done using the method of Hill and Wolff (1982). 1–5 independent experiments with the blood from up to 5 different donors were performed with each test compound, in one experiment in each series a positive control was included. Since in some cases blood from different donors was tested in one experimental series, the number of different blood probes does not always give the number of independent experiments. In cases where one experiment was run with the blood of one donor, the standard error (SE) is given. In cases where blood from different donors was tested with one test compound, pooled data are given with the standard error of the means of the single results (SEM).

(2) With metabolic activation

Cultures were set up as described in (1). After 47 h the cells were centrifuged and washed once with serum-free medium. The cells were resuspended in 4.5 ml medium, containing antibiotics, 1% S9 (Aroclor-induced rat liver, Litton Bionetics, Lot No. REL 091), 1.58 mg NADP, 0.76 mg G6P (both from Boehringer, Mannheim), and the highest concentration of the test compounds analyzed in (1), resulting in a final fluid content of 5.0 ml (see Perry et al., 1983). After 1 h, the cells were washed 2 times in medium and reincubated in complete medium as described in (1). Metaphases were prepared 24 h later, including a

TABLE 1

ACN (ACRYLONITRILE) WITHOUT METABOLIC ACTIVATION

Concentrations	Number of metaphases analyzed (number of blood probes used from different donors)	SCE per metaphase (± SE or SEM) [a]
0	90 (3)	6.08 (1.70)
DMSO, 1% (v/v)	60 (2)	7.92 (2.10)
ACN, 1 μg/ml	90 (3)	8.14 (1.63)
ACN, 10 μg/ml	30 (1)	4.60 (0.39)
DEB, 0.28 μg/ml	30 (1)	40.33 (1.84)

[a] SE, standard error in cases were 1 Expt. was done with the blood from 1 donor.
SEM, standard error of the means in cases were experiments were done with blood from different donors.

TABLE 2

BEN (BENZENE) WITHOUT METABOLIC ACTIVATION

Concentrations	Number of metaphases analyzed (number of blood probes used from different donors)	SCE per metaphase (± SE or SEM) [a]
0	90 (3)	6.49 (0.90)
BEN, 10 μg/ml	90 (3)	6.53 (0.31)
BEN, 100 μg/ml	90 (3)	5.55 (0.61)
BEN, 500 μg/ml	90 (3)	6.07 (0.77)
BEN, 600 μg/ml	60 (2)	6.88 (0.15)
DEB, 0.28 μg/ml	30 (1)	32.83 (1.32)

[a] SE, standard error in cases were 1 Expt. was done with the blood from 1 donor.
SEM, standard error of the means in cases were experiments were done with the blood from different donors.

TABLE 3

ZOIN (BENZOIN) WITHOUT METABOLIC ACTIVATION

Concentrations	Number of metaphases analyzed (number of blood probes used from different donors)	SCE per metaphase (± SE or SEM) [a]
0	90 (3)	6.38 (1.32)
DMSO, 1% (v/v)	76 (3)	6.34 (0.88)
ZOIN, 1 μg/ml	90 (3)	7.09 (1.09)
ZOIN, 10 μg/ml	90 (3)	7.19 (1.20)
ZOIN, 50 μg/ml	30 (1)	9.10 (0.44)
ZOIN, 100 μg/ml	90 (3)	6.79 (1.35)
DEB, 0.28 μg/ml	30 (1)	47.67 (1.64)

[a] SE, standard error in cases were 1 Expt. was done with the blood from 1 donor.
SEM, standard error of the means in cases were experiments were done with the blood from different donors.

TABLE 4

CAP(CAPROLACTAM) WITHOUT METABOLIC ACTIVATION

Concentrations	Number of metaphases analyzed (number of blood probes used from different donors)	SCE per metaphase (± SE or SEM) [a]
0	115 (4)	5.23 (0.34)
CAP, 10 μg/ml	85 (3)	5.42 (0.48)
CAP, 100 μg/ml	85 (3)	5.21 (0.49)
CAP, 1000 μg/ml	108 (4)	5.96 (0.45)
DEB, 0.28 μg/ml	30 (1)	30.73 (0.96)

[a] SE, standard error in cases were 1 Expt. was done with the blood from 1 donor.
SEM, standard error of the means in cases were experiments were done with the blood from different donors.

TABLE 5

DEHP (DIETHYLHEXYLPHTHALATE) WITHOUT METABOLIC ACTIVATION

Concentrations	Number of metaphases analyzed (number of blood probes used from different donors)	SCE per metaphase (± SE or SEM) [a]
0	90 (3)	6.59 (1.13)
DMSO, 1% (v/v)	90 (3)	5.95 (0.78)
DEHP, 10 μg/ml	90 (3)	5.89 (1.10)
DEHP, 100 μg/ml	90 (3)	5.42 (0.77)
DEHP, 1000 μg/ml	90 (3)	6.10 (0.50)
DEB, 0.28 μg/ml	30 (1)	57.50 (2.49)

[a] SE, standard error in cases were 1 Expt. was done with the blood from 1 donor.
SEM, standard error of the means in cases were experiments were done with the blood from different donors.

TABLE 6

HMPA (HEXAMETHYLPHOSPHORAMIDE) WITHOUT METABOLIC ACTIVATION

Concentrations	Number of metaphases analyzed (number of blood probes used from different donors)	SCE per metaphase (± SE or SEM) [a]
0	150 (5)	6.78 (0.69)
HMPA, 1 μg/ml	108 (4)	8.09 (0.43)
HMPA, 10 μg/ml	107 (4)	7.70 (1.10)
HMPA, 100 μg/ml	99 (4)	8.08 (0.99)
HMPA, 1000 μg/ml	118 (5)	7.19 (0.73)
DEB, 0.28 μg/ml	30 (1)	30.73 (0.96)

[a] SE, standard error in cases were 1 Expt. was done with the blood from 1 donor.
SEM, standard error of the means in cases were experiments were done with the blood from different donors.

TABLE 7

PB (PHENOBARBITONE) WITHOUT METABOLIC ACTIVATION

Concentrations	Number of metaphases analyzed (number of blood probes used from different donors)	SCE per metaphase (± SE or SEM) [a]
0	120 (4)	5.34 (0.45)
DMSO, 1% (v/v)	120 (4)	5.60 (0.56)
PB, 10 μg/ml	120 (4)	6.42 (0.72)
PB, 100 μg/ml	120 (4)	6.27 (0.71)
PB, 500 μg/ml	120 (4)	6.61 (0.62)
DEB, 0.28 μg/ml	30 (1)	59.50 (2.52)

[a] SE, standard error in cases were 1 Expt. was done with the blood from 1 donor.
SEM, standard error of the means in cases were experiments were done with the blood from different donors.

TABLE 8

SAF (SAFROLE) WITHOUT METABOLIC ACTIVATION

Concentrations	Number of metaphases analyzed (number of blood probes used from different donors)	SCE per metaphase (± SEM) [a]
0	90 (3)	4.90 (1.23)
SAF, 10 μg/ml	90 (3)	5.64 (0.83)
SAF, 50 μg/ml	90 (3)	7.25 (1.65)
SAF, 90 μg/ml	90 (3)	4.47 (1.27)
DEB, 0.28 μg/ml	60 (2)	37.27 (4.44)

[a] SEM, standard error of the means from the experiments run with the blood from different donors.

TABLE 9

TOL (*o*-TOLUIDINE) WITHOUT METABOLIC ACTIVATION

Concentrations	Number of metaphases analyzed (number of blood probes used from different donors)	SCE per metaphase (± SE or SEM) [a]
0	120 (4)	5.94 (1.05)
TOL, 1 μg/ml	30 (1)	5.53 (0.47)
TOL, 10 μg/ml	60 (2)	6.74 (1.57)
TOL, 100 μg/ml	60 (2)	7.47 (1.37)
TOL, 500 μg/ml	60 (2)	8.34 (0.87)
TOL, 600 μg/ml	60 (2)	10.10 (0.10)
DEB, 0.28 μg/ml	60 (2)	46.70 (15.97)

[a] SE, standard error in cases were 1 Expt. was done with the blood from 1 donor.
SEM, standard errors of the means in cases were experiments were done with the blood from different donors.

TABLE 10

DES (DIETHYLSTILBOESTROL) WITHOUT METABOLIC ACTIVATION

Concentrations	Number of metaphases analyzed (number of blood probes used from different donors)	SCE per metaphase (± SE or SEM) [a]
0	120 (4)	6.63 (0.53)
DMSO, 1% (v/v)	120 (4)	6.78 (0.78)
DES, 1 μg/ml	120 (4)	7.73 (0.28)
DES, 5 μg/ml	60 (2)	7.56 (0.34)
DES, 10 μg/ml	60 (2)	7.68 (1.09)
DEB, 0.28 μg/ml	30 (1)	24.40 (0.79)

[a] SE, standard error in cases were 1 Expt. was done with the blood from 1 donor.
SEM, standard error of the means in cases were experiments were done with blood from different donors.

TABLE 11

WITH METABOLIC ACTIVATION

Concentrations	S9 present (+) absent (−)	Number of metaphases analyzed (number of blood probes used from different donors)	SCE per metaphase (± SE or SEM) [a]
0	−	90 (3)	7.89 (1.41)
	+	115 (4)	7.04 (1.17)
DMSO, 1% (v/v)	−	74 (3)	7.61 (2.12)
	+	90 (3)	8.13 (0.98)
CP [b], 3.3×10^{-5} M	−	129 (5)	7.41 (1.98)
	+	124 (5)	36.08 (3.37)
ACN, 10 μg/ml	−	60 (2)	9.47 (1.44)
	+	60 (2)	9.13 (0.60)
BEN, 600 μg/ml	−	60 (2)	5.70 (0.80)
	+	60 (2)	5.20 (0.83)
ZOIN, 100 μg/ml	−	60 (2)	10.42 (0.29)
	+	60 (2)	10.75 (3.32)
CAP, 1000 μg/ml	−	60 (2)	5.87 (0.90)
	+	60 (2)	5.22 (0.49)
DEHP, 1000 μg/ml	−	60 (2)	10.17 (2.04)
	+	60 (2)	8.92 (1.05)
HMPA, 1000 μg/ml	−	60 (2)	5.42 (0.52)
	+	60 (2)	5.12 (0.72)
PB, 500 μg/ml	−	60 (2)	9.05 (1.85)
	+	60 (2)	8.69 (0.29)
SAF, 60 μg/ml	−	60 (2)	6.45 (0.78)
	+	60 (2)	6.34 (1.04)
TOL, 600 μg/ml	−	60 (2)	6.37 (0.47)
	+	60 (2)	6.64 (0.44)

[a] SE, standard error in cases were 1 Expt. was done with the blood from 1 donor.
SEM, standard error of the means in cases were experiments were done with the blood from different donors.
[b] CP, cyclophosphamide, for other abbreviations see Tables 1–9.

TABLE 12

DES WITH METABOLIC ACTIVATION

For abbreviations see Table 11.

Concentrations	S9 present (+) absent (−)	Number of metaphases analyzed (number of blood probes used from different donors)	SCE per metaphase (±SEM)
0	−	60 (2)	6.99 (0.39)
	+	60 (2)	7.80 (0.03)
DMSO, 1% (v/v)	−	60 (2)	7.72 (0.65)
	+	60 (2)	7.12 (1.19)
CP, 3.3×10^{-5} M	−	60 (2)	8.02 (1.49)
	+	60 (2)	23.17 (1.54)
DES, 5 μg/ml	−	60 (2)	6.63 (1.40)
	+	60 (2)	7.08 (1.05)

4.5 h colcemide treatment (see (1)). As positive control, cyclophosphamide (CP, final concentration, 3.3×10^{-5} M; gift from Prof. N. Brock, Asta-Werke, Bielefeld) dissolved in medium was tested in the same way as the test compounds. The experiments were done 1–2 times independently with the blood from different donors.

As can be seen from Tables 1–12, all test compounds were negative. Without metabolic activation, *o*-toluidine led to a slight elevation of the SCE rates, which did not even reach a doubling of the control values (Table 9).

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Tests for the induction of chromosomal aberrations in Chinese hamster ovary (CHO) cells in culture

F. Palitti, M. Fiore, R. De Salvia, C. Tanzarella, R. Ricordy, R. Forster [1], P. Mosesso [1], S. Astolfi [2] and N. Loprieno [2]

Centro di Studio Genetică Evoluzionistica, Istituto di Genetica, Citta Universitaria, Rome (Italy), [1] LSR, Roma Toxicology Centre, Roma (Italy) and [2] Istituto di Biochimica Biofisica e Genetica, Università di Pisa (Italy)

Summary

The ability of 5 chemicals to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells in vitro was determined. Benzene and safrole gave a positive result. HMPA, *o*-toluidine and caprolactam gave a negative response. The results demonstrated the value of multiple sampling times and that further extension of sampling times might have improved responses.

As a contribution to the International Program on Chemical Safety, we have assayed 5 chemicals for the induction of chromosomal aberrations in Chinese hamster ovary cells, after treatment (in the absence and presence of S9 metabolic activation) in vitro. The chemicals tested were hexamethylphosphoramide (HMPA), *o*-toluidine (TOL), safrole (SAF), benzene (BEN), and caprolactam (CAP). These chemicals (except CAP) are well-known carcinogens, not easily detected in the Salmonella assay. Since the mechanisms of action of these chemicals were not well understood, we used a protocol that included multiple sampling times, allowing us to detect clastogenic effects at each stage of the cell cycle.

Materials and methods

The Chinese hamster ovary cell line was obtained from A.T. Natarajan (Leiden). This cell line has a stable aneuploid karyotype with modal number 21–22 chromosomes, and a generation time of 10–12 h. The cells were grown at 37 °C in a 5% carbon dioxide atmosphere (100% humidity) in Ham's F10 medium supplemented with 15% newborn calf serum.

Metabolic activation

Rat-liver S9 homogenate. The S9 homogenate was prepared from the livers of young male Sprague–Dawley/CD rats, whose hepatic enzymes had been induced by mixed induction with phenobarbital and β-naphthoflavone, as follows:

Day 1	i.p.	Phenobarbital	30 mg/kg
Day 2	i.p.	Phenobarbital	60 mg/kg
Day 3	i.p.	Phenobarbital	60 mg/kg
	i.p.	β-Naphthoflavone	80 mg/kg
Day 4	i.p.	Phenobarbital	60 mg/kg
Day 5		Sacrifice	

The homogenate was prepared using chilled sterile equipment. The liver was homogenized in Hank's solution and Hepes buffer (4 ml/g) and centrifuged at 9000 *g*/av. for 10 min. The supernatant was then stored at −80 °C for subsequent use.

S9 mix. The mixture of S9 tissue fraction and cofactors (S9 mix) was prepared as follows:

S9 fraction		3 ml
NADP	40 mM	1 ml
G-6-P	50 mM	1 ml
Hepes buffer	20 mM	2 ml
$MgCl_2$	40 mM	1 ml
KCl	330 mM	1 ml
Distilled water		1 ml
Total		10 ml

Test chemicals

The test chemicals were received from J. Ashby (ICI plc). All the chemicals tested were dissolved in dimethyl sulphoxide (DMSO) and added to culture medium such that the final concentration of solvent did not exceed 1%.

Experimental design

Each experiment included negative and positive controls and 3 dose levels of the test substance, both in the presence and absence of S9 metabolic activation. Duplicate cultures were prepared at each experimental test point. The negative controls consisted of both untreated and solvent vehicle treated cultures. The positive controls used were both indirect: cyclophosphamide (Asta-Werke, W. Germany) or aflatoxin B1 (Fluka A.G.). The 3 concentrations of the chemical under test were set at half-log intervals. The top dose was selected as that giving approximately 50% inhibition of the mitotic index, compared with control values.

Assay procedure

Preparation of test cultures. Approximately 24 h before treatment an appropriate number of flasks for the experiment was prepared. Each 25-cm^2 flask was seeded with 7×10^5 cells/ml in 5 ml of supplemented Ham's F10. In the case of HMPA, glass bottles were used to avoid any interaction with the plastic flasks otherwise used.

Treatment of the cultures. The medium was aspirated from the flasks, the cells were washed with Hank's solution and then treated with treatment media prepared as follows:

Without metabolic activation	
Supplemented Ham's F10	4.95 ml
Test chemical solution	0.05 ml
With metabolic activation	
Supplemented Ham's F10	4.45 ml
S9 mix	0.5 ml
Test chemical solution	0.05 ml

The treatment period was 3 h, after which the medium was aspirated and the cultures were washed 3 times with phosphate-buffered saline. At this time group 1 cultures were fixed (3 h). Fresh medium was added to the remaining cultures, and

TABLE 1

EFFECT OF TESTED COMPOUNDS ON INDUCTION OF CHROMOSOMAL ABERRATIONS WITH AND WITHOUT METABOLIC ACTIVATION AFTER 3 h TREATMENT

Concentration (μg/ml)	S9 + / −	Number of cells	Chromosomal aberrations (%)						
			Gaps	Breaks		Chromatid exchanges	Dicent. + rings	% abnormal cells	Total aberrations + gaps (%)
				B′	B″				
HMPA									
0	−	200	3.5	1.5	2	0.5	2	9.5	7.5 + 3.5
	+	200	2	3	1.5	0.5	1	8	6.5 + 4.5
1% DMSO	−	200	4	3.5	1.5		1.5	10.5	7.5 + 4
	+	200	6.5	1	4			11.5	8.5 + 6.5
10000	−	175	8	2.28	2.86	1.14	1.14	14.28	7.42 + 8
	+	10	20	10				30	20.0 + 10
3000	−	200	6.5	3.5	3.5	1	1	11	9.0 + 6.5
	+	141	7	2.13	0.71		2.13	10.64	4.97 + 7.09
1000	−	57	3.5	3.5	7		1.75	15.8	12.27 + 3.5
	+	100	5	2	7	3	1	18	13.0 + 5
TOL									
0	−	200	1.5	1	1.5		0.5	4.5	3.0 + 1.5
	+	200	4		0.5			4.5	3.0 + 1.5
1% DMSO	−	200	1.5	0.5	1.5			3.5	2.0 + 1.5
	+	200	2	2	1			5	3.0 + 2.5

TABLE 1 (continued)

Concentration (μg/ml)	S9+/−	Number of cells	Chromosomal aberrations (%)						
			Gaps	Breaks		Chromatid exchanges	Dicent. + rings	% abnormal cells	Total aberrations + gaps (%)
				B′	B″				
900	−	200	4	1	2.5			7.5	7.5 +4
	+	200	5.5	2	1	1.5		4.5	4.5 +5.5
300	−	200	4	1	1.5			6.5	2.5 +4
	+	200	7	4.5	3			13.5 **	7.5 +7
100	−	200	2.5	1.5	0.3		0.5	5	2.5 +2.5
	+	200	3.5	1.5	4			9	4.5 +3.5
SAF									
0	−	200	5	2.5	1			8.5	3.5 +5
	+	200	1.5	0.5	2			4	2.5 +1.5
1% DMSO	−	200	4.5	0.5	1.5			6.5	1.5 +5
	+	200	2.5	2	3.5		0.5	8.5	6.0 +2.5
250	−	32	1.5	0.5		0.5		2.5	1.0 +1.5
	+	11	9					9	0.0 +9
83.33	−	200	4	2	0.5	0.5		7	3.0 +4
	+	200	4	2	2		1	9	5.0 +4
27.77	−	200	2	2	3			7	5.0 +2
	+	200	2	3	5			10	8.0 +2
BEN									
0	−	200	2.5	1	0.5		0.5	4.5	2.0 +2.5
	+	200	2.5	0.5	0.5			3.5	1.0 +2.5
1% DMSO	−	200	5	0.5	1.5			7	2.0 +5
	+	200	3		1			4	1.0 +3
1800	−	200	7	2	4.5		0.5	13.5 *	6.5 +7
	+	144	6.9	4.17	1.39			7	5.5 +6.9
600	−	200	2.5	2	3.5			7.5	5.5 +2.5
	+	200	4	3.5	0.5	0.5		8.5 *	4.5 +4
200	−	200	6	3.5			1	10	4.5 +6
	+	200	4.5	3		0.5		7	3.5 +4.5
CAP									
0	−	200	2	0.5	1		1.5	5	3.0 +2.5
	+	200	1	2.5	0.5			4	3.0 +1
1% DMSO	−	200	1.5	1	1		1.5	5.5	3.5 +1.5
	+	200	4	1.5	2		0.5	8	4.5 +4
2500	−	200	1.5	4	1.5		1	7.5	5.5 +1.5
	+	135	2.2		2.2		2.9	7.4	5.1 +2.2
1666.6	−	200		2.5	1			3.5	3.5 +0
	+	200	5.5	2	5		1	9	8.0 +5.5
500	−	200	3.5	0.5	4		1	9	5.5 +3.5
	+	200	3.5	3.5	3		3	12.5	9.5 +3.5

* $p < 0.05$.
** $p < 0.005$.

they were returned to the incubator. After a further 6 h, colchicine was added to group 2 cultures, and 3 h later they were fixed (12 h). Similarly, 12 h after their return to the incubator, colchicine was added to group 3 cultures, and 3 h later they were fixed (18 h). This procedure was used for treatments both with and without S9 metabolic activation.

Harvesting and slide preparations. The medium was aspirated from the flasks, and the cells were brought into suspension with trypsin. The cell suspension was centrifuged (1000 rpm for 10 min) and the cell pellet was resuspended in 5 ml of hypotonic trisodium citrate solution for 13 min at 37 °C. The cells were fixed in freshly prepared methanol : acetic acid fixative and washed 3 times with fixative. A few drops of the cell suspension obtained in this way were dropped onto clean, wet, grease-free glass slides and air-dried to produce metaphase chromosome spreads. The slides were stained in 3% Giemsa in Sorensen's buffer, and rinsed twice with distilled water. The slides were then immersed in xylene for 5 min and made permanent with Eukitt.

Scoring of the preparations. At least 2 slides per dose level were coded and examined under oil immersion at 1250 magnification. 200 well-spread metaphases were analyzed at each dose level (100 from each parallel culture).

Analysis of the results. The number of aberrant cells (including gaps) was utilized for statistical analysis. To determine the statistical significance, Fisher's exact test was used. A compound was considered positive only when a dose-related increase in the frequency of chromosomal aberrations was observed.

Results and discussion

The detailed chromosome analyses of the cell cultures fixed at 3, 12 and 18 h are shown in Tables 1–3.

Negative controls

In the experiments with HMPA and CAP, the negative control values for aberrant cells were

TABLE 2

EFFECT OF TESTED COMPOUNDS ON INDUCTION OF CHROMOSOMAL ABERRATIONS WITH AND WITHOUT METABOLIC ACTIVATION AFTER 12 h TREATMENT

Concentration (μg/ml)	S9 + / −	Number of cells	Chromosomal aberrations (%)						
			Gaps	Breaks		Chromatid exchanges	Dicent. + rings	% Abnor. cells	Total aberrations + gaps (%)
				B′	B″				
HMPA									
0	−	200	3.5	1.5	2		1.5	8	8 +3.5
	+	200	15	1	3		1.5	13	5.5+8
1% DMSO	−	200	7.5	1	3		1.5	8.5	5 +4
	+	200	4	1.5	1.5		0.5	7.5	3.5+4.5
10000	−	200	3	4.5	3	0.5	1.5	12.5	9.5+3
	+	200	4.5	4.5	1.5	1.5		12	7.5+4.5
3000	−	200	8	0.5	6.5	0.5	1.5	17	9 +8
	+	200	6	2	2		1	11	5 +6
1000	−	200	3	2	2.5	1	0.5	9	6 +3
	+	200	2.5	1	1		1	5.5	3 +2.5
TOL									
0	−	200	0.5	0.5	0.5			1.5	1 +0.5
	+	200	2		0.5	0.5	0.5	3.5	1.5+2
1% DMSO	−	200	4	0.5	1.5		0.5	6.5	2.5+4
	+	200	1.5	1.5	1.5		0.5	5	3.5+1.5

TABLE 2 (continued)

Concentration (μg/ml)	S9 + / −	Number of cells	Chromosomal aberrations (%)						
			Gaps	Breaks		Chromatid exchanges	Dicent. + rings	% Abnor. cells	Total aberrations + gaps (%)
				B′	B″				
900	−	200	4.5	0.5	1			6	1.5 + 4.5
	+	200	1.5	1	2		1	5.5	4 + 1.5
300	−	200	5.5	4.5	1.5			11.5	6 + 5.5
	+	200	2.5	0.5	0.5			3.5	1 + 2.5
100	−	200	1.5	2.5				4	2.5 + 1.5
	+	200	1.5	2.5	4		0.5	8.5	7 + 1.5
SAF									
0	−	200	1	0.5	0.5			2	1 + 1.5
	+	200	0.5	1	1.5		0.5	3.5	3 + 0.5
1% DMSO	−	200	1.5	1.5	0.5		0.5	4.5	3 + 1.5
	+	200		2	2		1	5	5
250	−	200	7	6.5	3.5			17 **	10 + 7
	+	200	8	2.5	0.5	1		11.5 *	4 + 8
83.33	−	200	3		3.5			6.5	3.5 + 3
	+	200	6	1.5	3.5		0.5	11 *	5.5 + 6
27.77	−	200	1	1.5	1			3.5	2.5 + 1
	+	200	3.5	2	1.5		1	8	4.5 + 3.5
BEN									
0	−	200	1.5	2	1			4.5	3 + 1.5
	+	200	2.5	0.5	0.5		1	4	1.5 + 2.5
1% DMSO	−	200	2.5	3	1.5		0.5	7.5	5.5 + 3
	+	200	3.5	1.5	2			7.5	4 + 3.5
1800	−	200	2	0.5	1			3.5	1.5 + 2
	+	200	2	2.5	2	0.5		7	5 + 2
600	−	200	2.5	0.5	1.5		0.5	5	2.5 + 2.5
	+	200	4	2	2.5		0.5	9	5 + 4
200	−	200	2	0.5	1			3.5	1.5 + 2
	+	200	1.5	2	1		0.5	5	3.5 + 1.5
CAP									
0	−	200	4	4	1.5		0.5	10	6 + 4
	+	180	3.3	2.8	2.2	0.5	1.1	10	3.3 + 6.7
1% DMSO	−	200	4	4.5	2	1.5	1.5	12.5	10 + 4
	+	200	2	0.5	5.5	0.5	1	9.5	7.5 + 2
2500	−	200	2.5	1.5	4.5	0.5		7.5	6.5 + 2.5
	+	200	4.5	1.5	4.5	0.5	2	9.5	8.5 + 4.5
1666.6	−	200	2.5	1	1		1	5.5	3 + 2.5
	+	200	4	1.5	2		1.5	8.5	5 + 4
500	−	200	5.5	3	3.5		1	12	7.5 + 5.5
	+	200	0.5	3.5	3		0.5	6	7 + 0.5

* $p < 0.05$.
** $p \ll 0.001$.

TABLE 3

EFFECT OF TESTED COMPOUNDS ON INDUCTION OF CHROMOSOMAL ABERRATIONS WITH AND WITHOUT METABOLIC ACTIVATION AFTER 18 h TREATMENT

Concentration (μg/ml)	S9 + / −	Number of cells	Chromosomal aberrations (%)						
			Gaps	Breaks		Chromatid exchanges	Dicent. + rings	% Abnor. cells	Total aberrations + gaps (%)
				B′	B″				
HMPA									
0	−	200	1.5	3	1		0.5	6	5 + 1.5
	+	200	8.5	3.5	0.5	1	1	14.5	7 + 8.5
1% DMSO	−	200	4	3.5	0.5	1	1.5	10.5	7.5 + 4
	+	200	6.5	0.5	0.5			7.5	1 + 8
10000	−	200	1.5	2	5	1.5	1	10	9.5 + 1.5
	+	200	2.5	0.5	3.5	0.5	0.5	7.5	5 + 2.5
3000	−	200	6	0.5	2		1.5	9	4 + 6
	+	200	4.5	4	4.5		0.5	13	9 + 4.5
1000	−	200	5	2				7	2 + 5
	+	200	6	0.5	1		0.5	6	2 + 8
TOL									
0	−	200	2	1	2			5	3 + 2
	+	200	1.5	1.5	1			4	2.5 + 1.5
1% DMSO	−	200	3	1.5	1			5.5	2.5 + 3
	+	200	2.5	2.5	2			7	4.5 + 2.5
900	−	200	0.5	1.5	0.5			2.5	2 + 0.5
	+	200	4.5		3			7	3 + 4.5
300	−	200	5.5	1	1.5			6.5	2.5 + 5.5
	+	200	9	9.5	3			10	3.5 + 9
100	−	200	1.5		1		0.5	3	1.5 + 1.5
	+	200	4.5	1.5	4.5			10.5	6 + 4.5
SAF									
0	−	200	2		1			3	1 + 2
	+	200	4.5	1.5	1.5			7.5	3 + 4.5
1% DMSO	−	200	2	1.5	1		0.5	5	3 + 2
	+	200	2	2	2			6	4 + 2
250	−	200	5	1	4			9	5 + 5
	+	200	4	1.5	4.5	0.5	1.5	12	8 + 4
83.33	−	200	3	1	1			5	2 + 3
	+	200	9	2	2.5			13 *	4.5 + 9
27.77	−	200	3	0.5	1		0.5	5	2 + 3
	+	200	0.5	1	2			3.5	3 + 0.5
BEN									
0	−	200	0.5	0.5	1		0.5	4.5	1.5 + 3
	+	200	3	0.5		0.5	0.5	2.5	2 + 0.5
1% DMSO	−	200	2.5	1	1.5			3	3
	+	200		2	1			5	2.5 + 2.5
1800	−	200	1.5	1.5	1		0.5	4.5	3 + 1.5
	+	200	2	2.5	1			5.5	3.5 + 2

TABLE 3 (continued)

Concentration (μg/ml)	S9 + / −	Number of cells	Chromosomal aberrations (%)						
			Gaps	Breaks		Chromatid exchanges	Dicent. + rings	% Abnor. cells	Total aberrations + gaps (%)
				B′	B″				
600	−	200	4.5	2	0.5	0.5		7.5	3 +4.5
	+	200	5.5	3	4		0.5	13 **	7.5 + 5.5
200	−	200	2.5	1.5	2			6	3.5 + 2.5
	+	200	6.5	4	4			13.5 **	8 +6.5
CAP									
0	−	200	4.5	2	1.5	0.5		7.5	5 +3.5
	+	200	4	1.5	2.5	0.5		8.5	5 +4
1% DMSO	−	200	1.5	0.5	2			4	3.5 + 1.5
	+	200	2	4	2		2.5	10.5	9 +2
2500	−	200	1.5	2.5	2.5	1.5	0.5	6.5	7 +1.5
	+	200	5.5	3	1	3	1	11.5	8 +5.5
1666.6	−	200	7.5	3	7	1	1	13	12 +7.5
	+	200	7.5	1	6		1.5	13.5	8.5 + 7.5
500	−	200	2.5	1	1.5		2.5	7	5 +2.5
	+	200	5	3.5	3.5		1.5	12.5	8.5 + 5

* $p < 0.05$.
** $p < 0.005$.

much higher than in the other experiments. Accordingly for the statistical analyses, comparisons were made between treated cultures and the controls prepared in the same experiment.

Solvent controls

DMSO, at a final concentration of 1%, did not affect the frequency of chromosomal aberrations, in either the presence or absence of S9 metabolic activation.

Positive controls

Cyclophosphamide increased the frequency of chromosomal aberrations in the presence of metabolic activation, in the cultures fixed at 12 and at 18 h. There was little variation between experiments. Aflatoxin B1 was used as a positive control only in the tests with benzene, and produced an increase in chromosomal aberrations in the presence of S9 metabolic activation in cultures fixed at 18 h.

HMPA

This compound gave a negative response in the assay in both the presence and absence of S9 metabolic activation. In the cultures fixed after 3 h it was not always possible to score 200 metaphases.

TOL

An increased chromosomal aberration frequency was observed only at the intermediate dose (300 μg/ml) in the presence of S9 metabolic activation in those cultures fixed after 3 h.

SAF

In cultures fixed after 3 h no positive response was observed with safrole. At the highest dose level it was difficult to score an adequate number of metaphases since there were few mitoses. Safrole produced an increased chromosomal aberration frequency at the 2 higher dose levels (250 and 83.3 μg/ml) in the presence of S9 metabolic activation, in cultures fixed after 12 h. An increase was also seen in the absence of S9 at the same fixation time but at the top dose only. The effect was a little greater in the absence of S9. The results in the presence of S9 did not demonstrate a dose–effect relationship. In cultures fixed after 18 h there was

an increased frequency of aberrations in the presence of S9 at the intermediate dose (83.3 μg/ml).

BEN

In cultures fixed after 3 h, increases in the frequency of chromosomal aberrations were seen in the absence of S9 metabolic activation (at the highest dose level, 1800 μg/ml), and in the presence of S9 (at the intermediate dose 600 μg/ml). Negative results were obtained in cultures fixed after 12 h. In cultures fixed after 18 h, increases were observed in the presence of S9, at the doses of 200 and 600 μg/ml.

CAP

Caprolactam gave negative results under all conditions.

Conclusions

A summary of our findings is given in Table 4. If a requirement for a dose response is one of the criteria for a positive result, then all of the data presented here must be considered negative, or at best equivocal in some cases. We have, however, considered safrole to have given a positive result, on the grounds that a marked increase in chromosomal aberrations was seen both with and without metabolic activation in the cultures fixed after 12 h. Similarly, benzene has been considered positive since marked increases were noted in the presence of metabolic activation, in cultures fixed after 18 h. The other compounds are considered to have given negative responses. Recently published guidelines for the performance of in vitro cytogenetic assays recommend that a dose response relationship should not form part of the criteria for a positive result (Scott et al., 1982).

TABLE 4

SUMMARY OF RESULTS

Compound	Concentration tested (μg/ml)	Result
HMPA	10000, 3000, 1000	Negative
TOL	900, 300, 100	Negative
SAF	250, 83.33, 27.77	Positive
BEN	1800, 600, 200	Positive
CAP	2500, 1666, 500	Negative

The results clearly demonstrate the value of multiple sampling times, and further extension of the sampling times might have improved responses, or allowed the detection of other clastogenic effects. Similarly, attempts to optimize metabolic activation, using multiple concentrations of S9, might have assisted in the detection of some agents, for example HMPA, where the IPCS study has clearly emphasized the importance of effective activation. Some of the differences noted between our results, and those obtained by other investigators contributing in vitro cytogenetics data to this study, may be explained by specific features of the protocol that we have used (for example, the mixed induction method used instead of induction by Aroclor 1254).

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Tests for the induction of sister chromatid exchanges in Chinese hamster ovary (CHO) cells in culture

A.M. Lane, B.J. Phillips and D. Anderson

British Industrial Biological Research Association, Carshalton, Surrey (Great Britain)

Summary

The first 5 test compounds — HMPA, TOL, BEN, SAF and CAP, were assessed for their ability to induce SCE in cultured Chinese hamster ovary (CHO) cells. 4 concentrations of each chemical were employed with a 2-fold dose spacing. Each concentration was tested in the presence and absence of Aroclor-induced rat-liver S9 and each test was carried out twice. Cells were exposed for 5 h and arrested in metaphase after 26 h in bromodeoxyuridine (BrdUrd).

Only TOL in the absence of S9 caused significant dose-related increases in SCE frequency and was judged positive. 6 positive controls, including 3 requiring S9, gave consistent positive results.

Introduction

The CHO/SCE test has been widely used to detect mutagens. The important features of the protocol used in this study were as follows:

Dose range. Maximum dose established from preliminary toxicity test; 4 concentrations tested using 2-fold serial dilutions from maximum.

Treatment. 5 h in the absence or presence of Aroclor-induced S9 fraction.

SCE analysis. Metaphase arrest at 26 h after start of treatment. Bromodeoxyuridine (3 μg/ml) present for 26 h. 30 metaphases scored per treatment; mean SCE/cell analysed by Student's *t*-test (two-sided).

Repetition. Two totally separate SCE tests carried out on each chemical, rather than using duplicate cultures, to assess variation between tests.

Materials and methods

Materials

Chinese hamster ovary cells (CHO-K1-BH_4) were obtained from J.P. O'Neill, who has described the derivation of this line (O'Neill et al., 1977). They were grown in Ham's F12 medium, supplemented with 5% foetal calf serum, penicillin (100 i.u./ml) and streptomycin (100 μg/ml).

Cyclophosphamide (CPA), benzo[*a*]pyrene (BP), 2-acetamidofluorene (2AAF), *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), ethyl methanesulphonate (EMS), 4-nitroquinoline *N*-oxide (NQO), 5-bromo-2-deoxyuridine (BrdUrd) and colcemid were obtained from Sigma.

S9 mix was prepared as follows: Male Sprague–Dawley rats were injected i.p. with 500 mg/kg Aroclor 1254. After 4 days, they were starved overnight, killed by cervical dislocation and the livers homogenised (1 g liver: 3 ml tris/HCl buffer pH 7.4). The homogenate was centrifuged for 20 min at 10000 *g* and ampoules of supernatant frozen in liquid nitrogen. Thawed supernatant (1 ml) was mixed with 9 ml of 0.1 M phosphate buffer containing 4 mM NADP, 5 mM glucose 6-phosphate, 33 mM KCl and 8 mM $MgCl_2$.

Exposure of cultures

All test chemicals were dissolved in DMSO. The solutions were diluted 1 in 100 in medium or

medium containing 10% S9 mix.

Cultures were established with 5×10^5 CHO cells in 25-cm^2 flasks containing 5 ml medium. After 24 h, the medium was removed and 5 ml of medium containing the test chemical and 3 μg/ml BrdUrd was added. The cultures were incubated at 37 °C for 5 h and then washed with phosphate-buffered saline and refed fresh medium containing BrdUrd.

Preliminary toxicity tests

Cultures were treated, in the absence of BrdUrd, with the maximum attainable concentration of each chemical and 5 serial log dilutions. Cells were suspended and counted 24 h after the start of treatment. The cell counts were used to estimate the maximum concentration which allowed sufficient cell division for the SCE test.

SCE tests

Each test comprised 12 cultures, (6 without S9 mix and 6 with S9 mix). Each group consisted of 4 concentrations of the test chemical, (2-fold serial dilutions), one negative control (DMSO) and one positive control. Two tests were carried out on each chemical.

The cultures were treated with 0.1 μg/ml colcemid for 2 h prior to trypsinisation 26 h after the start of treatment. Cell suspensions were counted and resuspended in 0.075 M KCl for 15 min. After fixation in 3 changes of methanol : acetic acid (3 : 1), slides were prepared by air-drying. These were stained with 2% Giemsa soln. (Gurr R66) in 0.3 M phosphate buffer (pH 10.4) and washed in tapwater (Alves and Jonasson, 1978).

Exchanges were scored in 30 second-division metaphases from each culture. Mean SCE per cell for each treatment was compared with the concurrent negative control using the two-sided Student's t-test, accepting $P < 0.05$ as significant. Also, data from replicate tests were pooled and analysed in the same way. A final judgement on the result of tests on each chemical was made on the basis of the analysis of pooled data. A chemical was judged positive only if there was some evidence of a dose-related increase in SCE.

Results

The pooled results from 2 separate tests of each compound are shown in Tables 1–5. In the case of HMPA and CAP, no effect on cell growth was observed and the highest concentration tested is as shown in the tables. Toxicity was observed with TOL, BEN and SAF; although higher concentrations were tested, the highest concentration shown

TABLE 1

POOLED DATA FROM TWO TESTS ON HMPA

	Compound	Concentration (μg/ml)	Number of cultures	Total cells scored	Number of chromosomes	Number of SCEs	SCEs/chromosomes	SCEs/cell
−S9	HMPA	0	2	60	1185	493	0.416	8.22
		5200	2	60	1157	550	0.475	9.17
		1730	2	60	1153	501	0.434	8.35
		580	2	60	1159	524	0.452	8.73
		190	2	60	1165	519	0.445	8.65
	EMS	130	2	60	1172	1577	1.346	26.28 ***
+S9	HMPA	0	2	60	1159	492	0.424	8.20
		5200	2	60	1174	545	0.464	9.08
		1730	2	60	1136	552	0.486	9.20
		580	2	60	1179	536	0.455	8.93
		190	2	60	1154	509	0.441	8.48
	CPA	10	2	60	1168	1524	1.305	25.40 ***

Mean SCE/cell analyzed by Student's t-test.
Significantly higher than control at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***).

TABLE 2

POOLED DATA FROM TWO TESTS ON TOL

	Compound	Concentration (μg/ml)	Number of cultures	Total cells scored	Number of chromo-somes	Number of SCEs	SCEs/ chromo-somes	SCEs/ cell
−S9	TOL	0	2	60	1175	469	0.399	7.82
		1000	2	60	1170	680	0.581	11.33 ***
		500	2	60	1173	612	0.522	10.20 ***
		250	2	60	1165	523	0.449	8.72
		125	1	30	579	280	0.483	9.33 *
	MNNG	0.025	2	60	1163	1469	1.263	24.48 ***
+S9	TOL	0	2	60	1159	607	0.524	10.12
		1000	2	60	1164	601	0.516	10.02
		500	2	60	1150	631	0.549	10.52
		250	2	60	1158	626	0.541	10.43
		125	1	30	583	210	0.360	7.00
	BP	5	1	30	593	724	1.221	24.13 ***
	2-AAF	25	1	30	573	622	1.086	20.73 ***

is the maximum, using 2-fold dose spacing, which allowed slides to be scored for SCE.

Only TOL gave a dose-related significant increase in SCE and then only in the absence of S9 mix. The effect was seen in both tests on TOL. BEN caused a significant increase only at the highest concentration in the presence of S9 mix. When 2 tests on BEN were analysed separately, only one test showed this effect. Similarly, an effect of the highest concentration (with S9) of CAP in only one test resulted in a significant effect in the assessment of the pooled data. No significant effects were observed with either HMPA or SAF.

6 compounds were used as positive controls. All of these, NQO, MNNG and EMS without S9 and

TABLE 3

POOLED DATA FROM TWO TESTS ON BEN

	Compound	Concentration (μg/ml)	Number of cultures	Total cells scored	Number of chromo-somes	Number of SCEs	SCEs/ chromo-somes	SCEs/ cell
−S9	BEN	0	2	60	1162	508	0.437	8.47
		1000	2	60	1165	523	0.449	8.72
		500	1	30	586	260	0.446	8.67
		250	2	60	1155	547	0.474	9.12
		125	1	30	578	281	0.487	9.37
	EMS	130	2	60	1167	1348	1.155	22.5 ***
+S9	BEN	0	2	60	1137	593	0.521	9.88
		1000	2	60	1137	649	0.571	10.82 *
		500	2	60	1145	597	0.521	9.95
		250	2	60	1154	579	0.502	9.65
		125	1	30	572	261	0.458	8.70
	CPA	10	2	60	1151	967	0.840	16.1 ***

TABLE 4

POOLED DATA FROM TWO TESTS ON SAF

	Compound	Concentration (μg/ml)	Number of cultures	Total cells scored	Number of chromosomes	Number of SCEs	SCEs/ chromosomes	SCEs/ cell
−S9	SAF	0	2	60	1168	416	0.356	6.93
		100	2	60	1178	462	0.392	7.70
		50	2	60	1168	437	0.374	7.28
		25	2	60	1142	387	0.339	6.45
		12.5	1	30	578	227	0.395	7.57
	NQO	0.2	2	60	1182	983	0.832	16.4 ***
+S9	SAF	0	2	60	1157	426	0.368	7.10
		100	1	30	578	234	0.405	7.80
		50	2	60	1160	371	0.320	6.18
		25	2	60	1158	426	0.368	7.10
		12.5	1	30	580	234	0.421	7.80
	BP	5	1	30	592	612	1.040	20.5 ***
	2AAF	25	1	30	574	570	0.993	19.0 ***

TABLE 5

POOLED DATA FROM TWO TESTS ON CAP

	Compound	Concentration (μg/ml)	Number of cultures	Total cells scored	Number of chromosomes	Number of SCEs	SCEs/ chromosomes	SCEs/ cell
−S9	CAP	0	2	60	1167	450	0.386	7.50
		5000	2	60	1157	443	0.383	7.38
		2500	2	60	1163	519	0.446	8.65 *
		1250	2	60	1167	428	0.367	7.13
		625	2	60	1158	417	0.360	6.95
	MNNG	0.025	1	30	577	757	1.276	25.2 ***
	NQO	0.2	1	30	582	375	0.641	12.5 ***
+S9	CAP	0	2	60	1166	575	0.493	9.58
		5000	2	60	1179	651	0.552	10.85 *
		2500	2	60	1157	583	0.504	9.72
		1250	2	60	1168	550	0.471	9.17
		625	2	60	1173	539	0.459	8.98
	2AAF	25	2	60	1188	1186	0.998	19.8 ***

TABLE 6

CONCLUSIONS (POSITIVE OR NEGATIVE) FROM SCE TESTS

Compound	Without S9	With S9
HMPA	−	−
TOL	+	−
SAF	−	−
CAP	−	−

2AAF, CPA and BP with S9, gave consistent and large increases in SCE.

Discussion

The strong effects observed with 6 different positive controls, including 3 requiring metabolic activation, suggest that the system used was capa-

ble of detecting the genetic effects of a wide variety of mutagens.

Of the test chemicals, only TOL showed a statistically significant effect on SCE. The SCE frequencies observed were low compared with the positive controls. It is possible that in some cases an effect might have been missed because of the size of the dose interval (log dilutions) used in preliminary toxicity tests. Treatment with concentrations causing precisely 50% growth inhibition was not achieved in most of the SCE tests.

The use of separate tests rather than duplicate cultures resulted in some disagreement between results obtained with the same chemical tested on different occasions. In general, however, this did not affect the final conclusions.

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Tests for the induction of chromosomal aberrations in human peripheral lymphocytes in culture

Catherine A. Howard, Timothy Sheldon and Christopher R. Richardson

Imperial Chemical Industries PLC, Central Toxicology Laboratory, Alderley Park, Cheshire (Great Britain)

Summary

5 compounds were tested in the in vitro human lymphocyte cytogenetic assay, these were hexamethylphosphoramide (HMPA), benzene (BEN), diethylstilbestrol (DES), caprolactam (CAP) and benzoin (ZOIN). All chemicals were tested using the same experimental method in cells from both a male and a female donor, and their response compared to a direct clastogen mitomycin C, and a clastogen requiring metabolic activation, cyclophosphamide.

The positive control substances both gave the expected response in all experiments. The carcinogens HMPA, BEN and DES all gave a statistically significant dose-related positive response, however, this response was comparatively weak in the case of DES. Chromosomal interchanges having been observed in cells following treatment with either HMPA or BEN. Of the two noncarcinogens tested ZOIN gave the expected response. However CAP gave a comparatively strong dose-related increase in aberrations in the cells from both donors.

The prediction rate given by this assay on the 5 chemicals tested was 80%.

Introduction

It has been shown that certain chemical mutagens have the ability to produce chromosome damage in human lymphocytes after in vitro exposure to the chemical. This damage can be seen after stimulation of the lymphocyte into division by phytohaemagglutinin (PHA) and this phenomenon was utilised in an in vitro test system to determine the clastogenic (chromosome-breaking) potential of compounds, as part of the CSSTT project.

5 compounds were tested: hexamethyl phosphoramide (HMPA), benzene, diethylstilbestrol (DES), caprolactam and benzoin. All were tested using the same experimental method, and compared to a direct-acting mutagen mitomycin C (MMC), and a mutagen requiring metabolic activation, cyclophosphamide.

Materials and methods

Test compounds

As described in this volume by J. Ashby (Chapter 1, pp. 3–9).

Positive control substances

Mitomycin C was obtained from Sigma Chemical Co., St. Louis, MO. Cyclophosphamide was obtained from Ward Blenkinsop and Co. Ltd, London.

Working concentrations

Positive control substances were diluted in physiological saline immediately prior to use.

Working concentrations of test compounds were prepared in dimethyl sulphoxide, except for caprolactam, which was dissolved in physiological saline. In all experiments negative control cultures

TABLE 1

CYTOTOXICITY OF TEST CHEMICALS TO HUMAN LYMPHOCYTES

Compound	Top dose [a] ($\mu g/cm^3$)	Middle dose ($\mu g/cm^3$)	Low dose ($\mu g/cm^3$)
HMPA	2500.00	500.00	100.00
Benzene	88.00	44.00	9.00
DES	0.20	0.10	0.02
Caprolactam	2750.00	1370.00	270.00
Benzoin	2.00	1.00	0.20

[a] 50–80% reduction in mitosis when compared to negative controls.

were treated with a volume of solvent equivalent to that used in test cultures. This was 10 μl for DMSO and 100 μl for saline.

Media

Incubation of the cells was in RPMI 1640 (Gibco) tissue-culture medium containing 10% foetal bovine serum (FBS) 0.1% PHA and 100 units/cm^3 penicillin and streptomycin.

Chemicals for processing cells

Cells were arrested in metaphase using colchicine (Sigma (London) Chemical Co.), and fixed using glacial acetic acid and methanol (Hopkin

TABLE 2

HMPA

	Concentration ($\mu g/ml$)	Number of cultures	Number of cells analysed	% of cells showing						
				Polyploidy (1)	Chromatid gaps (2)	Chromatid aberrations (3)	Chromosome aberrations (4)	Multiple aberrations (5)	All aberrations excluding gaps (6)	All aberrations including gaps (7)
Male	2500	3	300	0	4.3	2.0	3.0	0	5.0 **	9.3
−S9	500	3	300	0.3	3.0	2.0	1.0	0	3.0 **	6.0
	100	3	300	0.6	7.0	1.0	0	0	1.0	8.0
	0	3	300	0	1.3	0	0	0	0	1.3
	MITC	1	94	0	2.0	23.0	35.0	0	56.4 **	58.0
Female	2500	3	300	0	3.3	0.6	2.6	0	3.3	6.6
−S9	500	3	300	0.3	3.6	1.6	4.0	0	5.6 **	9.3
	100	3	300	0	2.3	0.6	1.3	0	2.0	4.3
	0	2	200	0	9.0	0	1.0	0	1.0	10.0
	MITC	2	188	0	8.0	12.0	11.0	0	23.4 **	31.3
Male	2500	3	300	0	5.3	1.3	3.3	0	4.6 **	10.0
+S9	500	3	300	0	6.0	2.3	1.0	0	3.3 **	9.3
	100	3	300	0.3	3.0	0.6	1.0	0	1.0	3.0
	0	3	300	0	1.2	0	0	0	0	1.3
	CTX	2	200	0	11.5	20.0	33.0	0	53.0 **	64.0
Female	2500	2	200	0	5.0	1.0	2.5	0	4.0 **	9.0
+S9	500	3	300	0	3.0	1.0	1.3	0	2.3 *	5.3
	100	3	300	0	2.0	1.3	1.0	0	2.3 *	4.3
	0	2	200	0.5	1.5	0	0	0	0	1.5
	CTX	2	85	0	18.0	10.0	19.0	0	29.0 **	47.0

* Statistically significantly different from controls $p < 0.05$ (Fisher's exact test one-sided).

** Statistically significantly different from controls $p < 0.01$ (Fisher's exact test one-sided).

(1) Polyploidy or endoreduplication.

(2) Gaps and/or iso-gaps.

(3) Breaks or single fragments or exchange figures or acentric rings or chromatid minutes.

(4) Acentric fragments or dicentrics or translocations or rings or minutes.

and Williams). Preparations were stained with Geimsa Gurrs R66 (Difco).

S9 mix

Tests requiring auxiliary metabolic activation were carried out using 200 μl of S9 mix consisting of a 1 : 1 mixture of S9 and cofactors. The liver homogenate was derived from rats induced with Aroclor 1254.

Treatment of blood cultures

The method used was that of Richardson and Wildgoose (1982), with minor modifications. The required amount of blood was drawn aseptically by venapuncture from a donor of each sex (with a previously established low incidence of chromosomal damage). The blood was collected into lithium/heparin tubes and mixed well. The cultures were set up using 0.5 cm^3 of whole blood in 9.5 cm^3 growth medium, and incubated at 37 °C.

Cytotoxicity tests

Preliminary cytotoxicity tests of the chemicals were performed in the absence and presence of auxiliary metabolic activation, using a range of dilutions. The top dose level for each chemical was selected where biological activity was shown by inhibition of mitosis by 50–80% when compared with negative controls (Table 1).

Frequency per cell of					
Chromatid gaps (2)	Chromatid breaks (8)	Chromatid exchanges (9)	Chromosome aberrations (4)	All aberrations excluding gaps (6)	All aberrations including gaps (7)
0.06	0.01	0	0.04	0.05	0.12
0.03	0.02	0	0.01	0.03	0.06
0.07	0.01	0	0	0.01	0.08
0.01	0	0	0	0	0.01
0.02	0.09	0.13	0.48	0.70	0.72
0.04	0.01	0	0.03	0.03	0.07
0.05	0.01	0.01	0.04	0.06	0.09
0.03	0.01	0	0.01	0.02	0.05
0.09	0	0	0.01	0.01	0.10
0.08	0.05	0	0.16	0.29	0.39
0.06	0.01	0	0.03	0.05	0.10
0.06	0.01	0	0.01	0.03	0.09
0.03	0.01	0	0.01	0.02	0.05
0.01	0	0	0	0	0.01
0.15	0.13	0.08	0.44	0.64	1.28
0.05	0.02	0	0.03	0.04	0.09
0.03	0.01	0	0.01	0.02	0.05
0.02	0.01	0	0.01	0.02	0.04
0.01	0	0	0	0	0.01
0.17	0.05	0	0.19	0.29	0.47

(5) Any combination of identifiable chromatid or chromosome aberrations.
(6) All types of chromatid and chromosome-type aberrations excluding chromatid gaps.
(7) All types of chromatid and chromosome-type aberrations including chromatid gaps.
(8) Breaks and/or single fragments.
(9) All chromatid exchanges including centric rings and symmetrical and asymmetrical interchanges.

Test without metabolic activation

The cultures were treated with the test chemicals 44 h after initiation. The top dose level was predetermined for each chemical as, HMPA 2500 $\mu g/cm^3$, benzene 88.0 $\mu g/cm^3$, DES 0.2 $\mu g/cm^3$, caprolactam 2750 $\mu g/cm^3$ and benzoin 2.0 $\mu g/cm^3$. Two lower doses were also included (Table 1). The positive control substance mitomycin C was dosed at 0.5 $\mu g/cm^3$ final concentration, and the appropriate negative solvent control was added at an equivalent volume to that of the dosing solutions. The blood cultures were exposed to the test chemicals for 3 h after which the growth medium was replaced with fresh medium.

Tests with metabolic activation

The experimental procedure was similar to the test without metabolic activation with modifications, the positive control substance requiring metabolic activation was cyclophosphamide (100 $\mu g/cm^3$ final concentration) and dose levels of test chemical plus relevant controls were incubated for 30 min with 200 μl of S9 mix before being added to the blood cultures.

The blood cultures were exposed to the test chemicals for 3 h, after which the growth medium was replaced with fresh medium. Lymphocyte cultures treated with benzene were agitated throughout the exposure time to maintain homogenicity of the chemical through the culture.

Processing of blood cultures

Dividing lymphocytes were arrested in metaphase by treating with 0.01 mg/cm^3 colchicine. Hypotonic treatment was for 12 min in 0.075 M KCl followed by fixation in acetic/methanol (1 : 3). The slides were then stained in 10% Giemsa for 7 min.

TABLE 3

BENZENE

	Concentration (μg/ml)	Number of cultures	Number of cells analysed	% of cells showing						
				Polyploidy (1)	Chromatid gaps (2)	Chromatid aberrations (3)	Chromosome aberrations (4)	Multiple aberrations (5)	All aberrations excluding gaps (6)	All aberrations including gaps (7)
Male	88	2	200	0.5	2.5	2.5	4.0	0	6.5 **	9.0
−S9	44	2	200	0	0.5	1.0	3.0	0	3.5 **	4.0
	9	2	200	0	3.5	3.0	4.5	0	7.5 **	10.5
	0	3	300	0	0.3	0	0	0	0	0.3
	MITC	1	50	0	2.0	18.0	20.0	1.0	28.0 **	30.0
Female	88	2	200	0	4.5	3.0	1.0	0	3.5 **	8.5
−S9	44	2	200	0	2.0	0	1.0	0	1.5	3.0
	9	2	200	1.0	0	0	0.5	0	0.5	1.5
	0	3	300	0	1.6	0.3	0	0	0.5	2.0
	MITC	1	100	0	7.0	34.0	30.0	0	64.0 **	71.0
Male	88	3	200	1.0	3.0	1.0	3.5	0	5.0 **	7.0
+S9	44	3	200	0	2.5	2.0	2.0	0	5.0 **	7.0
	9	3	200	0	2.0	2.5	2.5	2.5	5.0 **	7.0
	0	3	300	0	1.0	0	0	0	0	1.0
	CTX	1	50	0	0	24.0	26.0	0	40.0 **	40.0
Female	88	3	200	0.5	0	2.5	1.5	0	4.0 **	4.0
+S9	44	3	200	0	2.5	1.5	1.0	0	2.5 *	5.0
	9	2	200	0.05	0.05	0.05	1.0	0	1.5	2.0
	0	2	200	0	0	0	0	0	0	0
	CTX	2	77	0	8.0	13.5	18.5	0	32.0 **	35.0

For footnotes see Table 2.

The slides were mounted in DPX and coded to avoid observer bias. Where possible 200 cells were analysed from each dose level of benzene, DES, caprolactam and benzoin. In the study of HMPA 300 cells were analysed from each dose level. In all studies sufficient cells were analysed from cyclophosphamide and mitromycin C treated cultures to establish a significant positive response.

Statistical analysis

The statistical analysis was confirmed to the evaluation of percentage abnormal cells excluding gaps. Each treated group was compared with the negative control group using Fisher's exact test (one-sided). In addition, a test for linear trend was carried out with dose for each experimental compound.

Results and discussion

Criteria for assessment of data

The data have been assessed excluding chromosomal gaps. This was due to their unpredictable incidence in control cultures: one set of control cells gave a high frequency (10%) of gaps and another set from the same donor gave a 0% incidence of gaps.

The positivity or negativity of data has been determined on the observation of a statistically significant increase in aberrations at one or more dose levels, combined with a comparison with historical negative control data from these laboratories.

Added weighting has been given to results where a dose response has been observed. However, it is not uncommon to observe reduced aberration frequencies at high doses of compound due to mitotic

Frequency per cell of					
Chromatid gaps (2)	Chromatid breaks (8)	Chromatid exchanges (9)	Chromosome aberrations (4)	All aberrations excluding gaps (6)	All aberrations including gaps (7)
0.03	0.02	0	0.04	0.07	0.09
0.01	0.01	0	0.04	0.05	0.05
0.04	0.03	0	0.05	0.08	0.11
0.01	0	0	0	0	0.01
0.02	0.08	0.10	0.20	0.28	0.30
0.05	0.02	0.01	0.02	0.05	0.09
0.02	0.01	0	0.01	0.02	0.04
0.01	0.01	0	0	0.01	0.02
0.01	0.01	0	0	0.01	0.02
0.08	0.07	0.27	0.39	0.75	0.83
0.03	0.01	0	0.05	0.07	0.10
0.03	0.01	0	0	0.05	0.08
0.02	0.02	0.01	0.03	0.05	0.07
0.01	0	0	0	0	0.01
0	0.10	0.12	0.26	0.40	0.40
0	0.03	0	0.02	0.05	0.05
0.03	0.01	0	0.01	0.03	0.05
0.01	0.01	0	0.01	0.02	0.02
0	0	0	0	0	0
0.10	0.19	0.35	1.88	2.43	2.53

delay (UKEMS Guidelines, 1983).

Due to the rarity of chromosomal interchanges in control cultures in our laboratories (zero incidence in excess of 10000 cells analysed), these aberrations were accorded greater significance when the clastogenic activity of a compound was in doubt after consideration of the other criteria of assessment.

Hexamethylphosphoramide (Table 2)

There was a clear dose-related increase in chromosomal aberrations observed in the male donor both with and without auxiliary metabolic activation and in the female donor plus metabolic activation. However, statistically significant increases in aberrations were observed with both donors for all treatments. This finding was strengthened by the observation of one cell with an interchange. It was therefore concluded that HMPA was clastogenic in human lymphocytes in vitro.

Benzene (Table 3)

There was a clear dose-related increase in chromosomal aberrations for both male and female donors with and without auxiliary metabolic activation. There were also statistically significant increases in aberrations with all treatment regimes but the response was more marked in the male donor than in the female.

Chromatid exchanges were observed in cultures from both donors. There appeared to be little or no influence in the magnitude of the response obtained, due to the addition of auxiliary metabolic activation. In our studies with benzene we found that unless cultures were agitated during exposure, vast differences in both cytotoxicity and aberration frequencies were obtained. It was there-

TABLE 4

DIETHYLSTILBESTROL + AUXILIARY METABOLIC ACTIVATION

	Concentration (μg/ml)	Number of cultures	Number of cells analysed	% of cells showing						
				Polyploidy (1)	Chromatid gaps (2)	Chromatid aberrations (3)	Chromosome aberrations (4)	Multiple aberrations (5)	All aberrations excluding gaps (6)	All aberrations including gaps (7)
Male	0.2	2	200	0	1.0	2.0	2.5	0	3.5 **	4.5
−S9	0.1	2	200	0.5	3.0	3.0	1.0	0	2.5 **	5.5
	0.02	2	200	0	2.0	2.0	0	0	0	2.0
	0	3	300	0	3.6	0	0	0	0	0
	MITC	1	94	0	2.0	20.0	33.0	0	53.0 **	55.0
Female	0.2	2	200	0	1	4	2	0	5.0 *	6
−S9	0.1	2	200	0	0	0	0	0	0	0
	0.02	1	100	0	0	0	0	0	0	0
	0	2	200	0	1	1	0	1	1	10
	MITC	1	188	0	8	12	10	0	23.0 **	27.6
Male	0.2	2	200	0	0	0.5	1.0	0	1.5	1.5
+S9	0.1	2	200	0	3.0	5.0	0.5	0	2.5 **	5.5
	0.02	2	200	0	0.5	0.5	0	0	0	0.5
	0	3	300	0	1.3	0	0	0	0	0
	MTC	2	200	0	11.5	20.0	33.0	0	53.0 **	64.5
Female	0.2	2	200	0	1.0	3.0	2.0	0	4.0 **	5.0
+S9	0.1	2	200	0	0.15	1.0	0	0	0.5	1.0
	0.02	2	200	0	0	0	1.5	0	1.5	1.5
	0	2	200	0.5	1.5	0	0	0	0	1.5
	MITC	2	85	0	18.0	13.0	19.0	0	25.0 **	39.0

For footnotes see Table 2.

fore concluded that benzene gave a positive clastogenic response in human lymphocytes in vitro.

Diethylstilbestrol (Table 4)

There were small but statistically significant increases in chromosomal aberrations in both male and female donors. However, these effects were only shown to be dose-related in cultures without metabolic activation. It was noted during these experiments that DES was extremely cytotoxic, the top dose of 0.2 μg/ml inhibiting mitosis by 80%. Unfortunately, insufficient time was available to do serial sampling time experiments.

It was therefore concluded that DES gave a very weak clastogenic response.

Caprolactam (Table 5)

There was a clear dose-related increase in chromosomal aberrations in both donors with and without auxiliary metabolic activation. These levels of damage induced were statistically significant in all cases with the exception of the male donor or in the 270 μg/ml dose group without activation. The test response was unusual in that the chromosomal aberrations observed were confined to chromatid and chromosome breaks. However, it was also noted that on the inclusion of chromosome and chromatid gaps the aberration frequencies were doubled.

It was concluded that caprolactam was clastogenic in human lymphocytes in vitro.

Benzoin (Table 6)

There were dose-related increases in levels of chromosomal damage observed in the cultures treated with benzoin in both donors. However,

Frequency per cell of					
Chromatid gaps (2)	Chromatid breaks (8)	Chromatid exchanges (9)	Chromosome aberrations (4)	All aberrations excluding gaps (6)	All aberrations including gaps (7)
0.01	0.01	0	0.2	0.04	0.05
0.03	0.02	0	0.01	0.03	0.06
0.02	0	0	0	0	0.02
0.04	0	0	0	0	0
0.02	0.09	0.13	0.48	0.70	0.72
0.01	0.03	0	0.02	0.05	0.06
0	0	0	0	0	0
0	0	0	0	0	0
0.01	0	0	0.01	0.01	0.02
0.08	0.05	0.07	0.06	0.23	0.28
0	0.01	0	0.01	0.02	0.02
0.03	0.02	0	0.01	0.03	0.06
0.01	0	0	0	0	0.01
0.01	0	0	0	0	0.01
0.15	0.18	0.08	0.45	0.69	0.83
0.01	0.02	0	0.02	0.04	0.05
0.01	0.01	0	0	0.01	0.01
0	0	0	0.02	0.02	0.02
0.02	0	0	0	0	0.02
0.18	0.02	0.03	0.19	0	0.39

TABLE 5

CAPROLACTAM

	Concentration (μg/ml)	Number of cultures	Number of cells analysed	% of cells showing						
				Polyploidy (1)	Chromatid gaps (2)	Chromatid aberrations (3)	Chromosome aberrations (4)	Multiple aberrations (5)	All aberrations excluding gaps (6)	All aberrations including gaps (7)
Male	2750	2	200	0.5	13.5	16.5	4.0	0	7.0 **	20.5
− S9	1370	2	200	1.5	4.5	6.0	3.5	0	5.0 **	9.5
	270	2	200	1.0	4.0	5.5	0.5	0	2.0	6.0
	0	2	200	0	1.5	0	0	0	0	1.5
	MITC	1	50	0	2.0	1.8	20.0	1.0	28.0 **	3.0
Female	2750	2	200	0.5	6.5	11.5	2.5	0	7.5 **	14.0
− S9	1370	2	200	0.5	7.0	8.0	4.5	0	5.5 **	12.5
	270	2	200	0	4.0	5.0	1.5	0	2.5 **	6.5
	0	2	200	0	1.0	0	0	0	0	0
	MITC	1	100	0	7.0	34.0	30.0	0	64.0 **	71.0
Male	2750	2	200	0	8.0	14.5	3.5	0	10.0 **	18.0
+ S9	1370	2	187	0	4.8	6.4	2.7	0	4.3 **	9.1
	270	2	200	0	4.0	7.0	0.5	0	3.5 **	7.5
	0	2	200	0	2.0	0	0	0	0	2.0
	CTX	1	50	0	0	24.0	26.0	0	40.0 **	40.0
Female	2750	2	200	0.5	10.5	12.5	6.0	0	8.0 **	18.5
+ S9	1370	2	200	0	2.0	3.5	3.5	0	5.0 **	7.0
	270	2	200	0	6.0	11.0	4.0	0	9.0 **	15.0
	0	2	200	0	2.5	0	0	0	0	2.5
	CTX	2	77	0	6.0	13.5	18.5	0	32.0 **	35.0

For footnotes see Table 2.

these did not achieve statistical significance and were within historical control frequencies.

It was therefore concluded that benzoin is not clastogenic in human lymphocytes in vitro.

Frequency per cell of					
Chromatid gaps (2)	Chromatid breaks (8)	Chromatid exchanges (9)	Chromosome aberrations (4)	All aberrations excluding gaps (6)	All aberrations including gaps (7)
0.17	0.03	0	0.04	0.07	0.22
0.05	0.02	0	0.04	0.05	0.10
0.05	0.01	0	0.01	0.02	0.07
0.03	0	0	0	0	0.03
0.02	0.08	0.10	0.20	0.28	0.30
0.08	0.05	0	0.03	0.08	0.16
0.07	0.01	0	0.05	0.05	0.11
0.01	0.01	0	0.02	0.03	0.08
0	0	0	0	0	0
0.08	0.07	0.27	0.39	0.75	0.83
0.08	0.07	0	0.04	0.10	0.18
0.05	0.01	0	0.03	0.04	0.12
0.05	0.03	0	0.01	0.04	0.08
0.02	0.01	0	0	0	0.01
0	0.01	0.12	0.26	0.40	0.40
0.14	0.02	0	0.07	0.09	0.23
0.03	0.02	0	0.04	0.06	0.09
0.06	0.11	0	0.04	0.1	0.19
0.03	0	0	0	0	0.03
0.10	0.19	0.30	1.88	2.43	2.53

TABLE 6

BENZOIN

	Concentration (μg/ml)	Number of cultures	Number of cells analysed	% of cells showing						
				Polyploidy (1)	Chromatid gaps (2)	Chromatid aberrations (3)	Chromosome aberrations (4)	Multiple aberrations (5)	All aberrations excluding gaps (6)	All aberrations including gaps (7)
Male −S9	2.0	3	200	0	0	0.5	1.5	0	2.0	2.0
	1.0	2	200	0	1.0	1.5	0.5	0	1.0	2.0
	0.2	2	200	0	1.0	1.0	2.0	0	2.0	3.0
	0	3	300	0	3.6	0	0	0	0	3.6
	MITC	1	94	0	2.0	10.0	33.0	0	53.0 **	55
Female −S9	2.0	2	200	0	2.5	3.0	1.5	0	2.0	4.5
	1.0	2	200	0	0	0	0.5	0	0.5	0.5
	0.2	2	200	0	0.5	0.5	0.5	0	0.5	1.0
	0	2	200	0	9.0	0	1.0	0	1.0	10.0
	MITC	2	188	0	8.0	6.9	10.0	0	23.0 **	27.6
Male +S9	2.0	3	200	0	0.5	1.5	1.5	0	2.5 **	3.0
	1.0	3	200	0	1.0	1.5	0	0	0.5	1.5
	0.2	3	200	0	0	0	0	0	0	0
	0	3	300	0	1.3	0	0	0	0	0
	CTX	2	200	0	11.5	20.0	33.0	0	53.0 **	65.4
Female +S9	2.0	2	200	0	0	0	0.5	0	0.4	0.4
	1.0	2	200	0	0	0	0.4	0	0.4	0.4
	0.2	2	200	0	1.0	1.0	0	0	1.0	2.0
	0	2	200	0.5	1.5	0	0	0	0	1.5
	CTX	2	85	0	18.0	13.0	19.0	0	25.0 **	39.0

For footnotes see Table 2.

Frequency per cell of					
Chromatid gaps (2)	Chromatid breaks (8)	Chromatid exchanges (9)	Chromosome aberrations (4)	All aberrations excluding gaps (6)	All aberrations including gaps (7)
0	0.01	0	0.02	0.03	0.03
0.01	0	0	0.01	0.01	0.02
0.01	0	0	0.03	0.03	0.04
0.04	0	0	0	0	0.04
0.02	0.09	0.13	0.18	0.70	0.72
0.03	0	0	0.02	0.02	0.05
0	0	0	0.01	0.01	0.01
0.01	0	0	0.01	0.01	0.02
0.09	0	0	0.01	0.01	0.10
0.08	0	0.07	0.16	0.23	0.28
0.01	0.01	0	0.02	0.03	0.04
0.01	0.01	0	0	0.01	0.02
0	0	0	0	0	0
0.01	0	0	0	0	0.01
0.14	0.18	0.08	0.45	0.69	0.83
0	0	0	0.01	0.01	0.01
0	0	0	0.01	0.01	0.01
0.01	0.01	0	0	0.01	0.02
0.02	0	0	0	0	0.02
0.18	0.02	0.03	0.19	0.25	0.39

Conclusions

The results indicate that HMPA, benzene, caprolactam and DES are weakly clastogenic to human lymphocytes in vitro, while benzoin did not induce any significant chromosome-breaking effects in the same assay.

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The test for sister-chromatid exchanges in Chinese hamster V79 cells in culture

G.F. van Went

Cytogenetics Department, Division of Toxicology and Chemical Analysis of Foods, National Institute of Public Health, Bilthoven (The Netherlands)

Summary

Hexamethylphosphoramide (HMPA), *o*-toluidine, benzene, safrole and caprolactam were tested in the in vitro SCE test using V79 cells. An increase in mean number of SCEs to 150% of the control value, in combination with a dose–response, was used as a criterion for the assignment positive in the assay system. A tentative judgment may thus be given to the chemical substances investigated.

Caprolactam and benzene were negative in the assay, with as well as without metabolic activation. Hexamethylphosphoramide and safrole needed metabolic activation to give a positive response, the latter at a lower molar level. *o*-Toluidine was positive both with and without the use of S9. In comparison to HMPA the dose level was lower and the dose–response curve steeper. The increases in the SCE values for the positive control substances — diethylnitrosamine and benzo[*a*]pyrene — were highly significant, which was an indication for a satisfactory activity of the metabolic system. The results of the duplicate experiments generally matched very well. The absence of mycoplasma in the V79 cells was verified.

In the test on sister-chromatid exchanges (SCE), exchanges between both chromatids of a chromosome are registered. SCEs are the reflection of exchanges between DNA strands at homologous loci.

The assay is based on the higher affinity for heavy atoms of some chromosome stains, like the fluorescent stain Hoechst 33258. By selective incorporation of such a heavy atom (bromium) in DNA, the fluorescence is quenched and a differential staining of the chromatids of one chromosome can be obtained. The bromium required is added to the cultures as bromodeoxyuridine (BrdUrd).

A great number of variables may lead to a change in the number of SCEs. A standardization was therefore worked out concerning especially brdUrd concentration, amount of S9 fraction added to the S9 mix and checking of the cells for the presence of mycoplasma. Arginine deprivation or inhibition of protein synthesis in general may be the cause for changes in the SCE rate (Macrae et al., 1979; Sono and Sakaguchi, 1981; Takehisa, 1982). As in our experience the cell lines used are rather frequently infected with *Mycoplasma arginini* — using arginine for its metabolism — the possibility of a change in SCE rate might also exist here.

For the evaluation of the system as a short-term screening test a wide range of compounds from different chemical classes, carcinogens and non-carcinogens, have thus far been tested (Abe and Sasaki, 1982; De Serres and Ashby, 1981; Latt et al., 1981, 1982).

The aim of the present study is both the use of the test for identification of genotoxic agents as well as the establishment of its predictive value in short-term testing for chemical carcinogens. 4 carcinogens found negative in the Salmonella assay — hexamethylphosphoramide (HMPA), *o*-toluidine (TOL), benzene (BEN), and safrole

TABLE 1

SURVEY OF (CYTO)TOXICITY RESULTS FOR THE 5 CHEMICALS TESTED FOR 24 h

(1)HMPA	control	c	10^{-2}	10^{-2}	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-5}	c	control
	++++	++++	++	++	+++	++++	++++	++++	++++	++++	++++	++++
(2) *o*-Toluidine	control	c	10^{-2}	10^{-2}	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-5}	c	control
	++++	++++	+	+	+++	++++	+++	+++	++++	++++	++++	++++
(3) Benzene	control	c	10^{-2}	10^{-2}	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-5}	c	control
	++++	++++	++/+++	++	++++	+++	+++	++++	++++	++++	++++	++++
(4) Safrole	control	c	10^{-2}	10^{-2}	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-5}	c	control
	++++	++++	?/0	?/0	++++	++++	++++	++++	+++	++++	++++	++++
(5) Caprolactam	control	c	10^{-2}	10^{-2}	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-5}	c	control
	++++	++++	++	++	++++	+++	++++	++++	+++	++++	++++	++++

++++, excellent growth.
+++, outgrowth ±3/4 of surface.
++, outgrowth ±1/2.
+, cellgrowth ±1/4.
0, no growth.
c, control.

(SAF) — together with the noncarcinogen caprolactam (CAP) were tested.

Material and methods

Cell cultures

V79 cells (NIGMS Human Genetic Mutant Cell Repository Institute for Medical Research, Camden, NJ) with a generation time of 11–12 h were used. The cells were tested for the presence of mycoplasma (A.A. Polak-Vogelzang, Laboratory for Control of Bacterial Vaccines, National Institute of Public Health) by cultivation methods as well as by the DNA–fluorochrome staining technique. No mycoplasmas were detected.

The cells were stored at −80 °C; they were thawed immediately before an experiment and cultured for 4 days in P140 petri dishes (Greiner) in a density of 0.35×10^6 cells per dish.

The cells were cultured in a medium consisting of 85% Ham's F10 without hypoxanthine (Flow) and 15% newborn calf serum (Gibco). To this 1 ml of folic acid (8.68 mg/ml in 14% $NaHCO_3$), 4 ml of glutamine (200 mM) and penicillin (100 i.u./ml) and streptomycin (100 μg/ml) were added. Culturing took place in a CO_2 atmosphere at 37 °C.

After trypsinization in a 0.25% trypsin solution, the cells were seeded in F150 culture flasks (Corning) in a concentration of 1×10^6 cells per flask. The cells were grown for 24 h and then treated with the test chemical for 2 h with and without metabolic activation. The S9 mix used for this purpose, in a final concentration of 10%, was derived from Aroclor-1254-treated rats.

Treatment procedure

Cytotoxicity. To obtain a general impression about the cytotoxicity of the test chemicals, the substances were added in different concentrations without metabolic activation, to microtiter plates (Dynatech) having 8 rows of 12 wells (Fig. 1; Michell et al., 1981); 0.2 ml of a cell suspension containing $6 \cdot 10^4$ cells/ml was placed into each well. The cells were cultured for 24 h, then the medium was carefully removed and replaced by medium in which the test chemical was dissolved in the appropriate concentration. After another 24 h exposure a semi quantitative method was used for the outgrowth of the cells. A survey of the test results of the 5 chemicals is given in Table 1. The concentration range was between 10^{-5} and 10^{-2} moles/l.

Chemical treatment in SCE experiments. The chemical substances tested were: HMPA, TOL, BEN, SAF and CAP. All were derived in highly purified form from J. Ashby, Imperial Chemical Industries Ltd. As a negative control dimethyl sulfoxide (DMSO, Merck) was used in a 1% concentration. As a positive control diethylnitrosamine (DEN, Fluka) was chosen, except for the first experiment ($HMPA_1$) in which benzo[*a*] pyrene was used.

The dose levels used were for HMPA, BEN and CAP 5×10^{-3}, 10^{-2}, 2.5×10^{-2} and 5×10^{-2} moles/l. For TOL 2.5×10^{-3}, 5×10^{-3}, 2.5×10^{-2} and 5×10^{-2} moles/l and for SAF 10^{-4}, 5×10^{-4}, 10^{-3} and 5×10^{-3} moles/l.

The cells were grown in the dark for 24 h at 37 °C, washed with prewarmed Hanks' solution that was replaced by 6 ml of medium. This medium consisted of (a) in the activated cultures: 3.94 ml of F10 culture medium, 2 ml of S9-mix (see below) and 0.06 ml of the appropriate chemical solution, and (b) in the cultures without activation: 5.94 ml of F10 culture medium and 0.06 ml of the chemical test solution.

Each dose level was tested with or without metabolic activation during 2 h. All substances were tested in duplicate experiments.

After the 2-h treatment, the cells were washed twice with Hanks' solution and then recultured for 24 h in complete medium with BrdUrd in a final concentration of 10 μmoles/l. All handling after BrdUrd addition to the cultures was done in semi-darkness. To each F150 flask containing 20 ml of culture medium 1 ml of a 0.00025% of colcemid solution was added 2 h before harvesting the cells.

The cells were trypsinized, suspended in fresh medium and spun down 10 min at 1000 rpm. They were then subjected to a hypotonic treatment of 5 min at 37 °C in a 0.075 moles/l KCl solution, respun and fixed in 3 changes of fixative: glacial acetic acid and methylalcohol 1 : 3.

Chromosome preparations were made by the air-drying method.

The slides were stained for 15 min in Hoechst 33258 in a final concentration of 6 mg/l and rinsed in phosphate balanced salt solution (PBS).

TABLE 2

SURVEY OF SCE DATA ON 5 REFERENCE COMPOUNDS, IN DUPLICATE EXPERIMENTS [a]

Compound dose (moles/l)	−S9 2 h		+S9 2 h	
	SCE ± S.E.		SCE ± S.E.	
	Expt. I	Expt. II	Expt. I	Expt. II
DMSO [b] 1%	7.9 ± 0.4 ***	6.4 ± 0.3	7.7 ± 0.4	6.4 ± 0.3
HMPA 5×10^{-3}	n.d. [c]	n.a.	10.2 ± 0.4 ***	n.a.
HMPA 10^{-2}	7.6 ± 0.4	7.1 ± 0.5	10.5 ± 0.6 ***	9.1 ± 0.4 ***
HMPA 2.5×10^{-2}	8.0 ± 0.6	7.9 ± 0.5 *	10.4 ± 0.5 ***	9.7 ± 0.5 ***
HMPA 5×10^{-2}	8.4 ± 0.6	7.3 ± 0.5	13.8 ± 0.7 ***	12.7 ± 0.6 ***
DMSO 1%	6.9 ± 0.4	7.5 ± 0.3	8.2 ± 0.5	7.3 ± 0.5
TOL 2.5×10^{-3}	8.9 ± 0.6 *	8.9 ± 0.8	11.6 ± 0.6 ***	10.9 ± 0.4 ***
TOL 5×10^{-3}	8.4 ± 0.7	8.0 ± 0.5	13.5 ± 1.7 **	13.0 ± 1.0 ***
TOL 10^{-2}	11.7 ± 1.6 **	16.3 ± 1.3 ***	14.2 ± 1.2 ***	16.5 ± 1.8 ***
TOL 2.5×10^{-2}	11.2 ± 0.6 ***	n.a.	15.8 ± 1.2	n.a.
TOL 5×10^{-2}	n.d.	n.a.	n.d.	n.a.
DMSO 1%	5.7 ± 0.3	7.3 ± 0.5	7.9 ± 0.4	6.4 ± 0.4
BEN 5×10^{-3}	n.a. [c]	n.a.	n.a.	n.a.
BEN 10^{-2}	6.6 ± 0.5	7.0 ± 0.5	6.4 ± 0.4 *	8.6 ± 0.5 **
BEN 2.5×10^{-2}	6.3 ± 0.4	7.5 ± 0.5	6.2 ± 0.4 **	7.7 ± 0.5
BEN 5×10^{-2}	6.3 ± 0.4	6.4 ± 0.5	8.0 ± 0.5	7.6 ± 0.6
DMSO 1%	8.0 ± 0.6	7.5 ± 0.4	5.5 ± 0.3	8.2 ± 0.5
SAF 10^{-4}	n.a.	n.a.	n.a.	n.a.
SAF 5×10^{-4}	6.3 ± 0.5 *	7.9 ± 0.5	7.1 ± 0.4 **	12.1 ± 0.9 ***
SAF 10^{-3}	6.2 ± 0.4 *	7.5 ± 0.6	8.1 ± 0.4 ***	10.9 ± 0.8 **
SAF 5×10^{-3}	7.8 ± 0.6	7.9 ± 0.6	8.8 ± 0.8 ***	13.3 ± 0.8 ***
DMSO 1%	6.3 ± 0.5	9.6 ± 0.5	7.8 ± 0.4	8.5 ± 0.6
CAP 5×10^{-3}	n.a.	n.a.	n.a.	n.a.
CAP 10^{-2}	7.0 ± 0.5	8.2 ± 0.9	8.0 ± 0.5	7.8 ± 0.5
CAP 2.5×10^{-2}	5.7 ± 0.4	8.0 ± 0.7	6.7 ± 0.4	8.2 ± 0.5
CAP 5×10^{-2}	6.7 ± 0.5	8.5 ± 0.7	9.5 ± 0.5 *	8.8 ± 0.7

[a] Each value is the mean of 30 cells.

[b] abbreviations: DMSO, dimethyl sulfoxide; HMPA, hexamethylphosphoramide; TOL, *o*-toluidine; BEN, benzene; SAF, safrole; CAP, caprolactam.

[c] n.d., culture not done; n.a., culture not analyzed.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

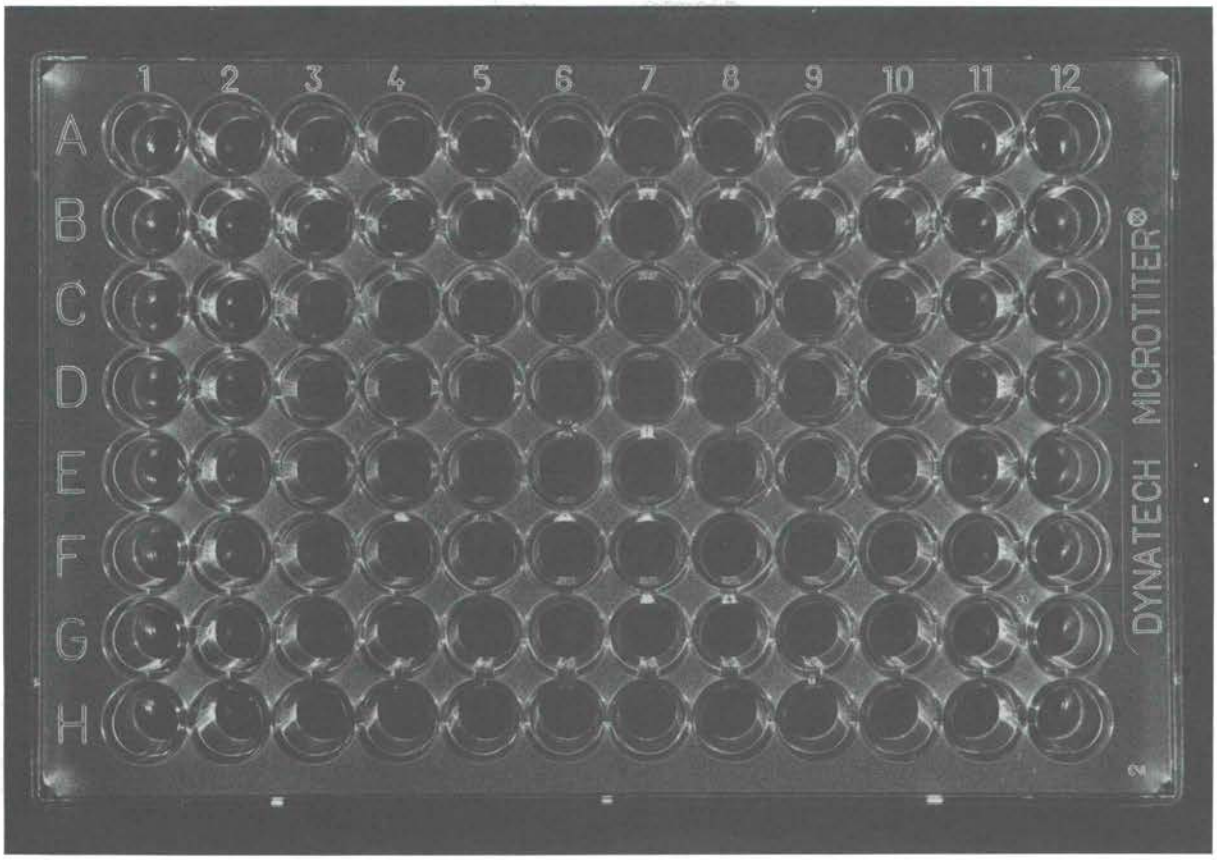


Fig. 1. Microtiter plate for (cyto)toxicity testing.

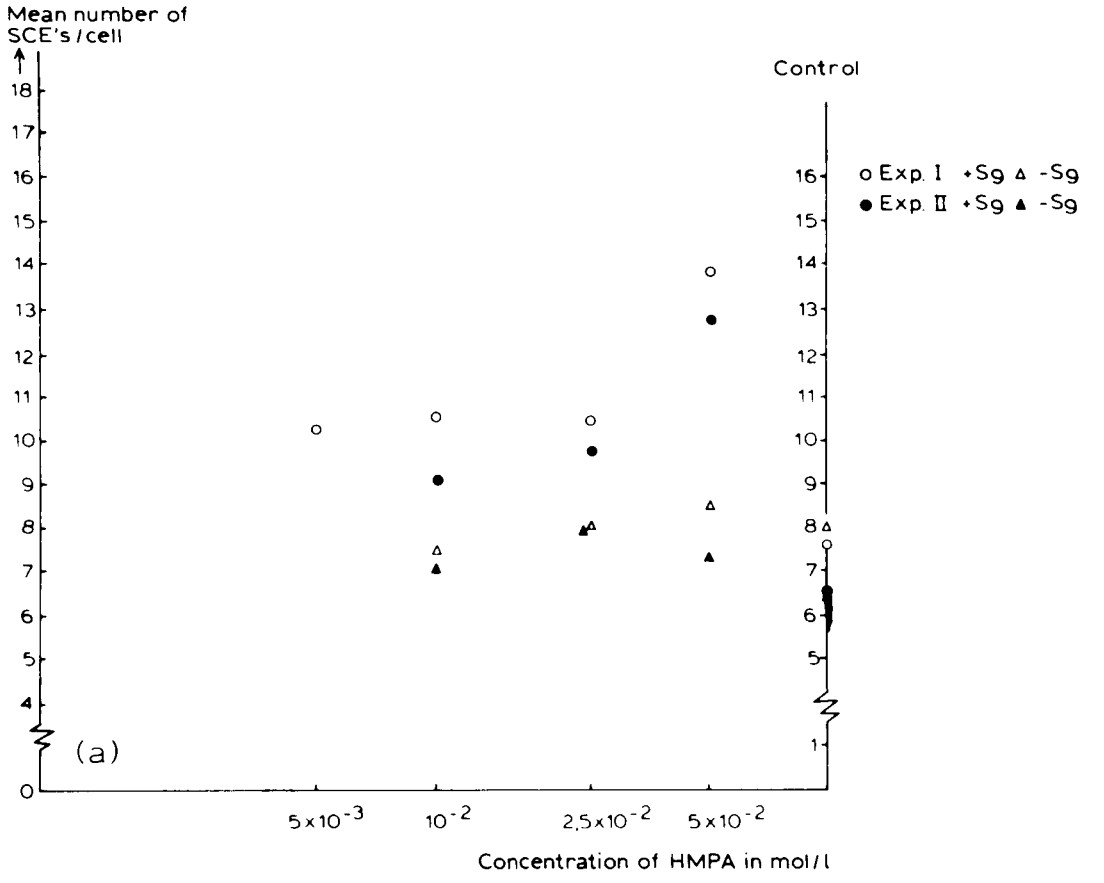
The fixed cells were immersed in PBS and coverslips were fixed with latex solution (Simson). The slides were left for about 15 h exposed to TL-light. After removal of the coverslips the slides were incubated in a waterbath with 2 × SSC (pH 7.0) at 60 °C for 15 min, rinsed in tap water and stained in a 3% Giemsa solution in Gurr buffer (pH 6.8) for 3 min. After drying, the slides were mounted in Entellan (Merck).

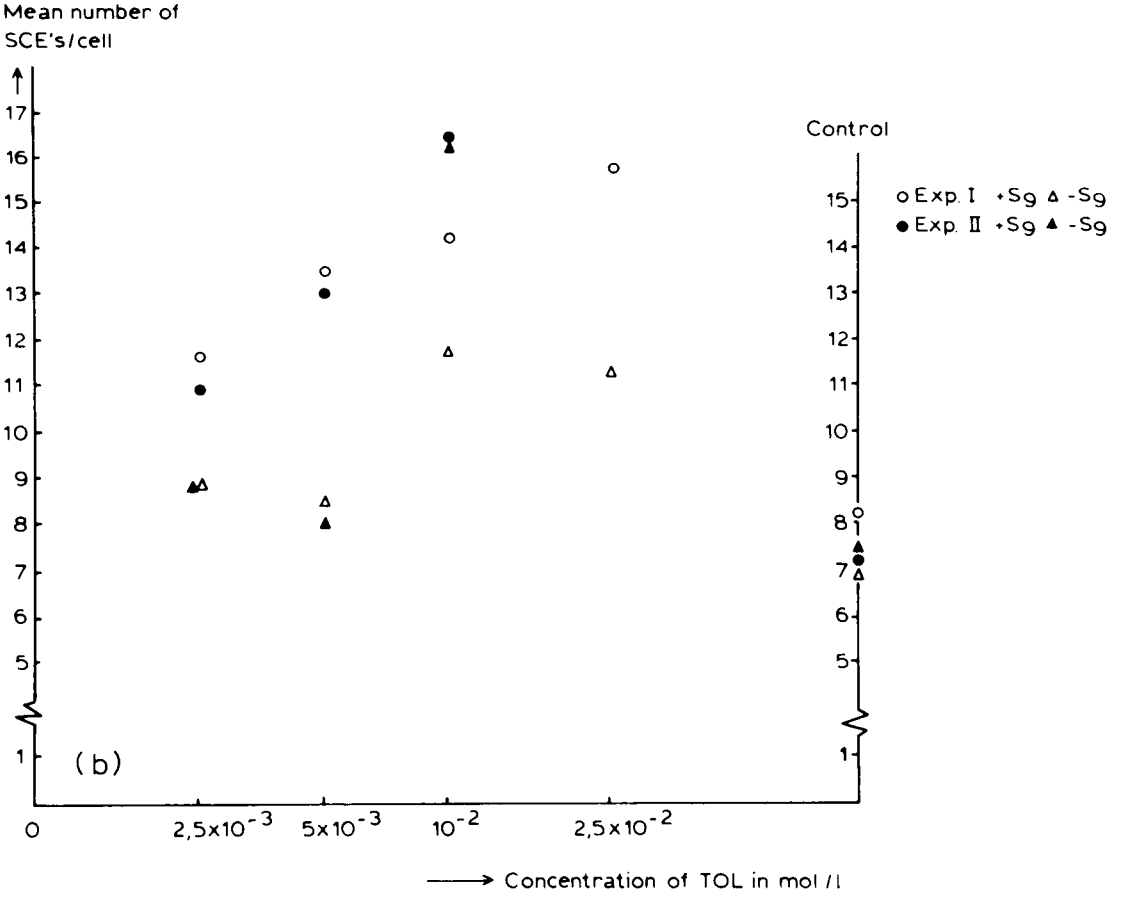
S9 preparation. Male Wistar (WUM) rats of 200–250 g were injected i.p. with 150 mg Aroclor 1254/kg body weight 5 days before being sacrificed. The vehiculum used for the Aroclor was corn oil. The livers were removed aseptically and rinsed in a 0.15 moles/l KCl-solution of 4 °C. The rest of the processing was also done at 4 °C. The S9 preparation was made by mincing the livers in 0.15 moles/l KCl solution, 3 ml/g of liver.

The material was then homogenized in a Potter–Elvehjem apparatus and the resulting homogenate was centrifuged at 9000 *g* for 15 min. The supernatant S9 fraction was stored at −80 °C in 2-ml aliquots in small screw-top bottles. The protein content of the S9 fraction was estimated using the method of Lowry, with BSA as a standard.

S9 mix was prepared immediately before use, containing per 10 ml: 2.0 ml, 20 mmoles/l Hepes buffer; 1.0 ml 330 mmoles/l KCL; 1.3 ml 50 mmoles/l $MgCl_2$; 1.0 ml 40 mmoles/l NADP (nicotinamide adenine dinucleotide phosphate); 1.0 ml 50 mmoles/l G6P (glucose 6-phosphate); 0.7 ml Ham's F10 medium (Flow) and 3.0 ml S9 fraction.

SCE analysis. Of the 4 concentrations used for the chemical treatment, in general the 3 highest possible levels were analyzed. The slides of the lowest concentration are available if analysis at a


Mean number of SCE's / cell
Control
o Exp. I +Sg Δ -Sg
● Exp. II +Sg ▲ -Sg
(a)
5x10⁻³
10⁻²
2,5x10⁻²
5x10⁻²
Concentration of HMPA in mol / l


Mean number of SCE's/cell
Control
o Exp. I +Sg Δ -Sg
● Exp. II +Sg ▲ -Sg
(b)
2,5x10⁻³
5x10⁻³
10⁻²
2,5x10⁻²
Concentration of TOL in mol / l

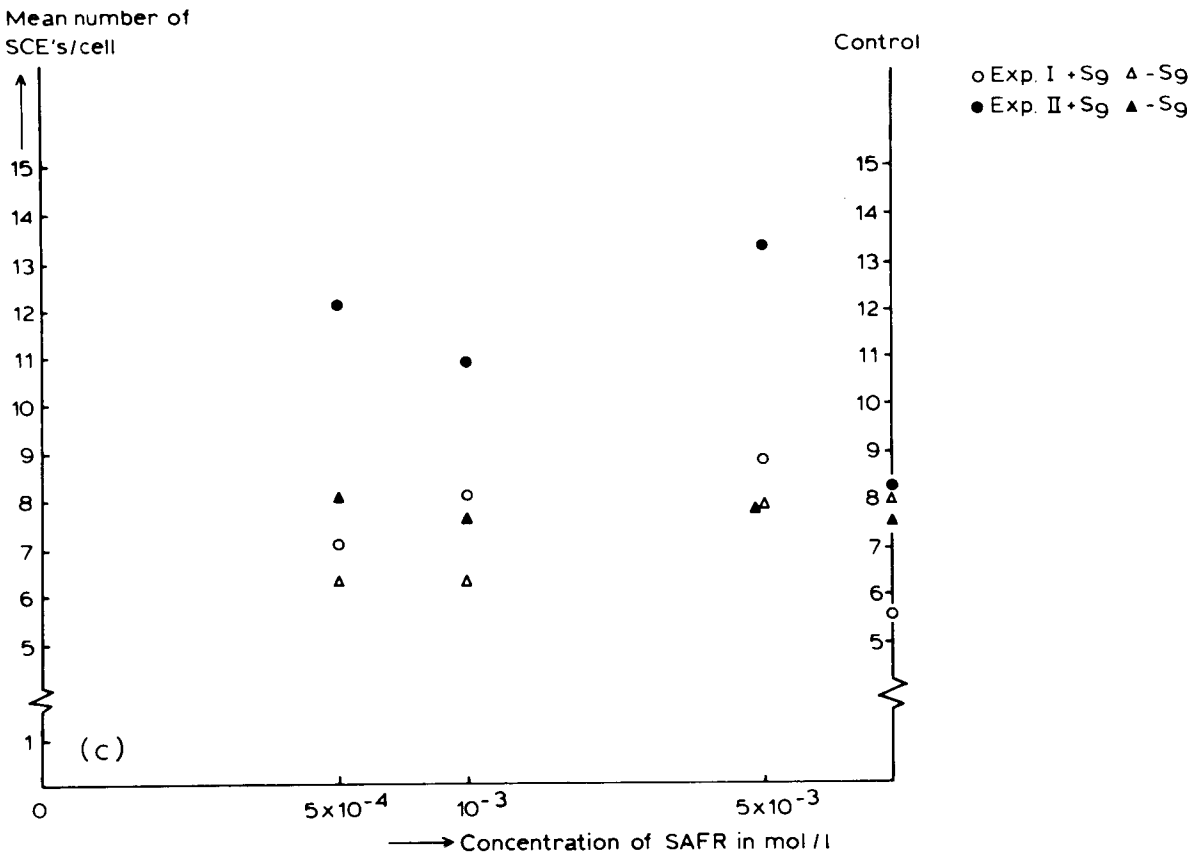


Fig. 2. Mean number of SCEs in V79 cells after exposure to different compounds, without (− S9) and with metabolic activation (+ S9) (a) HMPA; (b) TOL; (c) SAF.

later date might be necessary. All 5 chemicals and the control (DMSO) were tested with and without metabolic activation. The positive control (DEN) was tested with metabolic activation only. The majority of the cells contained 21 or 22 chromosomes; for each dose point 30 cells with 20–23 chromosomes were used for the analysis of SCEs. The total number of SCEs and the total number of chromosomes from the 30 cells analyzed were calculated.

The mean number of SCEs per cell and the mean number of SCEs per chromosome could thus be obtained.

Statistical analysis. To determine whether the increase in the mean number of SCEs was significant, a Student's *t*-test was used.

Results and discussion

A survey of the mean SCE values after treatment with the 5 chemicals, with and without metabolic activation, is given in Table 2. A good agreement was generally found between duplicate experiments.

In all cases the positive control experiments led to a highly significant increase in the mean number of SCEs ($P < 0.001$ using the Student's *t*-test), indicating that the metabolic activation system was functioning in a satisfactory way.

As on the one hand chemicals differ in their time of metabolic treatment, while on the other hand the S9-mix becomes toxic to the cells at longer treatment periods, a 2-h interval for the S9 addition was chosen instead of the 1 h used in many experiments from the literature. In the first

TABLE 3

SURVEY OF MEAN SCE VALUES IN POSITIVE CONTROL EXPERIMENTS WITH DIETHYLNITROSAMINE (DEN) [a]

Culture No.	DEN dose ($\times 10^{-2}$ moles/l)	SCE ± S.E.
IPCS-10 SAF II	3	20.2 ± 1.3
IPCS-11 CAP II	3	21.2 ± 0.9
IPCS-8 TOL II	3	25.2 ± 1.0 **
IPCS-4 SAF I	5	25.3 ± 1.5 *
IPCS-5 HMPA II	5	21.6 ± 1.1
IPCS-6 CAP I	5	22.5 ± 1.1
IPCS-7 BEN I	5	23.8 ± 1.5
IPCS-9 BEN II	5	20.6 ± 0.9
IPCS-2 TOL I	9	18.0 ± 0.7

[a] Treatment time was 2 h with metabolic activation.
* $P < 0.05$; ** $P < 0.01$.

experiment with HMPA (IPCS-1) benzo[*a*]pyrene was used as a positive control substance. However using this compound in the longer treatment period of 2 h, led to a mean value of SCEs of 9.5. This value was not as high as those for DEN, which amounted to around 20, although the significance was the same for both chemicals ($P < 0.001$).

DEN was used in a concentration of 3, 5 and 9 moles/l respectively. No consistent significant difference was found between the values of the different doses, as is seen in Table 3. When tested against the data of experiment IPCS-10 (3×10^{-2} moles/l) only the IPCS-8 value (3×10^{-2} moles/l; $P < 0.01$) and the IPCS-4 value (5×10^{-2} moles/l; $P < 0.05$) were significantly increased as compared to the IPCS-10 data.

For reasons of comparison, the doses of the individual chemicals were given in moles/l.

If a compound is qualified as positive when an increase in the mean number of SCEs to 150% of the correspondending control value is combined with a dose–response (see also Perry and Thomson, 1981), the following report can be given for the 5 test chemicals:

HMPA

In the 24-h toxicity experiments the cell growth was reduced to 50% only at the highest dose of 10^{-2} moles/l.

The results of the SCE analysis are given in Fig. 2a. Without metabolic activation at only one dose a small increase in the SCE rate was found. In the absence of a dose–response the compound can be considered negative without activation. After addition of S9 mix a highly significant dose-related increase was demonstrated. This compound clearly gave a positive response in this test system only after metabolic activation.

o-Toluidine

The 24-h toxicity studies led to a small growth retardation at doses of 10^{-4} and 10^{-3} moles/l, while for 10^{-2} moles/l cell growth was reduced to 25%. At the highest dose used in the SCE experiments no analysis was possible because no cells in mitosis were present. In the duplicate experiment at the second highest dose all mitoses present were in MI. Changes in the distribution of types of mitoses (MI, MII, MIII) are not uncommon (Van Went and Bergkamp, 1982). These shifts may be due to methodological aspects or to intrinsic factors of the cells cultured. Both with and without metabolic activation a dose-related increase in the mean number of SCEs was demonstrated, as is seen in Fig. 2b. We conclude that the compound is positive in the test system. The response was lower without activation in comparison to cells to which S9 mix had been added.

Benzene

In the toxicity studies the highest dose used, 10^{-2} moles/l gave a growth reduction of 50%. In the SCE test, none of the doses applied without metabolic activation resulted in any increase. In the activated cultures only at the lowest dose tested, 10^{-2} moles/l, was an elevated number of SCEs found. In the absence of a dose relationship, however, the substance should be regarded as negative in this system.

Safrole

This compound proved to have the highest toxicity; at 10^{-2} moles/l no cell growth was detected at all. In the SCE assay only with the addition of the metabolic system a dose-related increase in the mean number of SCEs was found. This chemical substance clearly was only positive after being metabolized (Fig. 2c).

Caprolactam

The toxicity experiment led to a reduction in growth of 50%, only at the highest dose used. Without metabolic activation in the SCE experiments no increase was found, while with activation only at the highest dose, 5×10^{-2} moles/l, a small but significant increase in SCEs was detected. In the absence of a dose relationship the compound can be regarded as negative.

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Tests for effects on mitosis and the mitotic spindle in Chinese hamster primary liver cells (CH1-L) in culture

Elizabeth M. Parry

Department of Genetics, University College of Swansea, Singleton Park, Swansea (Great Britain)

Summary

Table 2 summarises the results; a + or − is assigned to each compound. A compound was considered to have an effect on the spindle only when 3 or more of the criteria in columns 1–4 were satisfied. Thus DES, DEHP, PB and BEN were positive in these terms. DES was the most strongly positive agent.

Agreement was found between this study and the parallel study reported by Danford for aneuploidy for DES, DEHP and BEN. Danford also found that HMPA and TOL increased aneuploidy levels whereas this study also indicated that PB had an effect on the spindle. Thus, taken together, these results indicate that DES, DEHP and BEN are inducers of aneuploidy and that this may be produced by a spindle defect.

A differential staining technique described by Wissinger et al. (1981) that allows for the simultaneous visualisation of chromosomes and spindle fibres has been used to investigate the effects of the 10 compounds selected for the IPCS Collaborative Study/CSSTT on cell division and spindle formation. The cell line used is described by Danford (this volume); it is a low passage Chinese hamster fibroblast line derived from liver, showing some intrinsic metabolic activation capacity.

Materials and methods

Cells

Chinese hamster primary liver cells, CH1-L, were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% foetal calf serum and penicillin/streptomycin (50 units/50 μg/ml) (Gibco Europe Ltd.) in a 5% CO_2 : 95% air atmosphere at 37 °C. Subculturing was carried out with 0.25% trypsin solution using a split ratio of up to 1 : 9. The cell cycle time was approximately 18 h.

Test chemical concentration

The 10 chemicals were dissolved in dimethyl sulphoxide (DMSO) except for diethylhexylphthalate, which was dissolved in DMSO + 1% Tween 80 (BDH Ltd., Poole, U.K.). Benzoin and diethylhexylphthalate were handled in glass containers until added to the culture medium.

Each chemical was assessed for its toxicity to determine dose levels for further testing. Cell suspensions of 2 ml (25 000 cells/ml) were seeded into Linbro well plates (Flow Laboratories). After 24 h culture, the medium was removed and replaced with medium plus test agent (2 wells/concentration). When no toxicity data was available, an initial wide range of 5 concentrations (1–1000 μg/ml) was tested. A second test on an appropriate narrow range was carried out. After 36 h culture the medium was removed and the wells treated as follows: (a) Rinse with Hank's balanced salt solution. (b) Replace with methanol; 10 min. (c) Air dry. (d) Stain with 1 : 9 Giemsa diluted with phosphate buffer, 0.05 M, pH 6.8; 5 min. (e) Rinse with buffer and dry.

Cytotoxicity was assessed by noting the inten-

sity of staining; microscopic examination could further identify morphological abnormalities at doses that caused little reduction in staining.

The highest dose selected was either that causing approximately 50% growth inhibition, or up to a maximum solubility of 5 mg/ml. Lower doses were at 0.5, 0.25 and 0.1 times the highest dose. The lowest dose was important for the spindle stain, as the lack of spindle at toxic doses may be due to general effects on protein metabolism.

Mitotic profile and spindle studies

Slide cultures were set up by seeding 1 ml/slide of a 75000 cells/ml suspension onto sterile glass slides in sterile 9-cm petri dishes. After 2 h, to allow the cells to attach, 11 ml of medium was added. After approximately 24 h incubation, 3 ml of medium containing the test agent at 5 × the required concentration was added to each dish, 3 replicates per dose. The final volume of DMSO was 4 μl/ml culture medium in each case, except for caprolactam, for which it was 16 μl/ml. After a further 24 h incubation the slides were harvested in situ. The medium was removed and then replaced with fixative; 3:1, methanol:acetic acid. No hypotonic treatment was given, so that the cells were as undisturbed as possible. The cells were fixed in fresh 3:1, methanol:acetic acid containing magnesium and calcium at room temperature 3 ×, 14 min each, so that the spindle proteins and cellular integrity was maintained. Concentrations of either 4 mM $MgCl_2$ and 1.5 mM $CaCl_2$, or 5 mM $MgCl_2$ and 2 mM $CaCl_2$ in the fixative solutions were used. Following overnight air-drying, the slides were placed in 5% perchloric acid solution at 4°C for 24 h. The slides were rinsed for 10 min in several changes of distilled water and again air-dried. The cells were then stained with a solution of 0.5% brilliant blue R (Sigma Chemical Co.) and 0.5% safranin O (Harleco Co.) in 10% acetic acid for 24 h before being washed in distilled water and air-dried. The slides were dipped in xylene and mounted with DPX (BDH Ltd.).

All the slides were prepared within an 11-day period. The slides were then coded and scored for mitotic index (1000 cells were counted per slide, 3 replicas/dose), normal and abnormal division stages, spindle, chromosome dislocation from the spindle, multipolar spindles and anaphase lagging and bridges (100 dividing cells per slide, 3 replicas/dose).

These experiments were performed in parallel with those described by Natalie Danford measuring aneuploidy and chromosomal aberrations. The same cultures and test agent were used in these two studies.

Appropriate solvent controls were included throughout the study. Colcemid, a known spindle inhibitor, was used as a positive control chemical through this part of the study.

Analysis of data

The criteria used in assessing the potency of agents in causing spindle damage were as follows. (1) Direct effects on the spindle, e.g. pole-to-pole shortening of the spindle, or complete absence. (2) (a) An increase in the number of abnormal division stages. Previous studies indicate that of these stages the chromosome cluster group (CC) is the first to show an increase, and is therefore an indicator of possible spindle damage. (2) (b) An increase in this CC. (3) A reduction in the anaphase and telophase/metaphase (AT/M) ratio. (4) An increase in the frequency of multipolar spindles. (5) A change in the mitotic index (generally an increase). (6) An increase in chromosome dislocation from the spindle. (7) An increase in chromosome lagging and bridge formation at anaphase.

Previous work in our laboratory (Parry et al., 1982) indicates that criteria 1–4 are the most sensitive indicators of spindle damage. Criteria 5–7 were taken into account when assessing spindle effects only when 1–4 were positive.

Results and discussion

Results are shown in Figs. 1 and 2 for acrylonitrile and diethylstilboestrol as examples of the least and most potent of the agents with regards to spindle effects.

Hexamethylphosphoramide (HMPA)

The mitotic index was not changed by treatment. There was no change in the AT/M ratio but there was an increase in the chromosome cluster group and the abnormal division stages, indicating a very weak effect on the spindle.

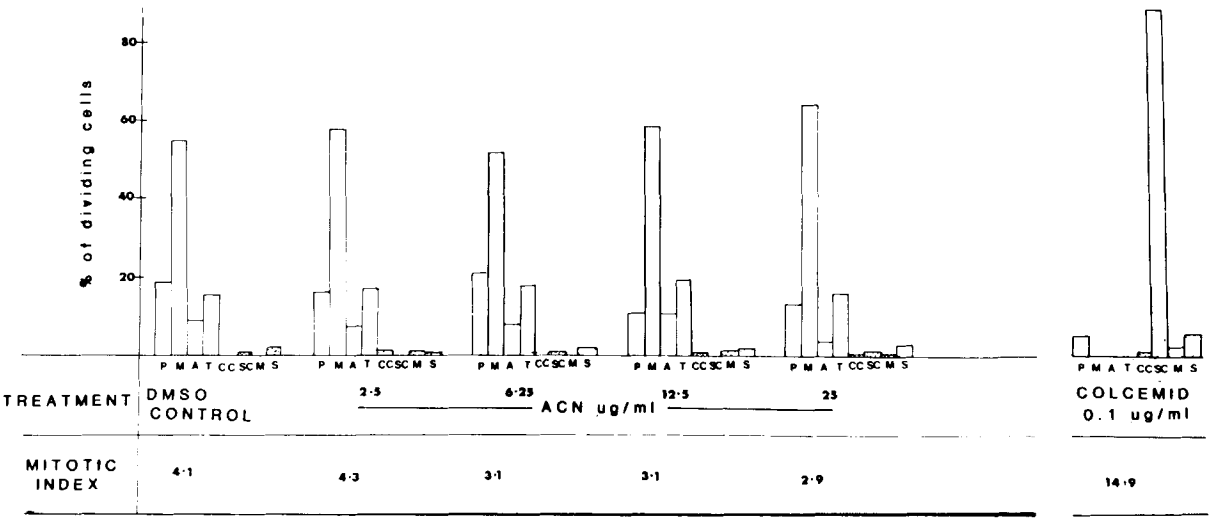

Fig. 1. Mitotic profile of CH1-L cells after exposure to acrylonitrile. Normal division stages: P, prophase; M, metaphase; A, anaphase; T, telophase. Abnormal division stages: CC, chromosome cluster; SC, scattered chromosomes; M. chromatin mass; S, scattered chromatin.

Chromosome dislocation was increased only at the top dose. There was no effect on either multipolar spindles or on anaphase lagging.

o-Toluidine (TOL)

A small increase in the mitotic index was seen with treatment. The AT/M ratio remained unaf-

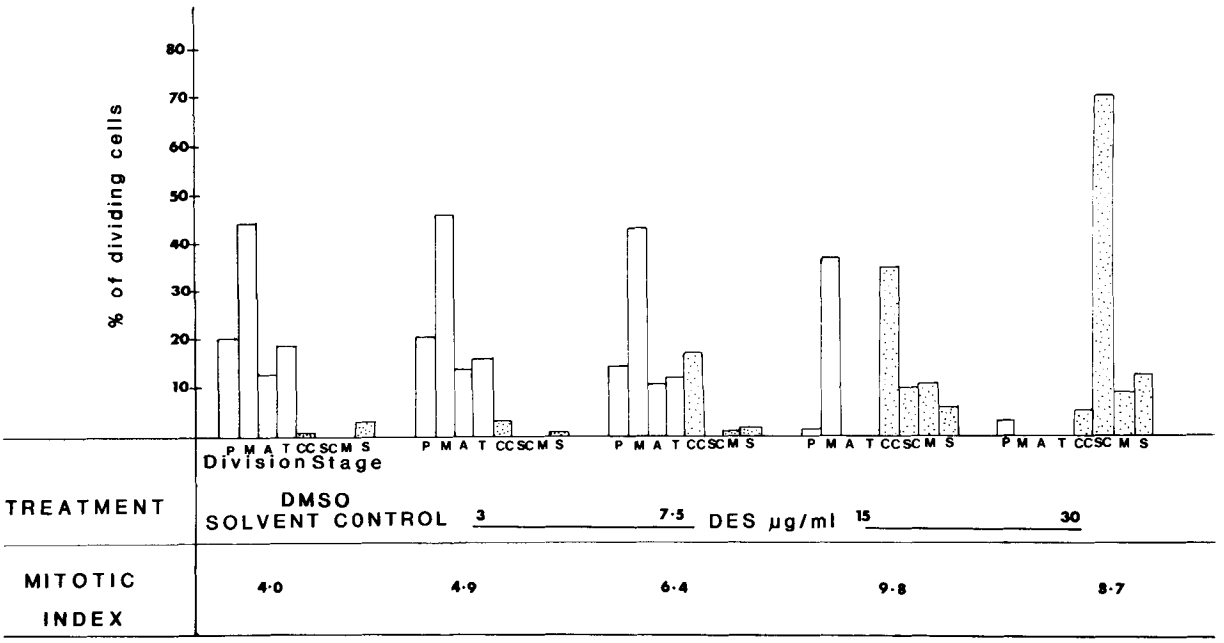

Fig. 2. Mitotic profile of CH1-L cells after exposure to diethylstilboestrol. Normal division stages: P, prophase; M, metaphase; A, anaphase; T, telophase. Abnormal division stages: CC, chromosome cluster; SC, scattered chromosomes; M, chromatin mass; S, scattered chromatin.

TABLE 1

EFFECTS OF TEST CHEMICALS ON MITOTIC DIVISION AND SPINDLES

Treatment	Mitotic index	Spindle	AT/M ratio	Percent abnormal division stages		Percent metaphases with chromosome dislocation	Percent metaphases anaphase and telophases with multipolar spindles	Percent anaphases with chromosome lagging and bridges
				Total	Chromosome cluster			
CH1-L passage 9								
0	2.9	+	0.44	1.3	0.7	0.58	1.21	32.1
2	3.1	+	0.54	2.6	2.0	0.58	0.76	35.5
5 HMPA	3.4	+	0.42	1.7	1.7	0	0.82	37.0
10 μg/ml	3.9	+	0.45	2.3	2.0	0	0	23.3
20	3.0	+	0.41	2.0	1.7	1.20	0.41	41.7
0.1 μg/ml colcemid	16.0	–	–	99.3	1.3	–	–	–
CH1-L passage 15								
0	4.0	+	0.72	4.0	0.7	3.8	0.9	39.5
12	4.7	+	0.42	2.0	1.7	0	0.9	4.0
30 TOL	4.1	+	0.71	2.3	1.0	0.7	1.3	8.1
60 μg/ml	5.0	+	0.71	1.7	1.7	4.2	0.4	21.3
120	5.2	+	0.72	1.3	0	3.5	0	15.9
0.1 μg/ml colcemid	14.9	–	–	95.0	0.3	–	–	–
CH1-L passage 10								
0	3.6	+	0.70	2.0	0.3	0.59	0.41	24.0
25	4.6	+	0.56	1.3	0.7	0	0	30.0
62.5 BEN	3.2	+	0.50	4.0	0.7	0.65	0	11.5
125 μg/ml	4.1	+	0.57	3.7	2.0	0	0.41	14.3
250	4.4	+	0.60	1.3	0.3	0	0.39	8.0
0.50 μg/ml colcemid	11.9	–	–	95.0	5.0	–	–	–
CH1-L passage 14								
0	2.9	+	0.44	1.3	0.7	0.58	1.21	32.1
6 SAF	2.8	+	0.23	1.6	1.3	0	0	28.6
15 μg/ml	3.1	+	0.34	1.6	1.4	0	0.41	21.4
30	3.2	+	0.39	2.3	1.7	1.80	1.28	43.8
60	2.8	+	0.30	1.4	0.7	1.12	1.25	13.4
0.1 μg/ml colcemid	16.0	–	–	99.3	1.3	–	–	–
CH1-L passage 11								
0	5.9	+	0.54	1.0	1.0	0	0.81	10.9
0.2	4.8	+	0.71	0	0	0	0.39	17.6
0.5 CAP	4.9	+	0.40	2.7	1.7	0	0	28.0
1.0 μg/ml	4.2	+	0.54	0.9	0.3	0	0.77	15.6
2.0	3.9	+	0.29	1.4	0	0.5	0	54.6
0.06 μg/ml colcemid	14.8	–	–	99.3	23.7	–	–	–
CH1-L passage 9								
0	4.1	+	0.44	2.5	0	1.8	3.2	10.0
2.5	4.3	+	0.42	2.0	1	2.3	2.4	22.7
6.25 ACN	3.1	+	0.48	2.3	0	3.2	1.7	37.5
12.5 μg/ml	3.1	+	0.47	2.7	0.3	4.6	2.7	14.3
25	2.9	+	0.31	3.6	0.3	4.2	3.6	100.0
0.05 μg/ml colcemid	13.4	–	–	98.3	12.3	–	–	–

TABLE 1 (continued)

Treatment	Mitotic index	Spindle	AT/M ratio	Percent abnormal division stages		Percent metaphases with chromosome dislocation	Percent metaphases anaphase and telophases with multipolar spindles	Percent anaphases with chromosome lagging and bridges
				Total	Chromosome cluster			
CH1-L passage 11								
0	5.7	+	1.1	0	0	0	0.40	11.1
5	6.6	+	0.54	4.3	4.3	0.63	0	12.1
12.5 DEHP	6.9	+	0.40	1.3	1.3	0	0	17.9
25 μg/ml	7.1	+	0.46	1.0	0.7	0	0.39	23.1
50	3.9	+	0.33	6.3	4.3	0	0	20.0
0.06 μg/ml colcemid	14.8	–	–	99.3	23.7	–	–	–
CH1-L passage 9								
0	4.1	+	0.44	2.5	0	1.8	3.2	10.0
20	2.7	+	0.46	1.0	0	3.9	2.7	44.0
50 ZOIN	3.2	+	0.37	2.3	0	1.7	0.8	40.0
100 μg/ml	2.7	+	0.40	2.3	0.3	3.4	2.8	52.0
200	2.2	+	0.43	1.3	0	1.1	1.2	12.0
0.05 μg/ml colcemid	13.4	–	–	98.3	12.3	–	–	–
CH1-L passage 15								
0	4.0	+	0.72	4.0	0.7	3.8	0.9	39.5
3 DES	4.9	+	0.65	3.3	3.0	0	0.4	11.9
7.5 μg/ml	6.4	+ short	0.52	19.6	17.3	10.8	0.5	15.6
15	9.8	+ short	0	61.4	34.7	8.1	0	–
30	8.7	–	0	97.0	5.0	–	–	–
0.1 μg/ml colcemid	14.9	–	–	95.0	0.3	–	–	–
CH1-L passage 10								
0	3.6	+	0.70	2.0	0.3	0.59	0.41	24.0
100	4.0	+	0.66	1.0	0.7	0.66	1.20	12.2
250 PB	4.2	+	0.54	0.3	0	0	0.40	14.3
500 μg/ml	3.0	+	0.26	3.6	3	0.49	1.56	28.6
1000	3.2	+	0.29	0.7	0.7	0	2.15	9.1
0.05 μg/ml colcemid	11.9	–	–	95.0	5.0	–	–	–

fected as did the numbers of abnormal division stages. There was no effect on either chromosome dislocation, multipolar spindles or anaphase lagging and bridges. Thus this agent did not affect the spindle.

Benzene (BEN)

A slight increase in the mitotic index was seen. There was an increase in the chromosome cluster group and other abnormal division stages at one dose. The AT/M ratio was reduced slightly and there was no effect on multipolar spindles. This may be described as a very weak spindle effect.

There was no effect on either chromosome dislocation or anaphase lagging.

Safrole (SAF)

No change in the mitotic index was found. The AT/M ratio was reduced by the treatment across the dose range and there was some increase in the numbers of abnormal division stages. Chromosome dislocation was increased at the two highest doses. Thus a possible small effect on the spindle was indicated. There was no change in either multipolar spindles or anaphase lagging.

Caprolactam (CAP)

The mitotic index was reduced only at the top dose probably as result of growth inhibition. The AT/M ratio was reduced only at this same dose and is disregarded for the same reason. The num-

TABLE 2

SUMMARY OF TEST CHEMICAL POTENCY

Test agent	Criteria of spindle disturbance								
	1 Spindle	2a Abnormal division stages	2b Chromosome cluster group	3 AT/M ratio	4 Multipolar spindles	5 Mitotic index	6 Chromosome spindle dislocation	7 Anaphase lagging and bridges	Spindle effect
DES	+	+ (×24)	+ (×50)	+	−	+	+	−	
DEHP	−	+ (×6)	+ (×4)	+	−	+	−	−	
PB	−	−	+ (×10)	+	+ (×5)	−	−	−	
BEN	−	+ (×2)	+ (×6)	+	−	+	−	−	positive
HMPA	−	+ (×2)	+ (×3)	−	−	−	−	−	
SAF	−	−	+ (×2)	+	−	−	+ (×2)	−	
ZOIN	−	−	−	−	−	+	+ (×2)	+	
ACN	−	−	−	−	−	−	+ (×2)	−	
TOL	−	−	−	−	−	−	−	−	
CAP	−	−	−	−	−	−	−	+	negative

+, when criteria is satisfied at 2 or more dosage points. Positive spindle effect when 3 or more + found in columns 1–4.

ber of abnormal division stages was unaffected by treatment. Thus no spindle effect was detected.

There was no effect on chromosome dislocation, multipolar spindles or on anaphase lagging (the higher value for the top dose can be explained by the low AT/M ratio).

Acrylonitrile (ACN)

No effect on the spindle was detected. There was a reduction in the mitotic index at the top dose; and this growth inhibition explains the apparent increase of anaphase lagging at that dose.

An increase in chromosome dislocation was seen; in some cases it was severe and associated with chromosome shattering. Chromosome dislocation was probably caused by chromosome damage rather than spindle damage.

Diethylhexylphthalate (DEHP)

The mitotic index was reduced only at the top dose which may reflect some growth inhibition (at this top dose cells appeared to be badly damaged). The AT/M ratio was reduced in a dose related manner. The chromosome cluster group and the abnormal division stage (mainly composed of CC) was increased by treatment. These observations indicate a positive spindle effect.

There was no effect on chromosome dislocation, multipolar spindles, or chromosome lagging and bridge formation.

Benzoin (ZOIN)

No effect on the spindle was detected. There was a reduction in the mitotic index throughout the doses. Chromosome dislocation, anaphase bridges and lagging were increased and some polyploidy was seen at the highest dose.

Diethylstilboestrol (DES)

The most potent effect on the spindle was found with this agent; it gave a very strong positive effect.

The mitotic index was increased in a dose-related manner; the spindle was also affected in this way. A shortening of the spindle pole-to-pole length was observed with increasing dose of DES. The spindle was abolished at the highest dose; while at the two intermediate doses the beginning of the shift from normal to abnormal division stages could be seen.

The number of abnormal stages increased with increasing dose; the chromosome cluster group was the first one of these to increase but at the highest dose there was a shift to other groups. For this reason it was used as an indicator of spindle damage.

The AT/M ratio was reduced to zero at the top two doses where no anaphase or telophase stages were found. This metaphase block and change to abnormal spindleless stages is similar to the effect of colcemid (Sawada and Ishidate, 1978; Parry et al., 1982).

There was an increase in chromosome dislocation with dose as far as it could be measured: few or no metaphases at the two highest doses. No effect was found on multipolar spindles or anaphase lagging or bridges.

Phenobarbital (PB)

There was no change in the mitotic index. The AT/M ratio was reduced with increasing dose. The chromosome cluster group and other abnormal division stages were increased at one dose. There was also a dose-related increase in multipolar spindles. These observations seem to indicate some effect on the spindle although it was not directly abolished.

There was no effect on chromosome dislocation or on anaphase lagging.

Controls

Positive control. Colcemid, a well known mitotic poison, that interferes with tubulin polymerization was used as a positive control throughout this study at concentrations of 0.05–0.1 μg/ml. In all cases the spindle was absent and the dividing cells were abnormal; the mitotic index was also elevated.

Negative controls. Although slight differences were found between subcultures in mitotic profiles, this was generally small and differences between replicates was very low. The most variable parameter was the number of anaphases showing chromosome lagging and bridges; this may be because the number of dividing cells at anaphase is always small and for this reason very little weight was given to this measurement.

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Tests for gene mutational activity in the L5178Y/TK assay system

David E. Amacher and Gail N. Turner

Drug Safety Evaluation, Pfizer Central Research, Groton, CT (U.S.A.)

Summary

10 chemicals supplied by IPCS were tested in L5178Y cells for gene mutational activity at the thymidine kinase locus as measured by trifluorothymidine resistance. Each chemical was tested with and without rat-liver postmitochondrial (S9) fraction using duplicate cultures at each test concentration. Of 22 completed tests (12 with S9, 10 without), 4, (3 with S9, 1 without) were positive, 3 were inconclusive, and 15 were negative. Acrylonitrile was unequivocally mutagenic under both test conditions (±S9). Hexamethylphosphoramide (HMPA) and safrole were weakly mutagenic, but only in the presence of rat-liver S9. The noncarcinogens benzoin and caprolactam were not mutagenic directly or when tested with S9 mix.

The L5178Y/TK assay system (Clive et al., 1972) detects gene mutations affecting the thymidine kinase (TK) locus and is a good predictor of animal carcinogenicity with few false positives or false negatives when both noncarcinogens and carcinogens (respectively) are tested (Amacher and Turner, 1982a). As part of the International Programme on Chemical Safety (IPCS) collaborative study, we tested 10 chemicals selected by IPCS in L5178Y cells, both directly and in the presence of an exogenous source of metabolic activation provided by rat liver postmitochondrial (S9) fraction.

These 10 chemicals were tested either to the point where obvious cellular toxicity was observed or up to a final concentration that exhausted the amount of chemical supplied. There were 3 inconclusive tests: benzoin and phenobarbitone with S9, and DEHP without S9. Phenobarbitone and benzoin were retested and both were without any mutagenic effect. Our supply of DEHP was exhausted, precluding a retest.

Using predetermined acceptability and response criteria, we found 1 chemical (acrylonitrile) mutagenic in the absence of S9 and 3 (acrylonitrile, safrole and HMPA) positive mutagens in the presence of S9. In general, 10 chemicals were relatively weak mutagens or nonmutagens compared to positive and solvent controls.

Materials and methods

Cell culture

Cells from clone 3.7.2C, originally obtained from D. Clive, were retrieved from liquid N_2 storage. These cells, negative for mycoplasma contamination, were grown for 24 h each week in medium containing amethopterin, thymidine, hypoxanthine and glycine (Amacher et al., 1980). Growth medium was RPMI-1640 (MA Bioproducts) supplemented with 0.05% Pluronic F68, antibiotics, and 5% horse serum. Test medium used during mutagen exposure was the same, but contained only 3% serum. Serum was not heat-inactivated and conditioned medium was not used in these studies. Cloning medium was RPMI-1640 plus 0.02% Pluronic F68, 5% serum and 0.37% Difco Noble agar. A single lot of horse serum was used for all studies. This serum had been preselected for its ability to permit colony growth to countable size in 7–8 days at 5% serum and a low constant mutant frequency in solvent controls at both 5 and 10% serum.

Exogenous metabolic activation

Two batches of postmitochondrial S9 fraction ($9000 \times g$) were prepared at different times during the test period from the homogenized livers of untreated male CrL:COBS CD rats by procedures previously described (Ames et al., 1973) and stored at $-80\,^{\circ}C$. S9 from Aroclor-pretreated rats was not used for reasons cited elsewhere (Amacher and Turner, 1982b). Analyses of these S9 preparations performed by Litton Bionetics indicated a protein content of 27.0 mg/ml and aryl hydrocarbon hydroxylase (AHH) activity of 2.8 nmoles/mg protein/20 min for batch 1 and 42 mg/ml protein and 2.1 nmoles/mg protein/20 min for batch 2. Final S9 concentration during testing was 5% (v/v) or 1.35–2.10 mg protein/ml (Amacher and Turner, 1982a).

Mutagenesis assay

A 3-h treatment period and a 48-h expression period were used throughout (Amacher and Turner, 1982a). Duplicate (independent) cultures of 6×10^6 cells were exposed to each concentration of test chemical in dimethyl sulfoxide (silylation grade DMSO, Pierce Chemical), DMSO only (solvent controls), or a positive control (5.37 μg/ml 3-methylcholanthrene or 621 μg/ml ethyl methanesulfonate, Sigma Chemical).

Cell colonies were counted 7–9 days after cells had been plated in soft-agar medium. Percent total relative growth, an estimate of cytotoxicity, and mutant frequencies were calculated as previously described (Clive and Spector, 1975).

Analysis and interpretation of test results

A test was acceptable if: (1) a positive control was included and produced a positive mutagenic response, and (2) two concomitant solvent controls were included and produced acceptable results. In this experimental series, absolute cloning efficiences of $93 \pm 9\%$ ($\bar{x} \pm 1$SD, $n = 44$) and spontaneous mutant frequencies of $17 \pm 4 \times 10^{-6}$ survivors ($\bar{x} \pm 1$SD, $n = 44$) were observed in solvent controls.

A positive mutagenic response was indicated when 3 or more concentrations of a test chemical satisfied all of the following: (1) total relative growth of 20–80%, (2) mutant frequencies that are both greater than the mutant frequencies for concurrent solvent controls and greater than the range of acceptable mutant frequencies for all solvent controls in this series, and (3) absolute mean mutant yields significantly greater ($P < 0.05$) than the corresponding average mutant yields for concurrent solvent controls within a particular trial.

Results

All solvent control and positive control mutant frequency data for this test series are summarized in Fig. 1. Low, stable background mutant frequencies were noted in the 46 individual solvent controls, a phenomenon made possible by careful prescreening of horse serum for use in this assay. Substantially greater, but increasingly variable mutant frequency responses were observed for the 30 3MCA (+S9) and 22 EMS (no S9) positive controls used in these trials. Variation among positive controls is most likely due to experimental error introduced during the weighing out or pipetting of small quantities of 3MCA or EMS, respectively.

Data for the 10 test chemicals are summarized in Table 1. 22 assays were completed and submitted, 12 with S9 metabolic activation and the

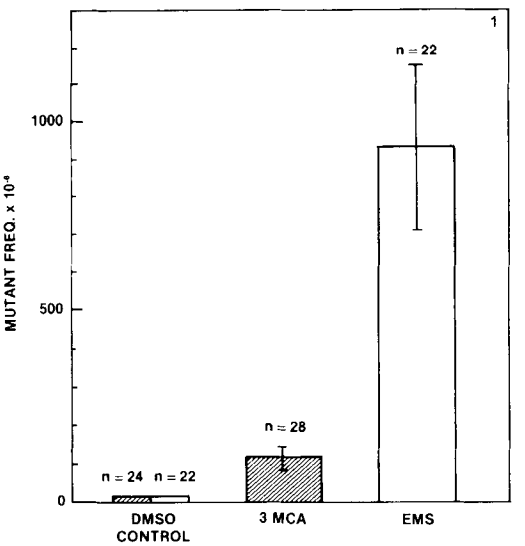


Fig. 1. Cumulative data for positive and negative (DMSO) controls. Test results obtained with rat-liver S9 are shown with hatched histobars. Bar heights are sample means ± 1 SD for the indicated number (n) of independent samples.

TABLE 1

SUMMARY OF MUTAGENICITY RESULTS FOR 10 CHEMICALS IN THE L5178Y/TK ASSAY

Chemical	S9 [a]	Concentration of test chemical	Total relative growth [b]	Mutagenic response [c]
Acrylonitrile	+	5–69 μg/ml	5–92%	Pos.
	–	22–43 μg/ml	28–73%	Pos.
Benzene	+	78–1042 μg/ml	42–99%	Neg.
	–	86–1148 μg/ml	6–94%	Neg.
Benzoin	+	32–424 μg/ml	12–96%	NC
	+	16–212 μg/ml	5–71%	Neg.
	–	3–44 μg/ml	27–108%	Neg.
Caprolactam	+	1.13–11.32 mg/ml	12–102%	Neg.
	–	1.13–11.32 mg/ml	51–109%	Neg.
DEHP	+	184–2468 μg/ml	1–98%	Neg.
	–	22–301 μg/ml	44–137%	NC
Diethylstilbuestrol	+	8–101 μg/ml	< 1–62%	Neg.
	–	12–165 μg/ml	0–57%	Neg.
HMPA	+	1.37–10.27 mg/ml	32–80%	Weak pos.
	–	1.16–8.73 mg/ml	66–101%	Neg.
o-Toluidine	+	120–903 μg/ml	6–89%	Neg.
	–	80–1072 μg/ml	5–92%	Neg.
Phenobarbitone	+	2.32–3.10 mg/ml	0–116%	NC
	+	1.6/9–3.251 mg/ml	0–79%	Neg.
	–	1.74–2.32 mg/ml	42–102%	Neg.
Safrole	+	4–50 μg/ml	16–85%	Weak pos.
	–	12–90 μg/ml	12–107%	Neg.

[a] The presence or absence of rat-liver S9 is indicated by + or –.

[b] Duplicate cultures (3 plates each) were tested at each dose. High and low survival values shown are averages for each pair.

[c] Responses were: Neg., negative; Pos., positive; NC, no conclusion reached.

remainder without. 4 of the tests were considered positive, 3 inconclusive, and 15 negative. Individual test results are as follows:

Acrylonitrile

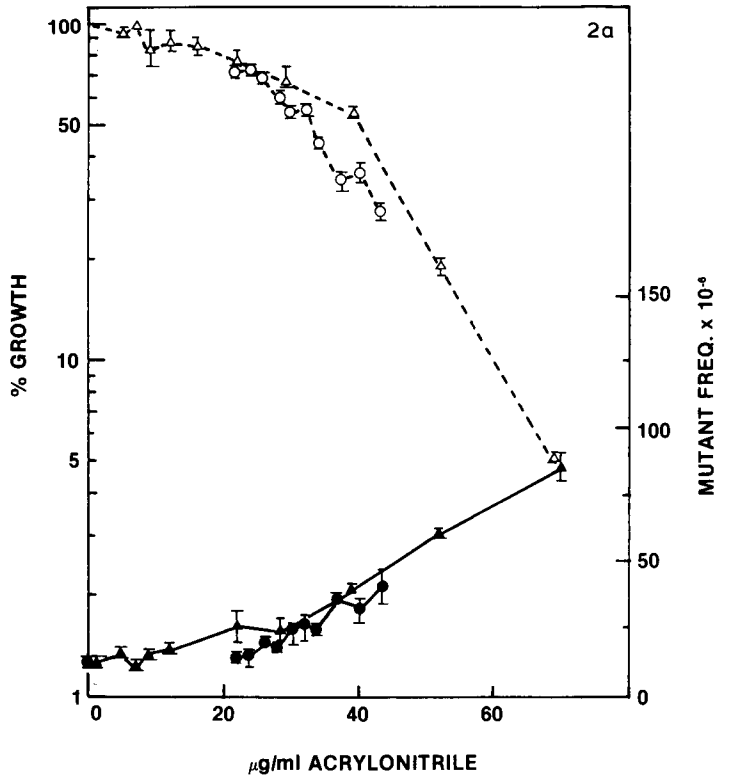
This chemical was clearly mutagenic in the presence or absence of S9 activation (Fig. 2a). The toxicity of this chemical was similar to that observed for diethylstilbestrol.

Benzene

The cells tolerated fairly high concentrations of this chemical and the presence of S9 further ameliorated benzene cytotoxicity (Fig. 2b). No evidence of mutagenicity was found.

Benzoin

Results with S9 (fig. 2c) were inconclusive (insufficient number of data points for analysis) although there was no obvious mutagenicity at 424 μg/ml which was quite toxic (~ 12% survival). In the absence of S9, benzoin was negative. When benzoin was retested with the same S9 preparation used in the first study, the results were clearly negative; however, benzoin-induced cytotoxicity was now much greater. Although the biological activity measured as the percent of total relative growth was variable in the presence of S9, benzoin was neither a direct-acting mutagen nor a promutagen in this assay system.



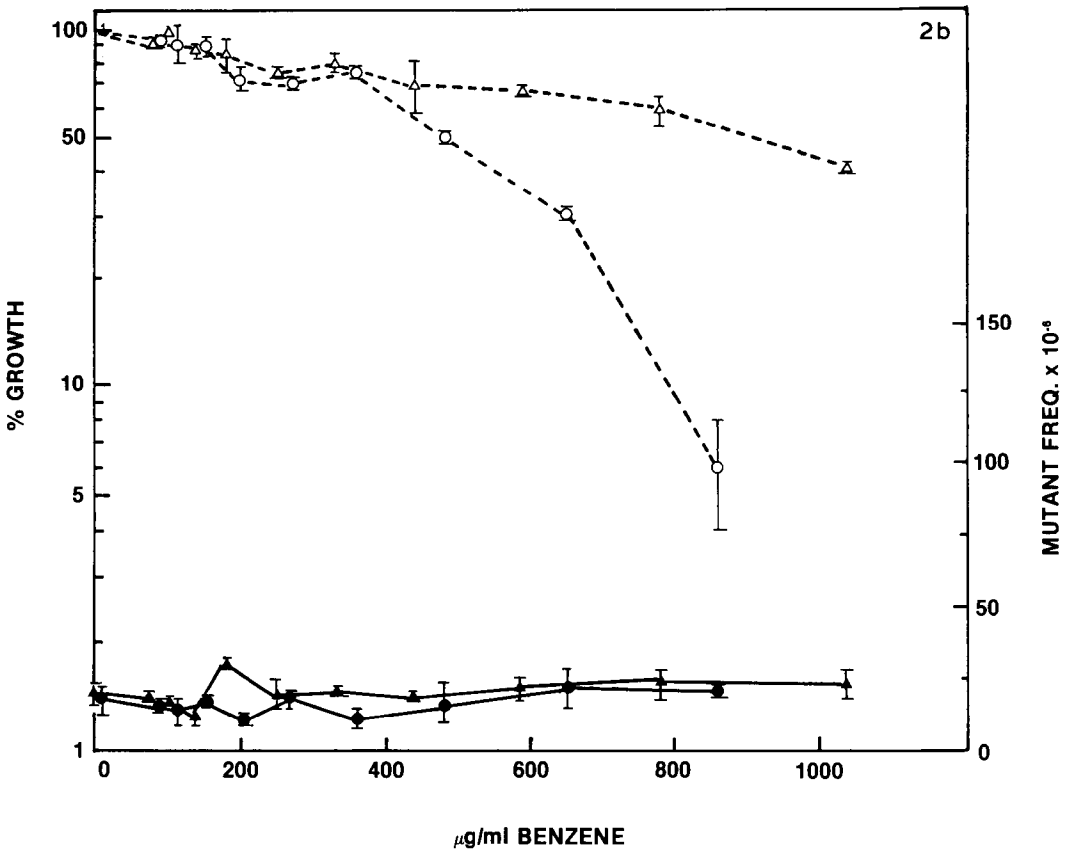
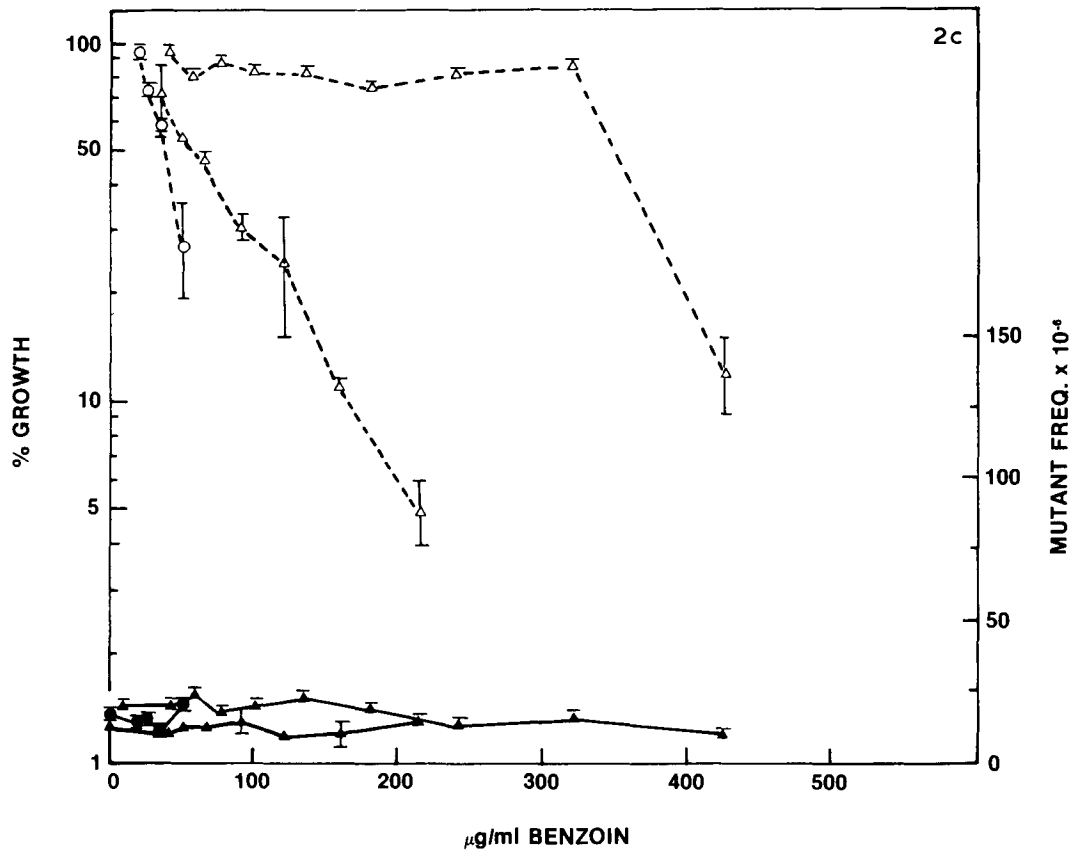
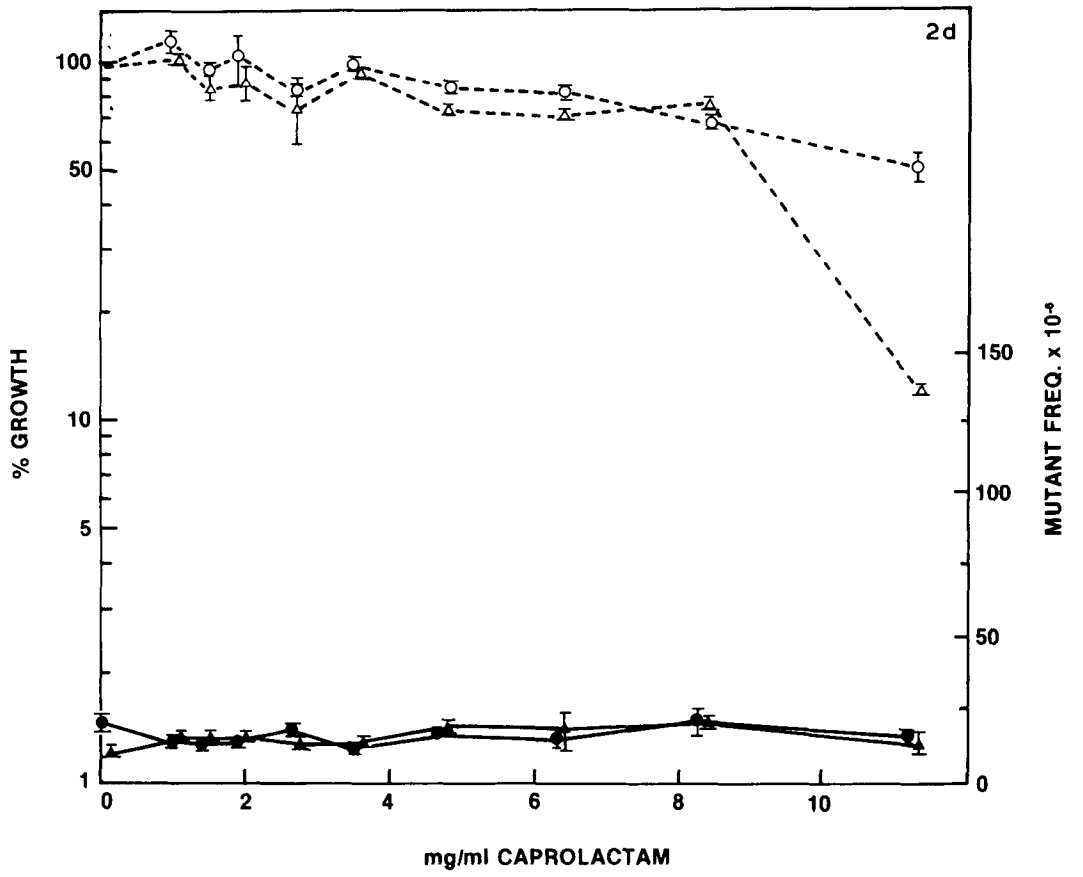
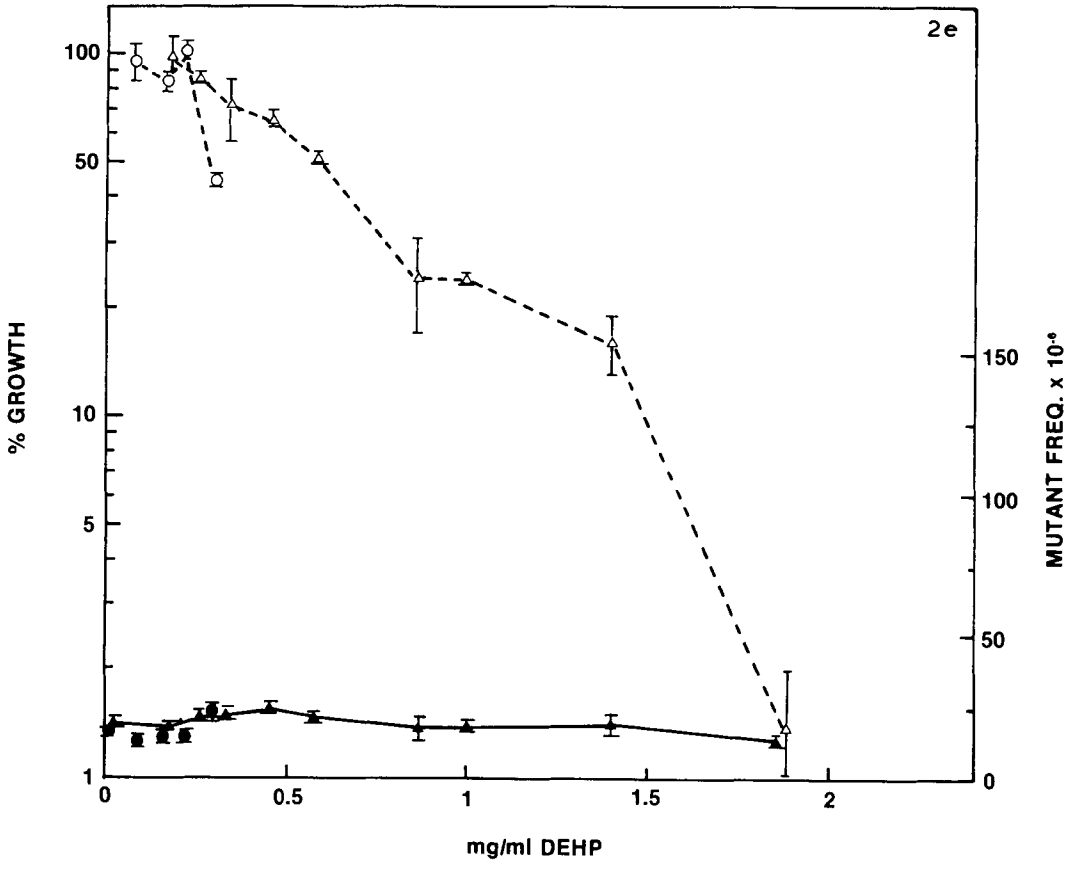


Fig. 2a–2j. Relative total cell survival (open symbols) and TFT-resistant mutant frequencies (closed symbols) for 10 chemicals in L5178Y/TK assay. Test results with S9 are shown with triangles; direct test results with circles.


2c
% GROWTH
MUTANT FREQ. x 10⁻⁶
100
50
10
5
1
150
100
50
0
0
100
200
300
400
500
μg/ml BENZOIN


2d
% GROWTH
MUTANT FREQ. x 10⁻⁶
100
50
10
5
1
150
100
50
0
0
2
4
6
8
10
mg/ml CAPROLACTAM


2e
100
50
10
5
1
% GROWTH
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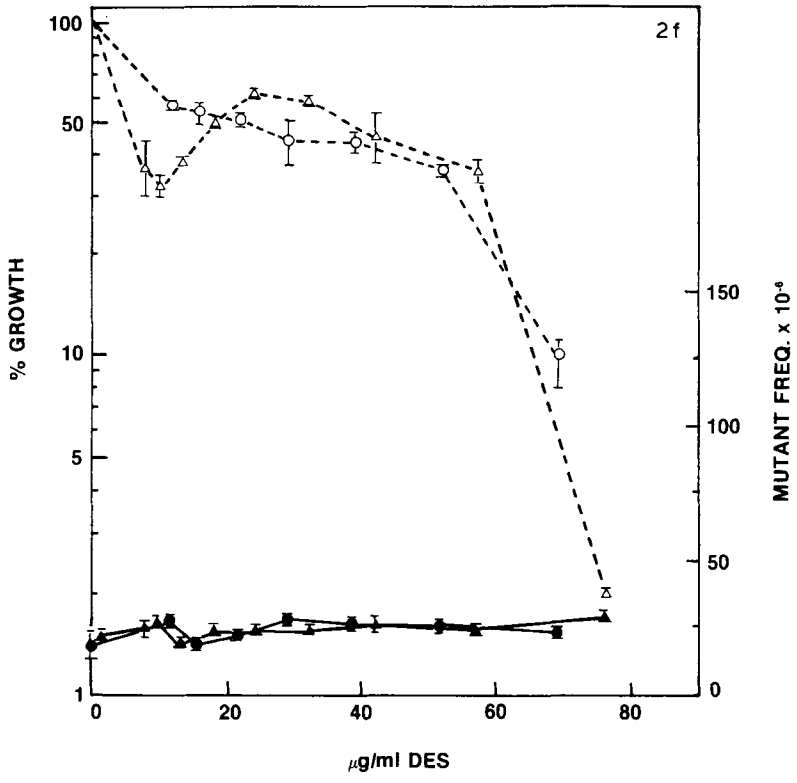
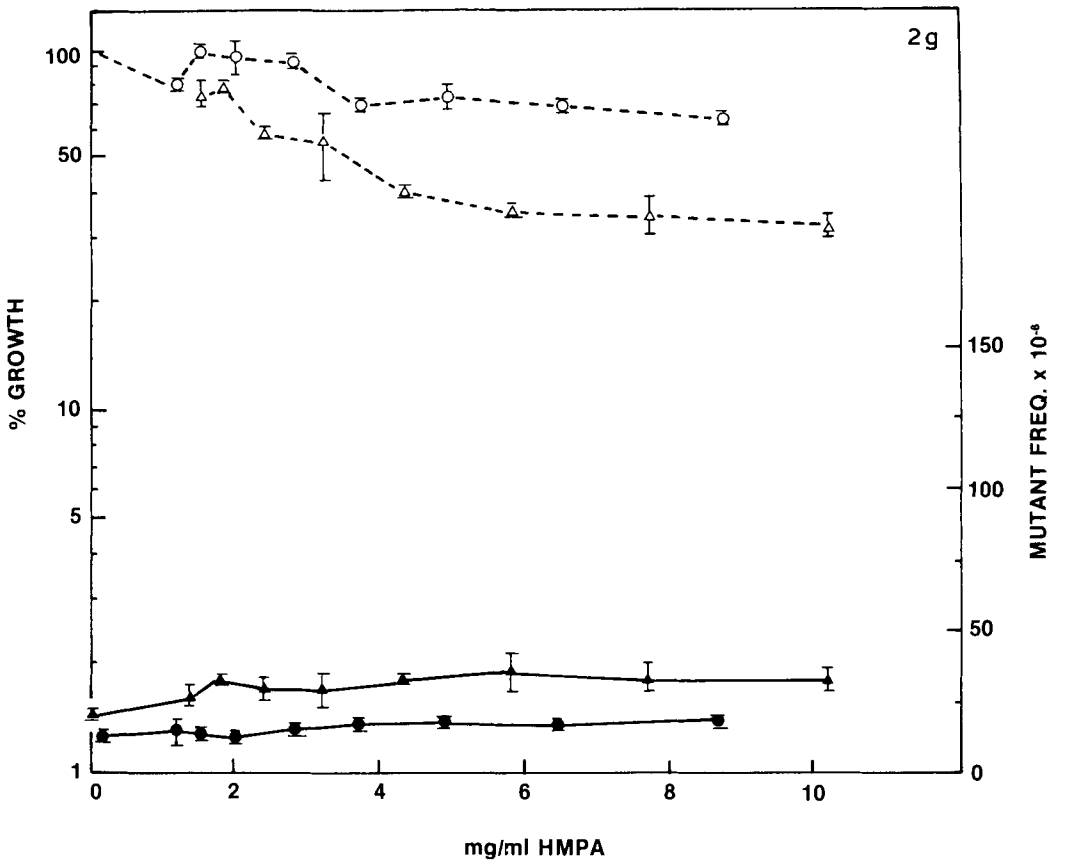
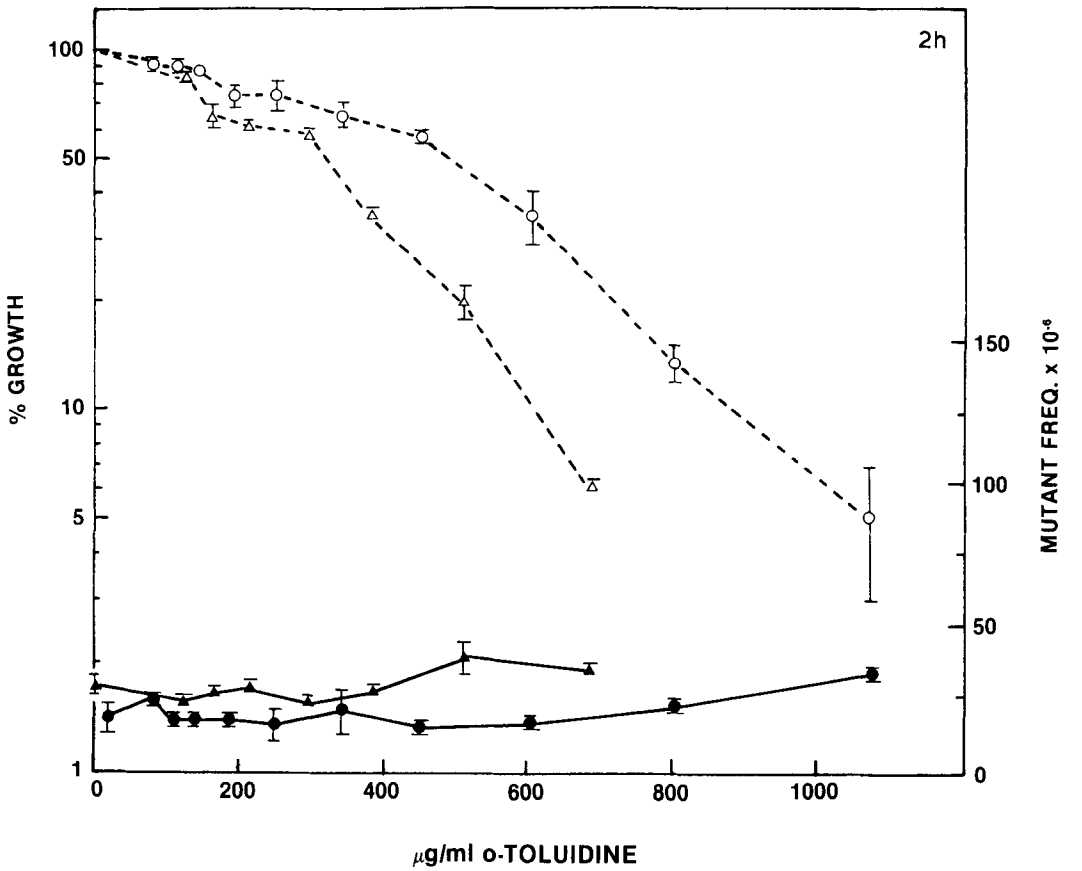
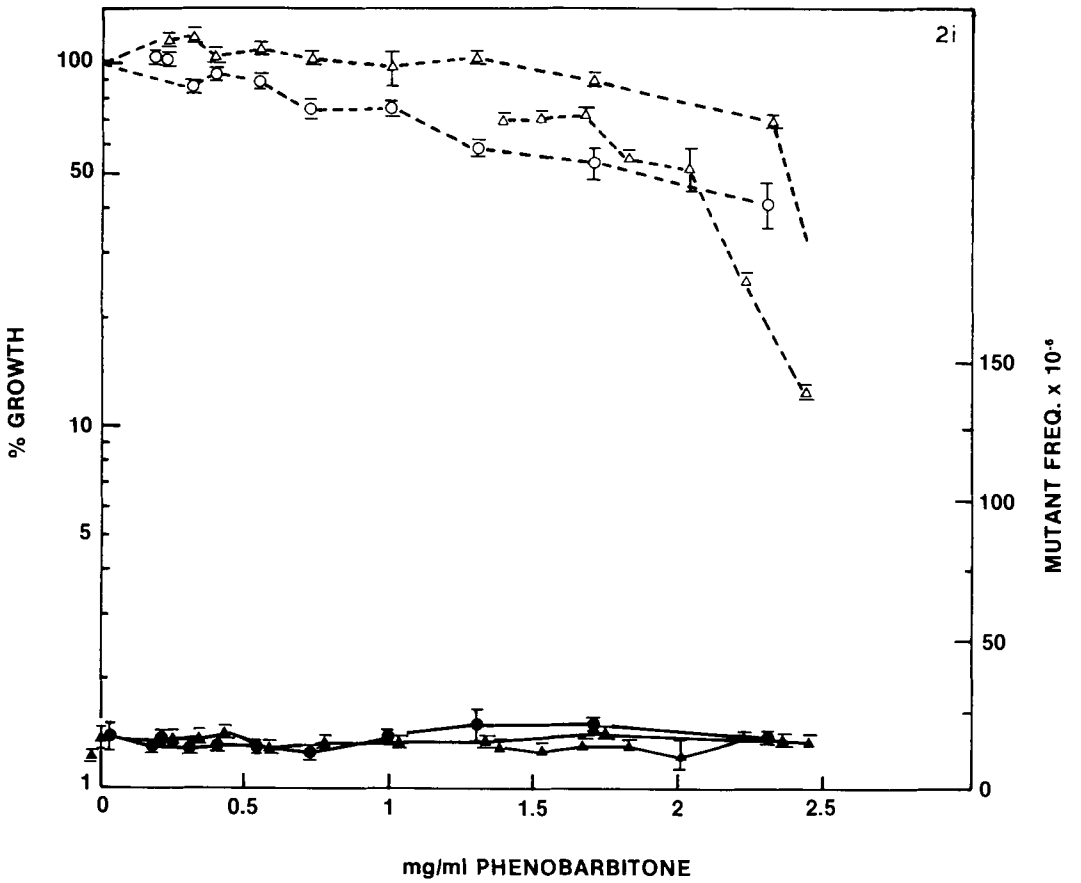
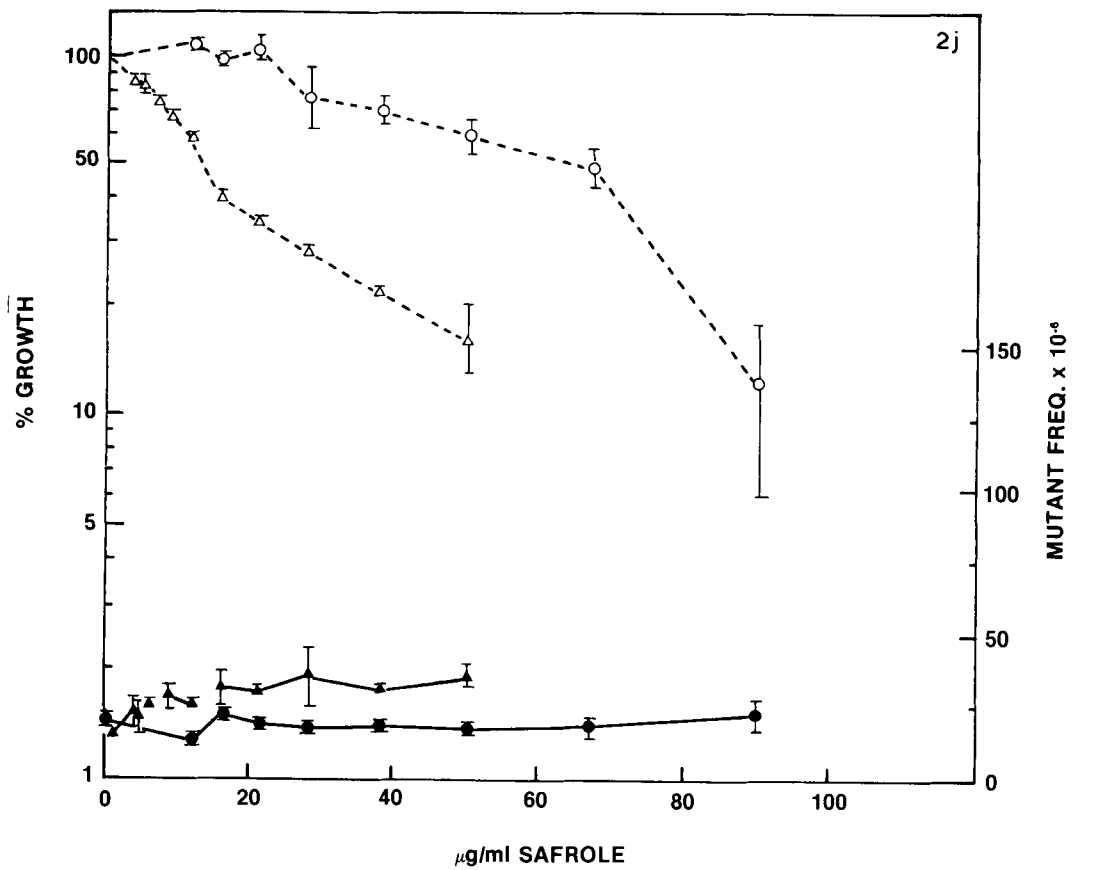
1
1.5
2
mg/ml DEHP
150
100
50
0
MUTANT FREQ. x 10⁻⁶


2f
100
50
10
5
1
% GROWTH
0
20
40
60
80
µg/ml DES
150
100
50
0
MUTANT FREQ. x 10⁻⁶


2g
100
50
10
5
1
% GROWTH
150
100
50
0
MUTANT FREQ. x 10⁻⁶
0
2
4
6
8
10
mg/ml HMPA


2h
100
50
10
5
1
% GROWTH
150
100
50
0
MUTANT FREQ. x 10⁻⁶
0
200
400
600
800
1000
μg/ml o-TOLUIDINE


2i
100
50
10
5
1
% GROWTH
150
100
50
0
MUTANT FREQ. x 10⁻⁶
0
0.5
1
1.5
2
2.5
mg/ml PHENOBARBITONE


2j
100
50
10
5
1
% GROWTH
150
100
50
0
MUTANT FREQ. x 10⁻⁶
0
20
40
60
80
100
μg/ml SAFROLE

Caprolactam

The chemical was virtually innocuous (Fig. 2d). No mutagenic affect was observed with or without S9 activation and cytotoxicity was appreciable only at > 11 mg/ml concentrations of this nonmutagen.

Diethylhexylphthalate (DEHP)

DEHP was clearly devoid of mutagenic activity in the presence of S9 at DEHP concentrations that produced considerable biological activity (Fig. 2e). Results for DEHP in the absence of metabolic activation were inconclusive due to lack of sufficient data points for analysis.

Diethylstilbestrol (DES)

The presence of S9 had little effect upon the cytotoxic or mutagenic effects of DES, suggesting that S9 proteins do not inactivate DES via protein binding nor activate DES via oxidative metabolism (Fig. 2f). DES was clearly not mutagenic even at toxic dose levels. DES was one of the most cytotoxic chemicals of the 10 assayed.

Hexamethylphosphoramide (HMPA)

Because this chemical was not very cytotoxic to L5178Y cells, all of the compound supplied was used up in the two trials described here (Fig. 2g). HMPA was a weak mutagen in the presence of rat-liver S9 when tested at concentrations up to 10 mg/ml. Oxidative demethylation of HMPA can produce formaldehyde (Jones and Jackson, 1968) which is strong mutagen in the L5178Y/TK assay at concentrations ≥ 5 μg/ml (our unpublished data).

o-Toluidine

When compared to concurrent solvent controls, *o*-toluidine was not considered mutagenic in either the presence or absence of liver S9 activation mix (Fig. 2h). Careful examination of the data does suggest, however, that this chemical was more cytotoxic in the presence of S9, along with a slight increase in mutant frequency.

Phenobarbitone

This chemical was not mutagenic directly when tested at concentrations > 2 mg/ml (Fig. 2i). The first test results in the presence of S9 were not conclusive due to the lack of sufficient data points for analysis, although we note that 3 mg/ml phenobarbitone produced zero cell survival, and thus was an upper test limit. Upon retesting, phenobarbitone was clearly negative in the presence of S9.

Safrole

Safrole, particularly in the presence of S9, was one of the most toxic chemicals in this series (Fig. 2j). This chemical was weakly mutagenic in the presence but negative in the absence of liver S9.

Conclusion

Only 3 of the 10 chemicals (HMPA, safrole and acrylonitrile) caused any significant gene mutational activity at the TK locus of L5178Y cells; two of these (HMPA and safrole) caused weak but reproducible (via duplicate test cultures) responses that were S9-dependent. No tallies of small TFT-resistant colonies were made during these trials because we have observed that: (1) trifluorothymidine-resistant colony populations are always bimodal, even in the absence of mutagen exposure, and (2) both large and small ouabain-resistant colonies can be recovered from EMS-treated L5178Y/TK cells (our unpublished data), an observation we are unable to reconcile with the general interpretation of small mutant colonies in this assay (Moore-Brown and Clive, 1979). In conclusion, of the 8 presumptive carcinogens (acrylonitrile, benzene, DEHP, DES, HMPA, *o*-toluidine, phenobarbitone, safrole) supplied by IPCS, only the 3 mentioned showed definite gene mutational activity in this study; 2 of the 3 were dependent upon exogenous metabolic activation. The noncarcinogens benzoin and caprolactam were without observable mutagenic activity in the presence or absence of exogenous metabolic activation provided by rat-liver S9.

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Tests for mutagenic activity using mutation assays at two loci in the human lymphoblast cell lines TK6 and AHH-1

Charles L. Crespi, Cynthia G. Ryan, Gerald M. Seixas, Thomas R. Turner and Bruce W. Penman

Gentest Limited Partnership, Woburn, MA (U.S.A.)

Summary

The 10 samples from the International Program for Chemical Safety Collaborative Study on Short-Term Tests were assayed for mutagenic activity in human lymphoblasts. Two distinct lines of human lymphoblasts were used in these assays; TK6, a human cell line which, like many cell lines, does not contain detectable mixed-function oxygenase activity; and AHH-1, a metabolically competent human cell line. Gene-locus mutations were measured at the thymidine kinase locus in TK6 cells and at the hypoxanthine guanine phosphoribosyl transferase locus in AHH-1 cells. The samples were assayed under 3 different conditions of metabolic activation. These conditions were: first, in TK6 cells without the addition of an extracellular metabolizing system; second, in TK6 cells with the addition of a rat-liver homogenate metabolizing system; and third, in the AHH-1 metabolically competent human lymphoblast cell line. A mutagenic response in one or more of the 3 assays performed under the differing conditions of metabolic activation defined a sample as mutagenic. Samples yielding positive mutagenic responses were *o*-toluidine, benzene, safrole, acrylonitrile, diethylstilbestrol and phenobarbital. Samples yielding non mutagenic responses were benzoin, caprolactam and diethylhexylphthalate. Inconclusive data were obtained that suggested that hexamethylphosphoramide was mutagenic; but in the absence of additional conclusive experiments, we must conclude that HMPA is not mutagenic.

The goal of genetic toxicology screening is to identify and eliminate potential mutagens to humans. To this end a number of test systems have been developed using a variety of cell types (bacterial, yeast, rodent, human, etc.). These systems are used as screens to detect chemicals that may be harmful to humans. A screening system should mimic the biochemistry of the species of interest, humans, to the greatest extent possible. There are important biochemical differences between cells of different species. For example, there are dramatic differences in the way the rodent and human cell types repair damage to their DNA. Cultured human cells have high levels of excision-repair activity, cultured rodent cells do not (Regan and Setlow, 1973; Lieberman et al., 1971; Klimek, 1966; Ben-Ishai and Peleg, 1975). The DNA repair in rodent cells is sensitive to the presence of caffeine, DNA repair in human cells is not (Trosko and Chu, 1973; Buhl and Regan, 1974; Fujiwara and Tasumi, 1976). Human cells continue to grow an additional cell generation after a lethal dose of an alkylating agent, rodent cells do not (Roberts, 1978). The biochemical differences between human cells and lower eukaryotic cells or prokaryotic cells are even greater. Given the differences between species, it is anticipated that mutagenicity evaluations performed in human cells will be more relevant to the actual effects of the chemical in humans than evaluations performed in cells from other species.

The human lymphoblast gene-locus mutation

assay system was developed at the Massachusetts Institute of Technology in the laboratory of William G. Thilly (Thilly et al., 1976; Skopek et al., 1978; Liber and Thilly, 1982; Furth et al., 1981; Thilly et al., 1980; Liber et al., 1982). This system has been demonstrated to be accurate, quantitative, and free of systemic bias. The human lymphoblast system is a versatile tool for the detection of many mutagens. Recently the human lymphoblast mutation assay system has been extended with the development of a mutation assay in a human lymphoblast cell line that has retained metabolic competence (Liber et al., 1982; Crespi and Thilly, 1984). A mutation assay in this metabolically competent cell line was included in our testing procedure. We report here that the human lymphoblast gene-locus mutation assay system detected as mutagenic most of the carcinogenic chemicals tested in this study. The noncarcinogenic chemicals were not mutagenic to human lymphoblasts.

Materials and methods

Cell lines

TK6 and AHH-1 human lymphoblasts were obtained from the Massachusetts Institute of Technology under a licensing agreement. The use of these cell lines in gene-locus mutation assays has been described (Thilly et al., 1976, 1980; Skopek et al., 1978; Liber and Thilly, 1982; Furth et al., 1981; Liber et al., 1982).

Preparation of mutagen stock solutions

All mutagens except caprolactam were dissolved in DMSO immediately before addition to the cells. Caprolactam was dissolved in RPMI medium 1640 and the solution was filter sterilized.

Preliminary cytotoxicity determination

The preliminary cytotoxicity determination was used to define the range of concentrations of the substances tested in the mutation assay.

The cytotoxicity of the test substance under each of the 3 exposure conditions was determined. The concentrations of the test substance in the mutation assays were chosen based on the results of the preliminary cytotoxicity determination and the aqueous solubility of the test substance. If the test substance was cytotoxic, the highest concentration of the test substance in the mutation assay was chosen to reduce cell survival to 10–20% relative to control cultures. If no cytotoxicity was observed for the test substance, the highest concentration of the test substance was 2 × the aqueous solubility or 10 mg/ml, whichever was less.

Pretreatment of cultures to eliminate preexisting mutants

The purpose of this step was to reduce the background frequency of mutants in the cell population. This reduction in the background noise increased the sensitivity of the test.

Exponentially-growing TK6 human lymphoblasts and AHH-1 human lymphoblasts (grown in RPMI medium 1640 supplemented with 10% v/v horse serum) were treated for 48 h with CHAT (1×10^{-5} M deoxycytidine, 2×10^{-4} M hypoxanthine, 2×10^{-7} M aminopterin, 1.75×10^{-5} M thymidine). Cells were then centrifuged ($1000 \times g$ for 5 min) and resuspended in medium containing THC (the same composition as CHAT without the aminopterin). The cultures were counted daily and scaled up depending on the number of cells required for the experiment. Cells were exposed to the test substance 2 or 3 days after the resuspension in THC-containing medium, depending on cell propagation.

Exposure to the test substance

In all mutation assays, multiple concentrations of the test substance were assessed for mutagenic activity. The negative control was the solvent vehicle, a known nonmutagen. The positive controls were known mutagens. The positive controls for TK6 cells without activation, for TK6 cells with activation and for AHH-1 cells were 70 ng/ml 4-nitroquinoline-*N*-oxide, 2.5 μg/ml benzo[*a*]pyrene and 3.1 μg/ml benzo[*a*]pyrene respectively.

During treatment with the test substance, enough cells were exposed that sufficient mutant cells survived the treatment. A larger number of mutant cells carried in an experiment decreased the variability between replicate cultures and hence increased the sensitivity of the test. For example, 4×10^7 AHH-1 cells were treated and the background mutant frequency in these cells was 3.5×10^{-6}, then $(4 \times 10^7) \times (3.5 \times 10^{-6})$ or 140 mutants

were present in the population. The expected standard deviation of the the number of mutants in this culture was 140 ± 12 or 8.5%. If only 1×10^7 cells had been treated, only 35 mutants would have been present in the population and the expected standard deviation would have been 35 ± 6 or 17%.

TK6 Cells Without Metabolic Activation. Each culture of 4×10^7 TK6 cells in 100 ml of medium was exposed to the test substance for 20 h (approximately one cell generation). Treatment was terminated by centrifuging ($1000 \times g$ for 5 min) from 4×10^7 to 5×10^7 cells and resuspending the cells in 100 ml of medium.

TK6 Cells With Metabolic Activation. In each culture, 4×10^7 TK6 cells in 40 ml of medium containing 5% v/v radiation sterilized arochlor 1254 induced rat-liver post-mitochondrial supernatant (PMS, commonly referred to as S9), 1 mg/ml $NADP^+$, 1 mg/ml glucose 6-phosphate (monosodium salt), 0.67 mg/ml magnesium chloride (hexahydrate) and 0.4 units/ml glucose-6-phosphate dehydrogenase were exposed to the test substance for 3 h. The treatment was terminated by centrifuging ($1000 \times g$ for 5 min) and resuspending the cells in 100 ml of medium.

AHH-1 Cells. Each culture of 4×10^7 AHH-1 cells in 80 ml of medium was exposed to the test substance for 28 h (approximately one cell generation). Treatment was terminated by centrifuging from 4×10^7 to 5×10^7 cells ($1000 \times g$ for 5 min) and resuspending the cells in 90 ml of medium.

Phenotypic expression of the induced mutations

When a mutation was induced at a particular locus in a cell, that cell became unable to produce the protein that was coded for by that locus. However, the cell initially had the normal amount of that protein. The amount of protein had to be reduced through cell division and protein turnover before the mutation appeared phenotypically. The time required for this phenotypic expression varies among different cell lines and among distinct gene-loci in the same cell line.

During the phenotypic expression period (3 days for the TK locus in TK6 cells and 6 days for the HGPRT locus in AHH-1 cells), the cell concentration was determined daily and cells were diluted with medium to 4×10^7 cells per culture.

Measurement of cytotoxicity

The measurement of cytotoxicity was important to ensure that sufficient mutant cells survived treatment.

Cytotoxicity was determined by the cell growth curve after treatment. The relative amount of cell growth in the test substance-treated cultures was divided by the relative cell growth in the negative control cultures to yield the relative survival. For example, if the test substance-treated cultures showed a 2.8-fold increase in cell number and the negative control cultures showed a 12.6-fold increase in cell number, the relative survival was 2.8/12.6 or 0.22.

Plating of the cells and scoring of plates

The mutant fraction is measured as the frequency of cells capable of forming colonies in 96-well microtiter plates in the presence of a selective agent divided by the fraction of cells which were capable of forming colonies with no selective agent present or the plating efficiency. Two parameters affect the accuracy of this measurement. First, the concentration of cells plated must not inhibit mutant colony formation. Second, the concentration of selective agent (trifluorothymidine or 6-thioguanine) used must completely suppress the growth of nonmutant cells but allow normal growth of mutant cells. These two parameters have been previously optimized. In addition, the cells were plated to determine mutant fraction only after the cells had recovered from any cytotoxicity and resumed exponential growth. This ensured that the plating efficiency in all cultures was comparable. If a culture had not recovered from the cytotoxicity by the 3rd day or 6th day after treatment for TK6 cells and AHH-1 cells respectively, plating of that culture was deferred an additional day to allow recovery.

For TK6 cells, cultures were plated to determine the mutant fraction on the third day after treatment. Cells were plated in 96-well microtiter plates at 30000 cells per well in medium containing 2 μg/ml trifluorothymidine (3 plates per culture) and 2 cells per well in medium (2 plates per culture). The serum supplement for plating was heat treated at 56 °C for 1 h. Plates were incubated for 12 days and scored for the presence or absence of a colony in each well.

For AHH-1 cells, cultures were plated on both the 6th and 7th days after treatment. Cells were plated in 96-well microtiter plates at 25 000 cells per well in medium containing 0.6 μg/ml 6-thioguanine (3 plates per culture) and 2.5 cells per well in medium (2 plates per culture). Plates were incubated for 14 days and then scored for the presence or absence of a colony in each well.

Calculation of mutant fraction

The number of negative wells and the total number of wells scored were recorded for each plate. The plate counts for each culture were pooled and the plating efficiency (PE) and the mutant fraction (MF) were calculated in accordance with the Poisson distribution.

$$PE = \frac{-\ln\left(\frac{\text{negative wells PE plates}}{\text{total wells PE plates}}\right)}{\text{cells per well plated in PE plates}}$$

$$MF = \frac{-\ln\left(\frac{\text{negative wells mutation plates}}{\text{total wells mutation plates}}\right)}{(\text{cells per well plated in mutation plates})(PE)}$$

For example, if 2 cells per well were plated in the PE plates and 60 negative wells of 192 total wells were observed, then the plating efficiency was $[-\ln (60/192)]/2$ or 0.58. If from the same culture 30 000 cells per well were plated in trifluorothymidine and 250 negative wells out of 288 total wells were observed, then the mutant fraction was $[-\ln (250/288)]/(30000 \times 0.58)$ or 8.1×10^{-6}.

Determination of mutational response

The data were analyzed to determine if the test substance was mutagenic or nonmutagenic. To accomplish this analysis, statistical tests were performed to determine whether the test substance had caused significant mutations in human cells.

The mean MF of the replicate cultures and the standard deviation of the MF determinations for each test condition were calculated. The means of the negative and positive controls for each assay had to be within the 99% confidence limits for each condition or that assay was repeated.

A mutagenic response was defined on the basis of two statistical criteria and checks of the assay performance. For a test substance to be defined as mutagenic, a mean mutant fraction, for a given test substance concentration, had to be greater than the 99% upper confidence limit of all historical negative control values and also greater than the concurrent negative controls at the 95% confidence level (t-test, one tail). If no mean mutant fraction fulfilled these criteria, then the response was defined as nonmutagenic.

The calculation of the 99% upper confidence limit of the historical negative control values was as follows. The number of mutant cells carried during chemical exposure and subsequent cell passaging was large (> 30), therefore the distribution can be approximated by a normal distribution. The number of mutant colonies on the plates was small, therefore the observations were not expected to be normally distributed, but instead should follow a Poisson distribution (a distribution skewed to the right). If the normal distribution were used to calculate the 99% upper confidence limit, the result would have been an underestimate. Therefore the negative control observations were fitted to a Poisson distribution. The 99% upper confidence limit was calculated as the value above which there was only 1% of the total area under the curve describing the Poisson distribution. The use of a 99% upper confidence limit of the historical controls for comparison to each of the 4 test substance concentrations yields an overall confidence level of 96%. Combination of the comparison to the historical controls with the *t*-test against the concurrent controls yields a rate of statistical false positive conclusions of less than 2%. This rate of statistical false positive conclusions can be further reduced by retesting and pooling the results before analysis. All positive conclusions (except acrylonitrile in AHH-1 cells) were based on at least two independent assays.

The following were the historical negative control data bases upon which the definitions of mutagenic or nonmutagenic were based.

TK6 cells

Number of observations: 72

Mean mutant fraction: 1.29×10^{-6}

Standard deviation: 0.61×10^{-6}

99% Upper confidence limit (triplicate cultures): 2.22×10^{-6}

99% Upper confidence limit (two sets of triplicate cultures): 1.85×10^{-6}

AHH-1 cells
Number of observations: 24
Mean mutant fraction: 3.91×10^{-6}
Standard deviation: 1.48×10^{-6}
99% Upper confidence limit (triplicate cultures): 6.5×10^{-6}
99% Upper confidence limit (two sets of triplicate cultures): 5.5×10^{-6}

Results and discussion

The results are summarized in Table 1. The response of the human lymphoblast gene-locus mutation assay under each condition of metabolic activation is given in this table. The highest concentration (in μg/ml) of the sample tested was given if the sample was found nonmutagenic. The lowest concentration of the sample that elicited a mutagenic response was given if the sample was found mutagenic. The following test samples (under the following treatment conditions) were mutagenic: *o*-toluidine (TK6 cells with PMS and AHH-1 cells), benzene (AHH-1 cells), safrole (TK6 cells with PMS), acrylonitrile (TK6 cells with PMS and AHH-1 cells), diethylstilbestrol (AHH-1 cells), and phenobarbital (AHH-1 cells). The following test samples were found to be nonmutagenic: caprolactam, benzoin and diethylhexylphthalate. The data we obtained were suggestive of a mutagenic response induced by HMPA. The observed mutant fractions were greater than the 99% upper confidence limit on the historical negative control; they were greater than the concurrent negative controls at the 90% confidence level, but not at the required 95% confidence level. We feel additional experiments are required to reach a definitive conclusion regarding HMPA mutagenicity. The primary data and calculated parameters for the individual mutation assays are presented in Table 2.

Mutagenic results were observed in the metabolically competent AHH-1 cell line and in the

TABLE 1

SUMMARY OF THE MUTAGENIC ACTIVITY OF THE 10 SAMPLES

Chemical	Assay system			Overall observed mutagenicity
	TK6	TK6+ activation	AHH-1	
Hexamethylphosphoramide	n.d.	– (10000)	– (10000)	–
o-Toluidine	– (750)	+ (450	+ (300)	+
Benzene	n.d.	– (2000)	+ (1000)	+
Safrole	– (1000)	+ (625)	– (160)	+
Caprolactam	– (8000)	– (8000)	– (5000)	–
Acrylonitrile	– (20)	+ (40)	+ (25)	+
Diethylhexylphthalate	– (1000)	– (1000)	– (1000)	–
Benzoin	– (500)	– (500)	– (400)	–
Diethylstilbestrol	– (25)	– (40)	+ (20)	+
Phenobarbital	– (1000)	– (1000)	+ (1000)	+

n.d., not done. Numbers in parentheses indicate the highest concentration of the chemical tested (in μg/ml) for a nonmutagenic response or the lowest chemical concentration tested that was mutagenic.

TABLE 2

HUMAN LYMPHOBLAST MUTATION ASSAY DATA

Treatment, the concentration of the sample tested, the negative control or the positive control; Point No., replicate points designated as "a, b, etc.", independent repeat experiments designated as "′, ″, etc."; Plate counts, listed for the plating efficiency and selective agent plates as the number of negative wells and the number of total wells; PE, the calculated plating efficiency; Mutant fraction, the calculated mutant fraction; Rel S, the calculated relative survival; Mean MF, the calculated mean of the replicate measurements of the mutant fraction; Std. Dev., the calculated standard deviation of the replicate measurements of the mutant fraction; Mutation within Expt., results of the *t*-test (one tail, 95% confidence) for significance (a "+" indicates that the mean MF for that treatment was greater than the mean MF of the concurrent negative controls); Comparison historical control, results of the comparison of the mean MF to the 99% upper confidence limit for the historical negative control observations (a "+" indicates that the mean MF was greater than the 99% upper confidence limit). Note: only when both statistical tests were "+" was the test sample defined as mutagenic.

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
Hexamethylphosphoramide, TK6 cells with activation										
Negative control	Oa	73/192	284/288	0.48	0.96					
	Ob	59/192	284/288	0.59	0.79					
	Oc	67/192	283/288	0.53	0.97					
	Oa′	119/192	287/288	0.24	0.48					
	Ob′	108/192	282/288	0.29	2.43					
	Oc′	122/192	284/288	0.23	2.06	1.00	1.28	0.78	−	−
2 mg/ml	1a	67/192	273/278	0.53	1.15					
	1b	64/192	281/288	0.55	1.49					
	1c	65/192	279/288	0.54	1.95	0.89	1.53	0.40	+	−
5 mg/ml	2a	55/192	287/288	0.63	0.19					
	2b	65/192	282/288	0.54	1.30					
	2c	72/190	282/288	0.49	1.45	0.84	0.97	0.69	−	−
10 mg/ml	3a	57/192	277/288	0.61	2.14					
	3b	67/192	284/288	0.53	0.89					
	3c	60/192	276/288	0.58	2.44					
	3a′	108/192	278/287	0.29	3.69					
	3b′	105/192	285/288	0.30	1.16					
	3c′	114/192	281/288	0.26	3.15	0.71	2.24	1.09	−	+
2.5 μg/ml B(a)P	4a	57/192	65/189	0.61	58.6					
	4b	74/192	83/192	0.48	58.6					
	4a′	100/192	246/288	0.33	16.1					
	4b′	107/192	226/288	0.29	27.6	0.62	40.2	21.7	+	+
HMPA, AHH-1 cells										
Negative control	Oa	241/384	572/576	0.19	1.50					
	Ob	234/384	565/575	0.20	3.54					
	Oc	227/384	552/565	0.21	4.42	1.00	3.15	1.50	−	−
2 mg/ml	1a	212/384	568/576	0.24	2.35					
	1b	220/384	569/576	0.22	2.20					
	Ic	229/384	552/576	0.21	8.23	0.80	4.26	3.44	−	−
5 mg/ml	2a	264/384	547/576	0.15	13.7					
	2b	233/384	563/576	0.20	4.57					
	2c	216/384	567/576	0.23	2.61	0.75	7.03	5.92	−	+
10 mg/ml	3a	257/384	570/576	0.16	2.61					
	3b	260/384	555/576	0.16	9.52					
	3c	259/384	569/576	0.16	3.10	0.53	5.08	3.86	−	−

TABLE 2 (continued)

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
3.1 μg/ml	4a	204/376	556/576	0.24	5.78					
B(a)P	4b	204/374	544/574	0.24	8.85	1.00	7.32	2.18	+	+
o-Toluidine, TK6 cells without activation										
Negative	Oa	67/192	280/288	0.53	1.78					
control	Ob	44/192	285/288	0.74	0.47					
	Oc	69/192	282/288	0.51	1.37					
	Od	36/192	283/288	0.84	0.70	1.00	1.08	0.60	−	−
150 μg/ml	1a	85/192	281/288	0.41	2.01					
	1b	27/192	261/288	0.98	2.31	0.90	2.20	0.27	+	−
300 μg/ml	2a	61/192	274/288	0.57	2.90					
	2b	41/192	279/288	0.77	1.37	0.74	2.13	1.08	−	−
450 μg/ml	3a	67/192	283/288	0.53	1.10					
	3b	54/192	280/288	0.63	1.48	0.48	1.29	0.26	−	−
600 μg/ml	4a	64/192	283/288	0.55	1.06					
	4b	58/192	275/288	0.60	2.57	0.54	1.82	1.07	−	−
750 μg/ml	5a	33/192	275/288	0.88	1.74					
	5b	19/192	273/288	1.12	1.54	0.40	1.64	0.14	−	−
70 ng/ml	6a	37/192	110/192	0.82	22.6					
4NQO	6b	17/192	75/192	1.21	25.8	0.63	24.2	2.33	+	+
o-Toluidine, TK6 cells with activation										
Negative	Oa	81/192	286/288	0.43	0.54					
control	Ob	63/188	281/288	0.55	1.50					
	Oc	75/192	277/288	0.47	1.99					
	Oa′	74/192	285/288	0.48	0.73					
	Ob′	65/192	282/288	0.54	1.30					
	Oc′	82/192	281/288	0.43	1.93	1.00	1.33	0.60	−	−
450 μg/ml	1a	72/192	277/288	0.49	2.65					
	1b	71/192	277/288	0.50	2.61					
	1c	62/192	275/288	0.57	2.72					
	1a′	56/192	279/288	0.62	1.72					
	1b′	87/192	279/288	0.40	2.67					
	1c′	62/192	279/288	0.57	1.87	0.98	2.37	0.45	+	+
900 μg/ml	2a	68/192	277/288	0.52	2.50					
	2b	72/192	282/288	0.49	1.43					
	2c	63/192	274/288	0.55	3.01	1.00	2.31	0.81	−	−
1350 μg/ml	3a	82/192	279/288	0.43	2.49					
	3b	80/192	282/288	0.44	1.60					
	3c	60/192	282/288	0.58	1.21	0.72	1.77	0.66	−	−
2.5 μg/ml	5a	87/192	92/192	0.40	69.8					
B(a)P	5b	49/192	46/192	0.68	73.2					
	5a′	50/192	84/192	0.67	41.0					
	5b′	70/192	82/192	0.50	56.2	0.79	57.2	12.2	+	+
o-Toluidine, AHH-1 cells										
Negative	Oa	193/384	567/576	0.27	2.28					

TABLE 2 (continued)

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
control	Ob	239/384	567/576	0.19	3.32					
	Oc	160/384	551/576	0.35	5.07					
	Oa′	252/384	571/576	0.17	2.07					
	Ob′	114/192	565/576	0.21	3.70					
	Oc′	113/192	568/576	0.21	2.64	1.00	3.18	1.11	–	–
100 μg/ml	1a	181/384	568/576	0.30	1.86					
	1b	184/384	536/576	0.29	9.78	0.65	5.82	5.60	–	–
200 μg/ml	2a	199/384	571/576	0.26	1.33					
	2b	218/384	557/576	0.23	5.92	0.50	3.63	3.25	–	–
300 μg/ml	3a	195/368	529/576	0.25	13.4					
	3b	228/384	562/576	0.21	4.72					
	3a′	255/384	555/574	0.16	8.22					
	3b′	142/192	565/576	0.12	6.39					
	3c′	115/192	538/556	0.21	6.42	0.48	7.83	3.35	+	+
400 μg/ml	4a	198/384	550/576	0.26	6.97					
	4b	199/384	550/567	0.26	4.63					
	4a′	251/384	568/576	0.17	3.29					
	4b′	303/384	565/576	0.10	8.14					
	4c′	286/384	522/534	0.12	7.71	0.50	6.15	2.10	+	+
500 μg/ml	5a	182/384	554/576	0.30	5.22					
	5b	218/384	538/576	0.23	12.1					
	5a′	148/192	564/576	0.10	8.08					
	5b′	293/384	564/576	0.11	7.78					
	5c′	292/384	543/557	0.11	9.29	0.50	8.49	2.49	+	+
3.1 μg/ml	6a	164/384	339/384	0.34	14.7					
B(a)P	6b	147/384	329/384	0.38	16.1					
	6a′	224/384	352/384	0.22	16.1					
	6b′	252/384	367/384	0.22	10.8	0.45	14.4	2.54	+	+
Benzene, TK6 cells with activation										
Negative	Oa	74/192	285/288	0.48	0.73					
control	Ob	65/192	282/288	0.54	1.30					
	Oc	82/192	281/288	0.43	1.93	1.00	1.32	0.60	–	–
0.5 mg/ml	1a	60/192	279/288	0.58	1.81					
	1b	73/192	286/288	0.48	0.48					
	1c	98/192	277/288	0.34	3.86	0.92	2.05	1.70	–	–
1 mg/ml	2a	47/192	283/288	0.70	0.83					
	2b	58/192	284/288	0.60	0.78					
	2c	82/192	284/288	0.43	1.10	0.94	0.90	0.17	–	–
1.5 mg/ml	3a	76/192	280/288	0.61	1.54					
	3b	45/192	280/288	0.73	1.29					
	3c	76/192	284/288	0.46	1.01	0.83	1.28	0.27	–	–
2 mg/ml	4a	57/192	276/288	0.61	2.33					
	4b	70/192	282/288	0.50	1.39					
	4c	68/192	282/288	0.52	1.35	0.61	1.69	0.56	–	–

TABLE 2 (continued)

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
2.5 μg/ml	5a	50/192	84/192	0.67	41.0					
B(a)P	5b	70/192	82/192	0.50	56.2	0.73	48.6	10.8	+	+
Benzene, AHH-1 cells										
Negative	Oa	105/192	282/288	0.24	3.49					
control	Ob	92/192	284/288	0.29	1.90					
	Oc	68/192	280/288	0.42	2.71					
	Oa′	183/384	563/576	0.30	3.08					
	Ob′	193/384	559/575	0.28	4.10					
	Oc′	189/384	547/568	0.28	5.31					
	Oa″	241/384	572/576	0.19	1.50					
	Ob″	234/384	565/575	0.20	3.54					
	Oc″	227/384	552/565	0.21	4.42	1.00	3.34	1.21	−	−
0.5 mg/ml	1a	79/192	282/288	0.36	2.37					
	1b	102/192	269/288	0.25	10.8	1.00	6.58	5.95	−	−
1 mg/ml	2a	101/192	276/287	0.26	6.08					
	2b	93/192	267/288	0.29	10.4					
	2c	78/192	278/288	0.36	3.92					
	2a′	167/384	544/568	0.33	5.18					
	2b′	163/384	557/574	0.34	3.51					
	2c′	170/384	547/576	0.33	6.34					
	2a″	230/370	559/576	0.19	6.30					
	2b″	233/381	543/576	0.20	12.0					
	2c″	234/373	552/576	0.19	9.12	1.00	6.99	2.92	+	+
1.5 mg/ml	3a′	149/384	566/576	0.38	1.85					
	3b′	138/384	547/576	0.41	5.04					
	3c′	188/384	554/576	0.29	4.98	1.00	3.96	1.82	−	−
2.0 mg/ml	4c	115/192	251/288	0.21	26.8	0.30	26.8	n/a	n/a	+
3.1 μg/ml	5a	86/192	174/192	0.32	12.2					
B(a)P	5b	89/192	175/192	0.31	12.1					
	5a′	161/384	355/380	0.35	7.83					
	5b′	179/384	342/378	0.31	13.1					
	5a″	204/376	556/576	0.24	5.78					
	5b″	204/374	544/574	0.24	8.85	1.00	9.98	2.93	+	+
Safrole, TK6 cells without activation										
Negative	Oa	44/192	279/288	0.74	1.44					
control	Ob	31/154	276/288	0.80	1.77					
	Oc	38/192	282/288	0.81	0.87	1.00	1.36	0.46	−	−
100 μg/ml	1a	33/192	277/288	0.88	1.47					
	1b	51/192	277/288	0.66	1.96	0.94	1.72	0.34	−	−
200 μg/ml	2a	45/192	278/288	0.73	1.62	0.78	1.62	n/a	n/a	−
500 μg/ml	3a	43/192	144/150	0.75	1.82					
	3b	47/192	280/288	0.70	1.33	0.74	1.58	0.34	−	−
1000 μg/ml	4a	62/192	277/283	0.57	1.26					
	4b	63/192	275/288	0.56	2.76	0.61	2.01	1.06	−	−

TABLE 2 (continued)

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
70 ng/ml	5a	79/192	118/190	0.44	35.8					
4NQO	5b	52/192	106/192	0.65	30.3	0.62	33.0	3.85	+	+
Safrole, TK6 cells with activation										
Negative	Oa	35/192	287/288	0.85	0.14					
control	Ob	46/192	280/288	0.71	1.31					
	Oa′	156/384	571/576	0.45	0.65					
	Ob′	126/384	558/576	0.56	1.90					
	Oc′	167/384	566/576	0.42	1.40	1.00	1.08	0.69	–	–
125 μg/ml	1a	61/192	280/288	0.57	1.64					
	1b	29/192	276/288	0.95	1.50	0.91	1.57	0.10	–	–
250 μg/ml	2a	31/192	292/288	0.91	0.77					
	2b	42/192	276/288	0.76	1.87	0.83	1.32	0.78	–	–
375 μg/ml	3a	27/192	273/288	0.98	1.82					
	3b	39/192	268/288	0.80	3.01	0.45	2.41	0.84	–	–
500 μgml	4a	41/192	281/288	0.77	1.06					
	4b	82/192	267/288	0.43	5.93					
	4a′	54/192	281/288	0.63	1.29					
	4b′	72/192	281/288	0.49	1.67					
	4c′	103/192	282/288	0.31	2.25	0.34	2.44	2.00	–	+
625 μg/ml	5a	74/192	269/288	0.48	4.77					
	5b	29/192	273/288	0.95	1.89					
	5a′	56/192	259/280	0.62	4.21					
	5b′	65/192	277/288	0.54	2.40					
	5c′	59/191	274/288	0.59	2.82	0.18	3.22	1.23	+	+
2.5 μg/ml	6a	29/192	48/192	0.95	48.9					
B(a)P	6b	24/192	44/192	1.04	47.2					
	6a′	65/192	104/192	0.54	37.7					
	6b′	78/192	98/192	0.45	49.8	0.70	45.9	5.55	+	+
Safrole, AHH-1 cells										
Negative	Oa	193/384	567/576	0.28	2.29					
control	Ob	239/384	567/576	0.19	3.32					
	Oc	160/384	551/576	0.35	5.07	1.00	3.56	1.40	–	–
40 μg/ml	1a	198/384	571/576	0.26	1.32					
	1b	202/384	559/576	0.26	4.66	0.87	2.65	2.84	–	–
80 μg/ml	2a	172/384	544/576	0.32	7.12					
	2b	226/384	565/576	0.21	3.64	0.86	5.38	2.64	–	–
120 μg/ml	3a	197/384	536/552	0.27	4.41					
	3b	205/384	558/576	0.25	5.06	0.80	4.73	0.47	–	–
160 μg/ml	4a	215/384	567/576	0.23	2.72					
	4b	231/384	566/576	0.20	3.45	0.60	3.08	0.52	–	–
3.1 μg/ml	6a	164/384	339/384	0.34	14.7					
B(a)P	6b	147/384	329/384	0.38	16.1	0.65	15.4	1.02	+	+
Caprolactam, TK6 cells without activation										
Negative	Oa	44/192	279/288	0.74	1.44					

TABLE 2 (continued)

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
control	Ob	31/154	276/288	0.80	1.77					
	Oc	38/192	282/288	0.81	0.87	1.00	1.36	0.46	–	–
1600 μg/ml	1a	65/192	283/288	0.54	1.08					
	1b	59/187	282/288	0.58	1.22	1.00	1.15	0.10	–	–
3200 μg/ml	2a	77/192	284/288	0.46	1.02					
	2b	56/182	281/288	0.59	1.39	0.77	1.21	0.26	–	–
4800 μg/ml	3a	57/192	285/288	0.61	0.57					
	3b	42/192	269/288	0.76	2.99	0.60	1.78	1.71	–	–
6400 μg/ml	4a	67/192	284/288	0.53	0.89					
	4b	69/192	281/288	0.51	1.60	0.40	1.24	0.51	–	–
8000 μg/ml	5a	63/192	284/288	0.56	0.84					
	5b	45/192	278/287	0.73	1.46	0.31	1.15	0.44	–	–
70 ng/ml	6a	79/192	118/190	0.44	35.8					
4NQO	6b	52/192	106/192	0.65	30.3	0.62	33.0	3.85	+	+
Caprolactam, TK6 cells with activation										
Negative	Oa	66/192	280/288	0.53	1.76					
control	Ob	77/192	278/288	0.46	2.58					
	Oc	70/192	280/288	0.50	1.86	1.00	2.07	0.45	–	–
1600 μg/ml	1a	67/192	282/288	0.53	1.33					
	1b	76/192	284/288	0.46	1.01	1.00	1.17	0.32	–	–
3200 μg/ml	2a	72/192	282/288	0.49	1.43					
	2b	56/192	286/288	0.62	0.38	1.00	0.90	0.75	–	–
4800 μg/ml	3a	85/192	287/288	0.41	0.28					
	3b	53/192	280/288	0.64	1.46	0.74	0.87	0.83	–	–
6400 μg/ml	4a	85/192	280/288	0.41	2.30					
	4b	56/192	285/288	0.62	0.57	0.92	1.44	1.23	–	–
8000 μg/ml	5a	57/192	277/288	0.61	2.14					
	5b	57/192	276/288	0.61	2.34	1.00	2.24	0.14	–	–
2.5 μg/ml	6a	64/192	108/192	0.55	34.9					
B(a)P	6b	56/192	113/192	0.62	28.7	0.69	31.8	4.42	+	+
Caprolactam, AHH-1 cells										
Negative	Oa	130/384	550/576	0.43	4.26					
control	Ob	133/384	562/576	0.42	2.32	1.00	3.29	1.37	–	–
1000 μg/ml	1a	132/384	548/576	0.43	4.67					
	1b	133/384	562/576	0.42	2.32	0.93	3.49	1.66	–	–
2000 μg/ml	2a	133/384	562/576	0.42	2.32					
	2b	137/384	560/576	0.41	2.73	0.94	2.53	0.29	–	–
3000 μg/ml	3a	175/384	556/576	0.31	4.50					
	3b	163/384	563/576	0.34	2.66	0.83	3.58	1.30	–	–
4000 μg/ml	4a	151/384	558/576	0.37	3.40					
	4b	162/384	562/576	0.35	2.85	0.65	3.13	0.39	–	–

TABLE 2 (continued)

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
5000 μg/ml	5a	172/384	561/576	0.32	3.29					
	5b	161/384	567/576	0.35	1.81	0.67	2.55	1.04	–	–
3.1 μg/ml	6a	132/384	412/480	0.43	14.3					
B(a)P	6b	150/384	420/480	0.38	14.2	0.93	14.3	0.07	+	+
Acrylonitrile, TK6 cells without activation										
Negative	Oa	48/192	279/288	0.69	1.53					
control	Ob	75/192	285/288	0.47	0.74					
	Oa′	60/192	283/288	0.58	1.00	1.00	1.09	0.40	–	–
5 μg/ml	1a	72/192	279/288	0.49	2.16					
	1b	64/192	277/288	0.55	2.36					
	1a′	56/192	282/288	0.62	1.14					
	1b′	13/192	284/288	1.35	0.35	0.91	1.50	0.94	–	–
10 μg/ml	2a	107/192	280/288	0.29	3.21					
	2b	57/192	281/288	0.61	1.35					
	2a′	32/192	288/288	0.90	0.00					
	2b′	35/192	280/288	0.85	1.10	0.67	1.42	1.33	–	–
15 μg/ml	3a	87/192	281/288	0.40	2.07					
	3b	76/192	277/288	0.46	2.80					
	3a′	49/192	279/288	0.68	1.55					
	3b′	64/192	278/288	0.55	2.14	0.26	2.14	0.51	+	–
20 μg/ml	4a′	46/192	275/288	0.71	2.16					
	4b′	26/192	285/288	1.00	0.35	0.18	1.25	1.28	–	–
70 ng/ml	5a	81/192	125/192	0.43	33.2					
4NQO	5b	94/192	146/192	0.36	25.6					
	5a′	49/168	85/192	0.62	44.1					
	5b′	69/192	89/192	0.51	50.1	0.39	38.2	11.0	+	+
Acrylonitrile, TK6 cells with activation										
Negative	Oa	35/192	287/288	0.85	0.41					
control	Ob	46/192	280/288	0.71	1.31					
	Oa′	66/192	280/288	0.53	1.76					
	Ob′	77/192	278/288	0.46	2.58					
	Oc′	70/192	280/288	0.50	1.86					
	Oa″	53/192	277/288	0.64	2.02					
	Ob″	64/192	280/288	0.54	1.71	1.00	1.63	0.76	–	–
10 μg/ml	1a	92/192	280/288	0.37	2.55					
	1b	43/184	279/288	0.73	1.46	1.00	2.00	0.78	–	–
20 μg/ml	2a	35/192	279/288	0.85	1.24					
	2b	49/192	280/288	0.68	1.38	0.86	1.31	0.09	–	–
30 μg/ml	3a	64/192	276/288	0.55	2.58					
	3b	9/192	269/288	1.53	1.49	0.62	2.03	0.77	–	–
40 μg/ml	4a	42/192	272/288	0.76	2.51					
	4b	37/192	273/288	0.82	2.17					
	4a′	100/192	259/288	0.33	10.8					
	4b′	80/192	262/288	0.44	7.20	0.37	5.68	4.14	+	+

TABLE 2 (continued)

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
50 μg/ml	5a	43/192	267/288	0.75	3.37					
	5b	28/192	270/288	0.96	2.23					
	5a′	157/192	279/288	0.10	10.5					
	5b′	105/192	280/288	0.30	3.11					
	5a″	76/192	259/288	0.46	7.63					
	5b″	83/192	263/288	0.41	7.22	0.26	5.68	3.27	+	+
2.5 μg/ml	6a	29/192	48/192	0.95	48.9					
B(a)P	6b	24/192	44/192	1.04	47.2					
	6a′	64/192	108/192	0.55	34.9					
	6b′	56/192	113/192	0.62	28.7					
	6a″	62/192	142/288	0.57	41.7	0.69	39.9	9.8	+	+
Acrylonitrile, AHH-1 cells										
Negative	Oa	193/384	567/576	0.28	2.29					
control	Ob	239/384	567/576	0.19	3.32					
	Oc	160/384	551/576	0.35	5.07	1.00	3.56	1.40	–	–
5 μg/ml	1a	185/384	572/576	0.29	0.95					
	1b	206/384	567/576	0.25	2.53	1.00	1.74	1.11	–	–
10 μg/ml	2a	172/384	562/576	0.32	3.06					
	2b	234/384	561/568	0.20	2.50	1.00	2.78	0.40	–	–
15 μg/ml	3a	179/384	549/576	0.31	6.29					
	3b	208/384	565/576	0.25	3.14	0.67	4.72	2.22	–	–
20 μg/ml	4a	238/384	538/576	0.19	14.3					
	4b	219/384	560/576	0.22	5.02	0.34	9.64	6.54	–	+
25 μg/ml	5a	265/384	524/576	0.15	25.5					
	5b	261/384	558/576	0.15	8.22	0.16	16.9	12.1	+	+
3.1 μg/ml	6a	164/384	339/384	0.34	14.7					
B(a)P	6b	147/384	329/384	0.38	16.1	0.65	15.4	1.02	+	+
Diethylhexylphthalate, TK6 cells without activation										
Negative	Oa	67/192	280/288	0.53	1.78					
control	Ob	44/192	285/288	0.74	0.47					
	Oc	69/192	282/288	0.51	1.37					
	Od	36/192	283/288	0.84	0.70	1.00	1.08	0.60	–	–
200 μg/ml	1a	61/192	272/282	0.57	2.10					
	1b	57/192	281/288	0.61	1.35	0.85	1.72	0.53	–	–
400 μg/ml	2a	71/192	280/287	0.50	1.65					
	2b	50/192	282/288	0.67	1.04	1.00	1.35	0.43	–	–
600 μg/ml	3a	60/192	274/288	0.58	2.86					
	3b	50/192	285/288	0.67	0.52	1.00	1.69	1.65	–	–
800 μg/ml	4a	44/192	280/288	0.74	1.27					
	4b	63/192	283/288	0.56	1.05	0.88	1.16	0.16	–	–
1000 μg/ml	5a	68/192	276/279	0.52	0.69					
	5b	49/192	279/288	0.68	1.55	1.00	1.12	0.60	–	–

TABLE 2 (continued)

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
70 ng/ml	6a	37/192	110/192	0.82	22.5					
4NQO	6b	17/192	75/192	1.21	25.8	0.67	24.2	2.33	+	+
Diethylhexylphthalate, TK6 cells with activation										
Negative	Oa	81/192	286/288	0.43	0.54					
control	Ob	63/192	281/288	0.56	1.47					
	Oc	75/192	280/288	0.47	2.00	1.00	1.34	0.74	–	–
250 μg/ml	1a	55/192	280/288	0.63	1.50					
	1b	42/192	281/288	0.76	1.08					
	1c	68/192	279/288	0.52	2.04	1.00	1.77	0.66	–	–
500 μg/ml	2a	65/192	280/288	0.54	1.73					
	2b	65/192	283/288	0.54	1.08					
	2c	57/192	282/288	0.61	1.16	0.99	1.33	0.36	–	–
750 μg/ml	3a	76/192	283/288	0.46	1.25					
	3b	55/192	281/288	0.63	1.31					
	3c	62/192	282/288	0.57	1.24	1.00	1.27	0.37	–	–
1000 μg/ml	4a	87/192	285/288	0.40	0.88					
	4b	82/192	281/288	0.43	1.93					
	4c	62/192	279/288	0.57	1.87	0.92	1.56	0.59	–	–
2.5 μg/ml	5a	87/192	92/192	0.40	62.0					
B(a)P	5b	49/192	46/192	0.68	69.8	0.79	65.9	5.51	+	+
Diethylhexylphthalate, AHH-1 cells										
Negative	Oa	242/384	560/566	0.18	2.30					
control	Ob	147/335	552/576	0.33	5.16					
	Oc	211/384	551/566	0.24	4.48					
	Od	213/384	555/575	0.24	6.01					
	Oa′	252/384	571/576	0.17	2.07					
	Ob′	114/192	565/576	0.21	3.70					
	Oc′	113/192	568/576	0.21	2.63					
	Oa″	96/383	548/576	0.55	3.60					
	Ob″	119/384	543/576	0.47	5.04					
	Oc″	127/375	550/576	0.43	4.26	1.00	3.93	1.31	–	–
200 μg/ml	1a	231/384	557/576	0.20	6.60					
	1b	187/384	540/576	0.29	8.97					
	1a′	253/384	574/576	0.17	0.83					
	1b′	267/384	568/576	0.15	3.85					
	1c′	296/384	572/576	0.11	2.68					
	1a″	119/384	557/576	0.47	2.86					
	1b″	96/384	557/572	0.55	1.92					
	1c″	134/384	536/576	0.42	6.80	0.93	4.31	2.83	–	–
400 μg/ml	2a	230/384	545/565	0.21	7.03					
	2b	160/384	550/576	0.35	5.27	0.91	6.15	1.24	–	–
600 μg/ml	3a	228/384	566/576	0.21	3.36					
	3b	204/384	556/576	0.25	5.59	0.92	4.47	1.58	–	–
800 μg/ml	4a	250/384	565/575	0.17	4.09					
	4b	157/384	545/572	0.36	5.41	0.85	4.74	0.93	–	–

TABLE 2 (continued)

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
1000 μg/ml	5a	270/384	562/576	0.14	6.99					
	5b	186/384	536/576	0.29	9.93					
	5a′	264/384	573/576	0.15	1.39					
	5b′	263/384	567/576	0.15	4.16					
	5a″	115/384	556/576	0.48	2.93					
	5b″	95/380	542/566	0.55	3.12					
	5c″	116/384	558/572	0.48	2.07	0.82	4.37	3.04	–	–
3.1 μg/ml	6a	136/192	163/192	0.14	47.5					
B(a)P	6b	185/384	325/384	0.29	22.8					
	6a′	224/384	352/384	0.22	16.1					
	6b′	252/384	367/384	0.17	10.8					
	6a″	123/376	358/384	0.45	6.27					
	6b″	107/384	338/384	0.51	9.99	0.65	19.1	15.4	+	+
Benzoin, TK6 cells without activation										
Negative	Oa	52/184	282/288	0.63	1.11					
control	Ob	78/184	282/288	0.43	1.64	1.00	1.37	0.37	–	–
100 μg/ml	1a	45/184	275/288	0.70	2.19					
	1b	67/192	284/288	0.53	0.89	1.00	1.54	0.92	–	–
200 μg/ml	2a	53/192	282/288	0.64	1.09					
	2b	53/192	281/288	0.64	1.27	1.00	1.18	0.13	–	–
300 μg/ml	3a	77/192	280/288	0.46	2.06					
	3b	45/192	285/288	0.73	0.48	1.00	1.27	1.11	–	–
400 μg/ml	4a	50/192	288/288	0.67	0.00					
	4b	47/192	278/288	0.70	1.67	1.00	0.84	1.18	–	–
500 μg/ml	5a	78/192	286/288	0.45	0.52					
	5b	34/192	284/288	0.87	0.54	1.00	0.53	0.02	–	–
70 ng/ml	6a	87/192	106/192	0.40	50.0					
4NQO	6b	73/192	90/192	0.48	52.2	0.69	51.1	1.56	+	+
Benzoin, TK6 cells with activation										
Negative	Oa	82/192	284/288	0.43	1.10					
control	Ob	47/192	280/288	0.70	1.33					
	Oc	17/192	280/288	1.21	0.77	1.00	1.07	0.28	–	–
100 μg/ml	1a	61/192	282/288	0.57	1.22					
	1b	77/192	276/288	0.46	3.11	1.00	2.16	1.33	–	–
200 μg/ml	2a	46/192	277/288	0.71	1.82					
	2b	38/192	286/288	0.81	0.29	1.00	1.05	1.08	–	–
300 μg/ml	3a	60/192	277/288	0.58	2.23					
	3b	55/192	283/288	0.63	0.93	1.00	1.58	0.92	–	–
400 μg/ml	4a	46/192	285/288	0.71	0.49					
	4b	90/192	280/288	0.38	2.48	1.00	1.48	1.41	–	–
500 μg/ml	5a	74/192	286/288	0.48	0.49					
	5b	81/184	284/288	0.41	1.14	1.00	0.81	0.46	–	–

TABLE 2 (continued)

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
2.5 μg/ml	6a	64/192	92/192	0.55	44.6					
B(a)P	6b	97/192	127/192	0.34	40.4	0.99	42.5	3.03	+	+
Benzoin, AHH-1 cells										
Negative	Oa	242/384	560/566	0.18	2.31					
control	Ob	147/335	552/576	0.33	5.17					
	Oc	211/384	551/566	0.24	4.49					
	Od	213/384	555/575	0.24	6.01	1.00	4.49	1.59	–	–
80 μg/ml	1a	183/384	558/566	0.30	1.92					
	1b	196/381	550/576	0.27	6.95	1.00	4.43	3.56	–	–
160 μg/ml	2a	197/384	552/575	0.27	6.12					
	2b	227/376	556/576	0.20	7.00	0.69	6.56	0.63	–	–
240 μg/ml	3a	204/384	559/576	0.25	4.74					
	3b	241/368	558/576	0.17	7.50	0.89	6.12	1.95	–	–
320 μg/ml	4a	199/384	557/576	0.26	5.10					
	4b	179/384	550/576	0.31	6.05	0.83	5.58	0.67	–	–
400 μg/ml	5a	207/384	568/576	0.25	2.22					
	5b	172/384	553/576	0.32	5.07	0.83	3.07	2.83	–	–
3.1 μg/ml	6a	185/384	325/384	0.29	22.8					
B(a)P	6b	136/192	163/192	0.14	47.5	0.65	35.2	17.4	+	+
Diethylstilbestrol, TK6 cells without activation										
Negative	Oa	52/184	282/288	0.63	1.11					
control	Ob	78/184	282/288	0.43	1.64	1.00	1.37	0.37	–	–
5 μg/ml	1a	70/192	286/288	0.50	0.46					
	1b	67/192	284/288	0.53	0.89	0.98	0.67	0.30	–	–
10 μg/ml	2a	51/192	280/288	0.66	1.42					
	2b	67/192	282/288	0.53	1.33	1.00	1.37	0.06	–	–
15 μg/ml	3a	71/192	276/288	0.50	2.85					
	3b	50/192	281/288	0.67	1.22	0.60	2.04	1.15	–	–
20 μg/ml	4a	64/192	284/288	0.55	0.85					
	4b	58/192	282/288	0.60	1.17	0.72	1.01	0.23	–	–
25 μg/ml	5a	69/192	284/288	0.51	0.91					
	5b	55/192	273/288	0.63	2.85	0.28	1.88	1.37	–	–
70 ng/ml	6a	87/192	106/192	0.40	50.0					
4NQO	6b	73/192	90/192	0.48	52.2	0.69	51.1	1.56	+	+
Diethylstilbestrol, TK6 cells with activation										
Negative	Oa	82/192	284/288	0.43	1.01					
control	Ob	47/192	280/288	0.70	1.33					
	Oc	17/192	280/288	1.21	0.77	1.00	1.07	0.28	–	–
8 μg/ml	1a	70/192	286/288	0.50	0.46					
	1b	98/192	285/288	0.34	1.04	0.80	0.75	0.41	–	–
16 μg/ml	2a	81/192	283/288	0.43	1.35					
	2b	95/192	284/288	0.35	1.33	0.88	1.34	0.02	–	–

TABLE 2 (continued)

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
24 μg/ml	3a	92/192	283/288	0.37	1.59					
	3b	88/184	284/288	0.37	1.26	0.78	1.43	0.23	–	–
32 μg/ml	4a	60/192	285/288	0.58	0.60					
	4b	65/192	281/288	0.54	1.51	0.70	1.06	0.65	–	–
40 μg/ml	5a	55/192	280/288	0.63	1.50					
	5b	55/192	282/288	0.63	1.12	0.57	1.31	0.27	–	–
2.5 μg/ml	6a	64/192	92/192	0.55	44.6					
B(a)P	6b	97/192	127/192	0.34	40.4	1.00	42.5	3.03	+	+
Diethylstilbestrol, AHH-1 cells										
Negative	Oa	242/384	560/566	0.18	2.31					
control	Ob	147/335	552/576	0.33	5.17					
	Oc	211/384	551/566	0.24	4.49					
	Od	213/384	555/575	0.24	6.01					
	Oa′	252/384	571/576	0.17	2.07					
	Ob′	114/192	565/576	0.21	3.70					
	Oc′	113/192	568/576	0.21	2.64	1.00	3.77	1.51	–	–
4 μg/ml	1a	160/377	561/576	0.34	3.08					
	1b	180/384	553/576	0.30	5.38	0.56	4.23	1.63	–	–
12 μg/ml	2a	190/382	562/576	0.28	3.52					
	2b	223/384	563/576	0.22	4.20	0.33	3.86	0.48	–	–
16 μg/ml	3a	203/384	554/576	0.26	6.11					
	3b	184/384	542/563	0.29	5.17					
	3a′	286/384	562/576	0.12	8.35					
	3b′	238/384	565/576	0.19	4.03					
	3c′	296/384	570/576	0.10	4.02	0.41	5.54	1.80	+	–
20 μg/ml	4a	216/384	551/576	0.23	7.71					
	4b	201/384	541/576	0.26	9.68					
	4a′	255/384	556/576	0.16	8.63					
	4b′	268/384	559/576	0.14	8.33					
	4c′	249/384	564/576	0.17	4.86	0.29	7.84	1.81	+	+
3.1 μg/ml	5a	136/192	163/192	0.14	47.5					
B(a)P	5b	185/384	325/384	0.29	22.8					
	5a′	224/384	352/384	0.22	16.1					
	5b′	252/384	367/384	0.17	10.8	0.65	24.3	16.2	+	+
Phenobarbital, TK6 cells without activation										
Negative	Oa	67/192	280/288	0.53	1.78					
control	Ob	44/192	285/288	0.74	0.47					
	Oc	69/192	282/288	0.51	1.37					
	Od	36/192	283/288	0.84	0.70	1.00	1.08	0.60	–	–
200 μg/ml	1a	65/192	275/288	0.54	2.84					
	1b	17/192	273/288	1.21	1.47	0.90	2.15	0.97	–	–
400 μg/ml	2a	68/192	284/288	0.52	0.90					
	2b	49/192	277/288	0.68	1.90	1.00	1.40	0.71	–	–
600 μg/ml	3a	47/192	279/288	0.70	1.50					

TABLE 2 (continued)

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
	3b	25/192	279/288	1.02	1.04	0.83	1.27	0.33	–	–
800 μg/ml	4a	69/192	282/288	0.51	1.37					
	4b	52/192	277/288	0.65	2.00	0.98	1.68	0.44	–	–
1000 μg/ml	5a	62/192	285/288	0.57	0.62					
	5b	31/192	282/288	0.91	0.77	1.00	0.69	0.11	–	–
70 ng/ml	6a	37/192	110/192	0.82	22.6					
4NQO	6b	17/192	75/192	1.21	25.8	0.67	24.2	2.33	+	+
Phenobarbital, TK6 cells with activation										
Negative	Oa	82/192	284/288	0.43	1.10					
control	Ob	47/192	280/288	0.70	1.33					
	Oc	17/192	280/288	1.21	0.77	1.00	1.07	0.28	–	–
200 μg/ml	1a	72/192	280/288	0.49	1.91					
	1b	67/192	286/288	0.53	0.44	0.99	1.17	1.04	–	–
400 μg/ml	2a	44/192	286/288	0.74	0.32					
	2b	50/176	282/288	0.63	1.11	1.00	0.72	0.57	–	–
600 μg/ml	3a	46/192	282/288	0.71	0.98					
	3b	50/192	276/288	0.67	2.11	0.90	1.55	0.80	–	–
800 μ/ml	4a	75/192	278/288	0.47	2.51					
	4b	38/192	284/288	0.81	0.58	1.00	1.54	1.36	–	–
1000 μg/ml	5a	42/192	278/288	0.76	1.55					
	5b	38/192	278/288	0.81	1.45	1.00	1.50	0.07	–	–
2.5 μg/ml	6a	64/192	92/192	0.55	44.6					
B(a)P	6b	97/192	127/192	0.34	40.3	0.99	42.5	3.0	+	+
Phenobarbital, AHH-1 cells										
Negative	Oa	242/384	560/566	0.18	2.31					
control	Ob	147/335	552/576	0.33	5.17					
	Oc	211/384	551/566	0.24	4.49					
	Od	213/384	555/575	0.24	6.00					
	Oa′	181/380	562/572	0.30	3.08					
	Ob′	194/384	553/575	0.27	5.71					
	Oc′	174/384	553/571	0.31	4.18	1.00	4.42	1.36	–	–
200 μg/ml	1a	197/384	558/570	0.27	3.19					
	1b	172/384	550/566	0.32	3.57	1.00	3.37	0.27	–	–
400 μg/ml	2a	231/384	566/576	0.20	3.44					
	2b	201/384	559/576	0.26	4.63	0.85	4.04	0.84	–	–
600 μg/ml	3a	190/379	556/576	0.28	5.12					
	3b	187/384	557/576	0.29	4.66	0.88	4.89	3.23	–	–
800 μg/ml	4a	197/384	560/576	0.27	4.22					
	4b	199/384	538/576	0.26	10.4					
	4a′	175/384	561/576	0.31	3.36					
	4b′	169/384	542/574	0.33	6.99					
	4c′	162/377	556/573	0.34	3.57	0.74	6.22	2.89	–	+

TABLE 2 (continued)

Treatment	Point No.	Plate counts		PE	Mutant fraction ($\times 10^6$)	Rel S	Mean MF ($\times 10^6$)	Std. dev. ($\times 10^6$)	Mutation within Expt.	Comparison historical control
		Plating efficiency neg/total	Selective agent neg/total							
1000 μg/ml	5a	217/384	550/576	0.23	8.09					
	5b	157/384	539/568	0.36	5.86					
	5a′	192/384	544/576	0.28	8.25					
	5b′	184/384	558/573	0.29	3.60					
	5c′	170/384	540/566	0.33	5.77	0.69	6.31	1.92	+	+
3.1 μg/ml	6a	185/384	325/384	0.29	22.8					
B(a)P	6b	136/192	163/192	0.14	47.4					
	6a′	158/358	342/384	0.33	14.1					
	6b′	156/350	318/384	0.32	23.3	0.65	27.0	14.3	+	+

TK6 cell line with the incorporation of a rat-liver PMS-activating system. No samples were found to be mutagenic to TK6 cells when treated without extracellular metabolism. Therefore, the TK6 cell system without PMS gave little additional information about the mutagenicity of these chemicals. However, we cannot recommend at this time that this part of the protocol be deleted from a screening program. The data base for "direct-acting" mutagens in AHH-1 cells is limited and the effects of intracellular oxidative metabolism on the mutagenicity of "direct-acting" chemicals is at present unknown. If AHH-1 cells prove sensitive to "direct-acting" mutagens, a comparable screening program could conceivably be carried out in a single cell line (AHH-1 cells treated with and without PMS).

The protocol used incorporated the use of two gene-loci, thymidine kinase (TK) in TK6 cells and hypoxanthine guanine phosphoribosyl transferase (HGPRT) in AHH-1 cells. We see little advantage to using two different loci over the use of the same locus in both cell lines; both loci are equally sensitive indicators of mutagenicity (Liber and Thilly, 1982). The phenotypic expression time for the TK locus is considerably shorter than for the HGPRT locus, making the TK locus a technically easier locus to use. The development of a mutation assay at the TK locus in AHH-1 cells is currently in progress.

We find the observed mutagenicity of benzene of considerable interest. Benzene is a human carcinogen (causing leukemia) with limited evidence for carcinogenicity in rodents. The metabolically competent human lymphoblastoid cell line (AHH-1) detected a mutagenic effect induced by benzene exposure. We feel that given the species, cell type, and metabolic function similarities between AHH-1 cells and the actual cells at risk for benzene-induced carcinogenicity, this observation should be followed up with additional experiments. Of particular interest would be the effects of a long-term exposure to low concentrations of benzene.

The statistical methods used to reach a conclusion of mutagenic or nonmutagenic take into account both the variability inherent in the assay system (a comparison to the large number of observations which constitute the historical data base) and the variability observed in the individual experiments (a comparison to the concurrent controls via a *t*-test). The frequency of statistical false positive results can be calculated for this test procedure. For the purpose of this calculation we use a protocol where 4 chemical concentrations and a negative control were tested in triplicate cultures, at least one concentration found to be mutagenic was retested under the same conditions, and the results of the 2 experiments were pooled and analyzed as described in the methods section. Taking into account the multiple dose levels and the retest, a false positive in the comparison to the concurrent control cultures (*t*-test) would be observed 7% of the time. A false positive in the

comparison to the historical negative control would be observed 0.44% of the time. Both tests must be positive for the chemical to be defined as mutagenic in an assay; and while both tests are not independent, the frequency of false positive results should be reduced to 0.3% or about 1 assay in 300. Since a mutagenic result in any of the 3 assays defines a chemical as mutagenic, the overall frequency of false positive conclusions is just under 1%. We consider this to be an acceptable frequency of false positive conclusions.

We conclude that this testing system has considerable promise as a screen for potential human mutagens.

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Tests for mutagenic activity at the HGPRT locus in Chinese hamster V79 cells in culture

Margaret Fox and G.F. Delow [1]

Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester and [1] Glaxo Group Research Ltd., Ware, Hertfordshire (Great Britain)

Summary

The HGPRT locus of V79 Chinese hamster cells was used to determine the mutagenicity of 5 chemicals from the IPCS study (benzene, caprolactam, HMPA, *o*-toluidine and safrole). These 5 chemicals were assayed for cytotoxicity, and also for colony-forming ability after 4 different treatment times, and in the presence and absence of serum within the treatment medium. The remaining IPCS compounds were assayed for cytotoxicity and colony-forming ability only.

All compounds were tested in the presence and absence of a rat-liver metabolising system (S9 mix), using cyclophosphamide and ethyl methanesulphonate respectively as positive controls.

We conclude that all 5 chemicals assayed for mutation were non-mutagenic both in the presence and absence of S9 mix.

The cytotoxicity and mutagenicity of 5 chemicals provided in the IPCS study was determined in V79 Chinese hamster cells, using the HGPRT locus. The cytotoxicity of the remaining 5 compounds was also determined, but insufficient time was available for mutagenicity testing. Each of the initial 5 compounds was assayed on more than one occasion in the presence and absence of a rat-liver metabolic activation system.

Materials and methods

Preliminary cytotoxicity studies

These were carried out using V79 cells plated into 96-well microtitre plates as follows. Each well in the microtitre plates was seeded with 2×10^5 V79 cells, contained in 200 μl Eagles minimal essential medium (MEM) with and without 5% foetal calf serum (FCS). The plates were then incubated for 24 h at 37 °C in 5% CO_2, for attachment and establishment of the monolayer. The test drugs were then added over a concentration range from 10 mg/ml to 5 μg/ml, with or without an exogenous metabolising system (S9 mix). Control wells included untreated cells, those treated with solvent/vehicle alone, and where appropriate, S9 mix alone and with solvent/vehicle. After addition of the compounds, wells were observed microscopically at intervals up to 4 h for evidence of disruption of the monolayer.

Assay for toxicity by inhibition of colony-forming ability

Based on initial cytotoxicity studies, a dose range was chosen for each compound for use in assays for inhibition of colony-forming ability. V79 cells were plated at 200 cells/50-mm diameter dish, in MEM + 5% FCS and allowed 4 h for attachment. Cells were then exposed to the test compounds in the presence and absence of serum and in the presence or absence of S9 mix, for periods of up to 4 h. The appropriate concentration of liver homogenate was chosen on the basis of a preliminary titration performed in microtitre

plates. After exposure, plates were rinsed with PBS, growth medium was replaced, and they were then incubated for 7 days prior to fixation and staining. Plates were scored for surviving colonies (> 50 cells) and the results expressed as percent of solvent control.

Mutation assay

This was performed essentially as previously described (McMillan and Fox, 1979; Fox, 1982) using thioguanine 0.025 mM to select for HGPRT-deficient mutants. Prior to initiation of the whole series of experiments, V79 cells were recloned, and cloned stocks frozen in liquid nitrogen. New stocks were reinitiated from frozen cultures at approximately 3-month intervals. All stocks were routinely tested for mycoplasma by the method of Chen (1977) and found negative. Exposure to the test compounds was for 60 min in the absence of serum and in the presence and absence of S9 mix. Cyclophosphamide (CYC) and ethylmethanesulphonate (EMS) were used as positive controls, in the presence and absence of S9 mix respectively. Doses of test compounds used in mutation assays were chosen to include at least one that induced measurable cytotoxicity in colony-forming assays, and one higher dose.

Metabolic activation system

The S9 used was derived from male albino rats (200–300 g bodyweight). Animals were induced with phenobarbital and β-naphthoflavone, a combination previously shown efficient in inducing a wide spectrum of activating enzymes within the liver (Gatehouse and Delow, 1979; Matsushima et al., 1976). Phenobarbital was administered at 1 mg/ml in drinking water (ad libitum) for 7 consecutive days prior to S9 preparation. β-Naphthoflavone (400 mg/20 ml) in arachis oil, was administered i.p. on days 4, 5 and 6 of the phenobarbital treatment; 0.5 ml per 100 g bodyweight was given.

Quality control. The folowing biochemical analyses were carried out on each batch of S9 used. (i) Protein determination (Lowry and Rosebrough, 1951). (ii) Determination of cytochrome P450 content (Omura and Sato, 1964). (iii) Determination of aniline hydroxylase activity (Zannoni, 1971). (iv) Determination of *p*-nitroanisole '*O*'-demethylase activity (Kamataki et al., 1979). (v) Determination of ethoxyresorufin '*O*'-de-ethytase activity (Burke and Mayer, 1974).

The liver homogenates from animals shown by these tests to have responded to the enzyme-induction process were pooled, 1 ml of the pooled preparation containing microsomes from 250 mg wet liver. The pooled liver was distributed into 2 ml volumes in sterile, screw-capped vials, and stored at approximately −80 °C for up to one month.

S9 mix. For use in an in vitro assay system, the S9 (liver homogenate) was combined with an NADPH-generating system to form an S9 mix. The composition of the S9 mix was as follows.

	Vol./wt	Final concentration (mM)
Glucose 6-phosphate	15.2 mg	5
NADP	30.6 mg	5
$MgCl_2 \cdot 6H_2O$ 0.40 M salt	0.2 ml	8
KCl 1.65 solution		
0.2 M phosphate buffer	5.0 ml	100
Medium (MEM)	3.55 ml	
Liver homogenate	1.0 ml	
	10.0 ml	

For routine use in V79 forward-mutation system, the liver homogenate was incorporated at 2.0%, and the S9 mix routinely filtered prior to use.

Compounds

Caprolactam (CAP), benzene (BEN) and hexamethylphosphoramide (HMPA) were found to be adequately soluble in DMSO and no precipitation was observed even at the maximum concentrations tested. *o*-Toluidine (TOL) and safrole (SAF) were soluble at high concentrations in DMSO, but when added to tissue-culture medium or other aqueous solutions, formed an oily suspension. Of the compounds not subjected to mutagenicity testing, acrylonitrile (ACY) was soluble in DMSO, but phenobarbitone (PB) only to 1 mg/ml. PB was more soluble in methanol (120 mg/ml) and this was used as solvent. Diethylhexylpthalate (DEH) formed an oily suspension when dissolved in DMSO and added to tissue-culture medium. Diethylstilbestrol (DES) was soluble in DMSO up to 60 mg/ml but formed flocculant suspensions

when added to tissue culture medium at greater than 600 μg/ml. Benzoin was only sparingly soluble in DMSO, but was more soluble in acetone; however it precipitated out when added to tissue-culture medium. The maximum DMSO concentration used in mutation assays was 0.2%.

Results

The results of the preliminary cytotoxicity studies on all 10 compounds tested in the presence and absence of serum, and in the presence and absence of S9 mix are summarised in Table 1. Under these conditions, all compounds except DES showed very little toxicity, and the toxicity was little affected by addition of S9 mix. Increasing the concentration of liver homogenate in the S9 mix from the usual 2.0% up to a maximum of 22.5% produced little evidence of further metabolism. On the basis of these microtitre tests, which could be considered to be relatively insensitive, doses were chosen for testing for inhibition of colony-forming abolity, with and without serum and S9 mix. Using 60-min exposure times for all test compounds, little evidence of toxicity (except at very high concentrations) was evident in this assay (Table 2). Under these conditions CYC was significantly activated only in the absence of serum. It was therefore decided to exposure V79 cells to test compounds in mutation assays in the absence of serum.

Extension of the exposure time in the presence of serum and presence or absence of liver homogenate for the first group of the 5 compounds produced little evidence of further metabolism. However, CYC became significantly more toxic with increasing time of expose to S9 mix in the absence of serum (Fig. 1).

The results of the mutation assays for the 5 compounds tested, with and without activation (including those for CYC and EMS) are presented in Table 3. The mean spontaneous frequency of $6TG^R$ colonies over the whole series was 4.9×10^{-6}; only in 3 of the 39 assays did the frequency exceed 1×10^{-5}.

EMS routinely produced a > 10-fold increase in mutant frequency in the absence of activation, as did CYC with activation when assayed at 8 and 12 day expression times.

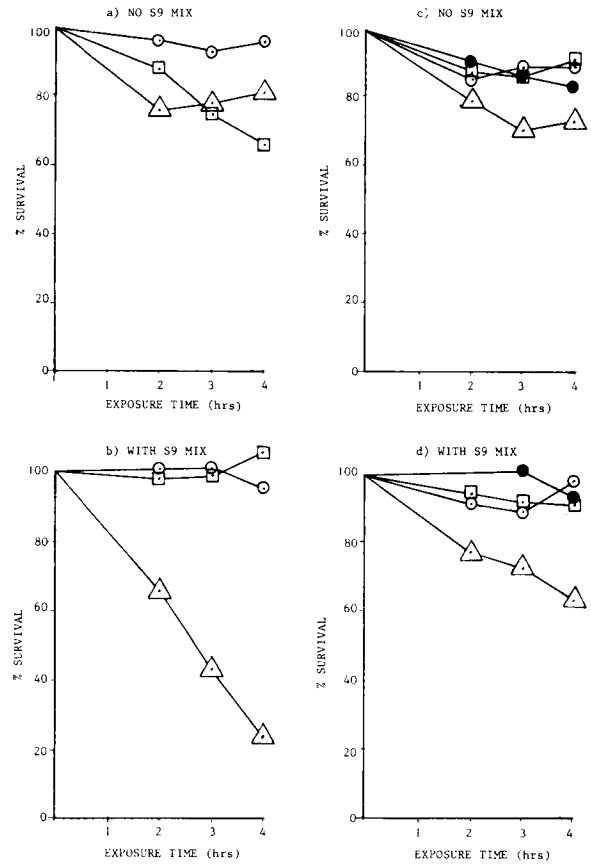

Fig. 1. Graphs to show the effects of extended treatment times on colony-forming ability. (a) ○, HMPA 1000 μg/ml; □, EMS 1000 μg/ml; △, cyclo 3000 μg/ml. (b) ○, HMPA 2000 μg/ml; □, EMS 2000 μg/ml; △, cyclo 20 μg/ml. (c) ●, *o*-toluidine 1000 μg/ml; ○, caprolactam 2000 μg/ml; □, benzene 300 μg/ml; △, safrole 400 μg/ml. (d) ●, *o*-toluidine 1000 μg/ml; ○, caprolactam 1000 μg/ml; □, benzene 300 μg/ml; △, safrole 400 μg/ml.

BEN did not induce a significant increase in mutant frequency over the spontaneous control at any of the doses tested or at any expression time, in the presence or absence of activation. A single result was obtained with CAP, which suggested a 5-fold increase at the highest dose tested, and at an 8-day expression time without activation, when the survival level fell to 44%. No other significant results were obtained in subsequent experiments, even when similar doses were tested at up to 12-day expression times, in the presence and absence of both serum and activation. HMPA also produced a single, isolated increase at an inter-

TABLE 1

PRELIMINARY CYTOTOXICITY STUDIES USING THE MICROTITRE ASSAY

Compound	Serum	Maximum non-toxic dose in microtitre assay (mg/ml) after exposure for					
		No S9 mix			With S9 mix		
		2 h	3 h	4 h	2 h	3 h	4 h
Benzene	+	0.31	0.31	0.31	0.16	0.31	0.31
	−	1.25	0.63	0.63	0.63	0.31	0.31
CAP	+	2.50	0.16	0.08	0.16	0.31	0.16
	−	2.50	2.50	2.50	2.50	2.50	2.50
HMPA	+	0.16	0.16	0.16	0.63	1.25	0.63
	−	1.25	0.63	0.63	2.50	1.25	1.25
TOL	+	1.25	0.63	1.25	0.08	0.16	0.31
	−	2.50	1.25	0.63	2.50	1.25	0.31
SAF	+	0.08	0.04	0.04	0.04	0.04	0.08
	−	5.00	5.00	2.50	2.50	0.63	0.04
CYC	+	5.00	1.25	1.25	0.16	0.02	0.04
	−	2.50	1.25	1.25	2.50	0.63	0.63
EMS	+	1.25	0.31	0.31	0.63	1.25	1.25
	−	2.50	1.25	0.63	5.00	5.00	2.50
ACR	+	0.01	0.01	0.01	0.01	0.01	0.01
	−	0.63	1.25	1.25	0.63	0.31	0.31
ZOIN	+	0.63	0.16	0.08	0.16	0.16	0.16
	−	0.04	0.04	0.04	0.04	0.04	0.04
DEH	+	5.00	0.63	0.63	0.63	0.63	0.63
	−	0.63	0.63	0.63	0.31	0.08	0.04
DES	+	0.02	0.01	0.01	0.01	0.02	0.01
	−	0.04	0.04	0.02	0.02	0.01	0.005
PB	+	2.50	1.25	0.16	0.31	0.31	0.31
	−	2.50	2.50	2.50	5.00	2.50	2.50

+, present.
−, absent.

mediate dose level of 2.0 mg/ml, again without activation, at 12 days. However a higher dose of 3.0 mg/ml, was not significant at the same expression time. One significant result was obtained in an experiment using TOL at 0.3 mg/ml at a 5-day expression time, without activation. There was however, no increase over the spontaneous level at a later expression time of 8 days, with the same dose. In the same experiment, no increases were seen with 1.0 mg/ml TOL at either expression time. Similarly in a repeat experiment without activation and with doses of up to 2.0 mg/ml TOL, no increases in mutation frequency were seen over control levels.

Exposure of cells plated on glass roller bottles to high concentrations of SAF (> 0.1 mg/ml) resulted in complete detachment of the monolayer, an effect not observed when cells were treated in plastic flasks at the same dose level. Thus SAF was assayed in both glass and plastic vessels. When cells were plated on glass and treated in the absence of activation, doses of up to 0.1 mg/ml SAF

TABLE 2

INHIBITION OF COLONY-FORMING ABILITY

Compound	Serum	Toxicity in colony-forming assay (1 h exposure) mg/ml			
		Highest non-toxic dose		Concentration for 50% kill	
		No S9 mix	With S9 mix	No S9 mix	With S9 mix
BEN	+	0.003	0.003	1.00	1.00
	−	0.003	0.003	> 1.00	> 1.00
CAP	+	0.03	0.03	> 3.00	1.00
	−	0.30	0.30	> 4.00	> 4.00
HMPA	+	0.03	1.00	> 0.30	> 3.00
	−	> 3.00	> 3.00	> 3.00	> 3.00
TOL	+	0.10	0.10	> 3.00	> 3.00
	−	0.10	0.10	3.00	2.00
SAF	+	0.10	0.10	0.50	0.50
	−	0.10	0.30	0.50	> 1.00
CYC	+	3.00	1.00	> 1.00	> 1.00
	−	> 3.00	0.03	> 3.00	> 0.25
EMS	+	1.00	> 3.00	> 3.00	> 3.00
	−	1.00	1.00	3.50	3.00
ACR	+	0.05	NT	> 0.10	NT
	−	> 0.03	NT	> 0.03	NT
ZOIN	+	NT	NT	NT	NT
	−	NT	NT	> 0.30 *	NT
DEH	+	1.00	NT	> 2.00	NT
	−	1.00	NT	> 2.00	NT
DES	+	0.003	NT	> 0.02	NT
	−	0.005	NT	> 0.02	NT
PB	+	2.00	NT	> 2.40	NT
	−	2.40	NT	> 2.40	NT

+, present.

−, absent.

NT, not tested.

* At concentrations > 300 μg/ml in tissue culture medium, ZOIN precipitates out and attaches to the surface of plastic culture vessels.

did not significantly increase mutation frequency compared to the vehicle control. When treated in the presence of S9 mix, a single significant result (4.5 × control levels) was seen at a 12-day expression time with the highest dose tested. Data from experiments whereby cells were exposed to SAF in plastic flasks in the presence of an activation system are incomplete. However the data suggest the possibility of a weakly positive result although further testing would be required to determine the significance of the data.

Discussion and conclusions

The compounds have not been as extensively tested as we would have desired or originally planned, due to the unfortunate receipt of a batch of 50-mm plastic plates to which V79 cells would not attach. This resulted in the loss of cell viability data (hence mutation frequencies) on repeat assays for SAF, HMPA, TOL and CAP. On the basis of the available data we conclude that all 5 compounds tested (SAF, CAP, BEN, TOL and HMPA)

TABLE 3

SUMMARY TABLE OF MUTATION FREQUENCIES

Treatment	Dose (mg/ml)	Serum	Mutation frequency at expression times of –					
			No S9 mix			With S9 mix		
			5 days	8 days	12 days	5 days	8 days	12 days
BEN	0.1	–	1.7×10^{-6}		NT	4.4×10^{-6}	5.8×10^{-6}	NT
BEN	0.3	–	3.2×10^{-6} [b]	8.5×10^{-6} [b]	NT	$<4.6\times10^{-6}$	$<7.0\times10^{-6}$	NT
BEN	1.0	–	3.8×10^{-6} [b]	5.7×10^{-6} [b]	NT	3.1×10^{-6}	7.7×10^{-6}	NT
BEN	2.0	–	2.4×10^{-6} [b]	3.1×10^{-6}	NT	NT	NT	NT
Vehicle	0.0	–	5.2×10^{-6} [b]	3.2×10^{-6} [b]	NT	4.7×10^{-6}	$<5.2\times10^{-6}$	NT
Positive	[a]	–	2.9×10^{-6} [b]	8.1×10^{-5}	NT	8.1×10^{-6}	9.3×10^{-5}	NT
CAP	0.3	–	NT	NT	NT	3.1×10^{-6}	6.8×10^{-6}	NT
CAP	1.0	–	1.7×10^{-6}	$<5.0\times10^{-6}$	NT	5.9×10^{-6}	3.2×10^{-6}	NT
CAP	2.0	–	2.1×10^{-6}	$<6.2\times10^{-6}$	NT	NT	NT	NT
CAP	3.0	–	NT	NT	NT	4.5×10^{-6}	1.2×10^{-5}	NT
CAP	4.0	–	8.3×10^{-6}	1.3×10^{-5}	NT	NT	NT	NT
Vehicle	0.0	–	1.9×10^{-5}	$<2.8\times10^{-6}$	NT	3.1×10^{-6}	6.3×10^{-6}	NT
Positive	[a]	–	3.0×10^{-6}	1.0×10^{-4}	NT	7.6×10^{-6}	9.3×10^{-6}	NT
CAP	2.0	–	NT	$<1.8\times10^{-6}$	2.6×10^{-6}	$<4.9\times10^{-6}$	$<3.4\times10^{-6}$	$<3.5\times10^{-6}$
CAP	3.0	–	NT	2.7×10^{-6}	5.4×10^{-6}	8.0×10^{-6}	3.6×10^{-6}	$<3.4\times10^{-6}$
Vehicle	0.0	–	NT	2.2×10^{-6}	8.8×10^{-6}	5.6×10^{-6}	$<2.7\times10^{-6}$	$<3.6\times10^{-6}$
CAP	2.0	+	NT	$<3.7\times10^{-6}$	$<6.2\times10^{-6}$	7.7×10^{-6}	$<2.7\times10^{-6}$	3.6×10^{-6}
CAP	3.0	+	NT	2.9×10^{-6}	$<6.5\times10^{-6}$	4.5×10^{-6}	3.0×10^{-6}	3.7×10^{-6}
Vehicle	0.0	+	NT	$<3.6\times10^{-6}$	1.9×10^{-5}	$<6.6\times10^{-6}$	3.5×10^{-6}	3.0×10^{-6}
HMPA	0.3	–	NT	NT	NT	$<4.4\times10^{-6}$	7.6×10^{-6}	NT
HMPA	1.0	–	2.0×10^{-6} [b]	1.7×10^{-6} [b]	-	$<4.2\times10^{-6}$	$<7.7\times10^{-6}$	NT
HMPA	2.0	–	$<3.3\times19^{-6}$	1.5×10^{-6} [b]	7.0×10^{-6} [b]	NT	NT	NT
HMPA	3.0	–	5.5×10^{-6} [b]	2.3×10^{-6} [b]	2.7×10^{-6} [b]	$<5.2\times10^{-6}$	1.8×10^{-5}	NT
Vehicle	0.0	–	2.1×10^{-6} [b]	1.3×10^{-6} [b]	$<1.3\times10^{-6}$ [b]	$<3.2\times10^{-6}$	$<3.7\times10^{-6}$	NT
Positive	[a]	–	2.2×10^{-5} [b]	2.3×10^{-4} [b]	4.7×10^{-4} [b]	4.2×10^{-6}	2.7×10^{-5}	NT
TOL	0.25	–	NT	NT	NT	3.9×10^{-6}	7.7×10^{-6}	NT
TOL	0.30	–	6.7×10^{-5} [b]	2.9×10^{-7} [b]	NT	NT	NT	NT
TOL	0.50	–	NT	NT	NT	$<5.5\times10^{-6}$	$<8.0\times10^{-6}$	NT
TOL	1.00	–	5.9×10^{-6} [b]	2.6×10^{-6} [b]	NT	$<5.0\times10^{-6}$	5.2×10^{-6}	NT
TOL	2.00	–	5.8×10^{-6}	3.6×10^{-6}	NT	NT	NT	NT
Vehicle	0.00	–	5.3×10^{-6} [b]	3.1×10^{-6} [b]	NT	$<3.5\times10^{-6}$	4.2×10^{-6}	NT
Positive	[a]		6.2×10^{-5} [b]	7.0×10^{-4} [b]	NT	6.3×10^{-6}	8.0×10^{-5}	NT
SAF In	0.025	–	$<1.2\times10^{-6}$	5.1×10^{-6}	NT	6.2×10^{-6} [b]	4.3×10^{-6} [b]	3.4×10^{-6} [b]
SAF glass	0.050	–	$<2.4\times10^{-6}$	1.6×10^{-6}	NT	6.6×10^{-6} [b]	7.5×10^{-6} [b]	1.0×10^{-5} [b]
SAF	0.100	–	3.2×10^{-6}	1.6×10^{-6}	NT	5.7×10^{-6} [b]	6.8×10^{-6}	
Vehicle	0.000	–	$<2.8\times10^{-6}$	1.9×10^{-6}	NT	5.0×10^{-6} [b]	5.6×10^{-6} [b]	2.2×10^{-6} [b]
Positive	[a]	–	1.4×10^{-6}	1.4×10^{-5}	NT	5.2×10^{-5} [b]	3.6×10^{-5} [b]	2.2×10^{-5} [b]
SAF	0.030	–				6.2×10^{-5}	NT	NT
SAF	0.063	–				NT	4.2×10^{-6}	5.4×10^{-6}
SAF In	0.100	–				3.8×10^{-5}	NT	NT
SAF plastic	0.125	–		Not tested		NT	$<7.1\times10^{-6}$	$<3.6\times10^{-6}$
SAF	0.250	–				NT	NT	$<2.4\times10^{-6}$
SAF	0.500	–				2.2×10^{-4}	NT	NT
Vehicle	0.00	–				3.5×10^{-6}	4.2×10^{-6}	2.3×10^{-6}
Positive	[a]	–				2.2×10^{-4}	2.2×10^{-5}	6.2×10^{-6}

NT, not tested.

[a] positive controls (i) +S9, Cyclophosphamide 0.2 mg/ml; (ii) no S9 = EMS 1.0 mg/ml.

[b] average of 2° Expts.

were non-toxic in the presence and absence of liver homogenate. Further, all of the compounds were also found to be nonmutagenic in the absence of activation and in the presence of the activation conditions that we used, which were adequate for CYC. The sensitivity of the V79/HGPRT system might be considered to be low and thus unable to detect weak mutagens, but was able to detect the activity of 4-chloromethyl-biphenyl (4CMB) (Fox, 1982). In the present series of tests the spontaneous frequency of TG^R was similar to that observed previously, i.e. 4.0×10^{-6} (Fox, 1982), we thus feel that we would have detected significant increases in mutagen frequency, had they occurred, particularly since we tested the compounds (except SAF) at some concentrations that produced measurable cytotoxicity.

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Tests for the induction of mutations to ouabain or 6-thioguanine resistance in mouse lymphoma L5178Y cells

R. Colin Garner and Jean Campbell [1]

Cancer Research Unit, University of York, York, and [1] Microtest Research Ltd., Heslington, York (Great Britain)

Summary

10 IPCS chemicals were tested in an in vitro mammalian cell mutation assay in the presence and absence of a rat-liver metabolizing system. None of the test chemicals were found to be potent mammalian cell mutagens to mouse lymphoma L5178Y cells using a "fluctuation" assay protocol when compared with potent carcinogens such as benzo(a)pyrene. 5 of the test chemicals safrole, *o*-toluidine, diethylstilboestrol, benzene and acrylonitrile, induced mutations to either ouabain or 6-thioguanine resistance. Of these only *o*-toluidine required liver S9 for mutation induction; acrylonitrile appeared to be the most active of the 10 chemicals tested. The 2 non-carcinogens, benzoin and caprolactam, failed to induce mutations. The results obtained reflect the marginal genotoxicity of the test chemicals in this in vitro assay and raise the question of whether or not some of the test chemicals are genuine tumour initiators.

In vitro assays to detect genotoxic chemicals are now widely accepted by regulatory authorities as providing useful information on the potential hazards of chemicals. Indeed some in vitro testing is already mandatory for new chemicals prior to their introduction onto the market. The most widely used short-term test, and the one which has been most extensively validated, is the Salmonella/microsome assay (Maron and Ames, 1983). Most known organic chemical carcinogens are active in this test, which is both cheap and reliable. However, a few compounds known to be animal carcinogens (but with no evidence of human carcinogenicity) are unable to induce mutations in Salmonella no matter how the system is modified (Garner, 1979, 1980). To partly circumvent this problem of false negative results as well as to reinforce the information obtained from the Salmonella/microsome assay, a number of in vitro mammalian cell tests have been suggested as a complement to bacterial assays (Garner, 1981; IARC. 1980). OECD guidelines suggest that an in vitro or in vivo cytogenetics assay be carried out in conjunction with the Salmonella test. While this will give some information about the clastogenic effects of the study compound, it gives no indication of whether or not cells containing aberrations would be viable. Mutation in cultured mammalian cells, on the other hand, provides an in vitro assay of the effect of mutagens on the genome.

Several mammalian cell assays can be used to study mutagenesis (Howard-Flanders, 1981; Clive et al., 1983; UKEMS, 1983). Many of these have not been examined in detail by more than one laboratory and so no recommendations can be made about which assay is superior to another. We, in our laboratories, have developed a mutation assay in mouse lymphoma L5178Y cells according to a protocal originally reported by Cole et al (1979). In this procedure a suspension culture of cells is treated with the agent, left to express the mutation, and then plated into drug containing media in "mictrotitre" wells. Drug-resistant clones are counted and used to calculated a mutation frequency in conjunction with a viability count; at the same time cells are plated into non drug-con-

taining medium to determine survival. We believe this technique has several advantages over other mammalian cell assays in that (1) cells can be grown in suspension culture; (2) plating efficiencies are high; (3) the "mictrotitre" method lends itself to automation; (4) scoring is easy; and (5) mutation at two or more gene loci can be studied. This report describes the results obtained using mouse lymphoma L5178Y cells after exposure to the 10 chemicals supplied by IPCS.

Materials and methods

Media and chemicals

Fischer's medium and horse serum were purchased from Gibco Biocult, Paisley, Scotland; ouabain, 6-thioguanine, benzo[*a*]pyrene and 4-nitroguinoline-1-oxide from Sigma Chemical Co. Ltd., Poole, Dorset; NADP and glucose 6-phosphate from Boehringer Ltd., Lewes, Sussex; all other chemicals were of laboratory reagent grade.

Routine culture of cells

Mouse lymphoma L5178Y cells (obtained from J. Cole, University of Sussex) were grown in 50 ml Fischer's medium containing 10% horse serum, 100 units/ml peniciilin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone and 200 μg/ml sodium pyruvate (FM10) from a density of 10–100 cells in a gassed incubator (5% CO_2/95% air) at 37 °C. Once cells had reached the required call density ($1-5 \times 10^5$/ml) they were used in an experiment (approximately 10–14 days).

Mutation assay

Dosing. Six 75-cm^2 flasks containing 50 ml of FM10 and L5178Y cells at a density of 5×10^5/ml were pooled and counted using a Coulter counter. Cells were centrifuged and resuspended in 20 ml FM10 (approximately 10^7 cells were used). Doses of either test or control compound or solvent (0.04 ml DMSO) were added to each 20-ml aliquot of cells. Each concentration was tested in duplicate. S9 mix was added when required and cells were treated in rocking cultures at 37 °C for 2 h. At the end of this time cells were spun down washed in 20 ml FM10, counted and finally resuspended to give 2×10^5 cells/ml in FM10 prior to placing in 75-cm^2 flasks.

Survival determination. 0.1 ml was removed from each flask, and diluted in FM10 to give 10 ml of medium containing 2×10^3 cells/ml. 0.25 ml of this cell suspension was further diluted in 50 ml FM10 and 0.2 ml of this suspension added to each well of two 96-well "microtitre" plates (average of 2 cells/well). Plates were incubated for 7–10 days at 37 °C before counting the number of wells containing clones.

Mutation to Ouabain Resistance. Treated cells were cultured in FM10 for 48 h and the cell density adjusted to 2×10^5/ml. 0.1 ml of this cell suspension was removed to determine viability as above. 120 ml of the cell suspension was then adjusted to 10^{-3} M with ouabain and 0.2-ml aliquots added to each well of two 96-well "microtitre" plates (approximately 4×10^4 cells/well). Wells were incubated for 2 weeks at 37 °C prior to counting the number of wells containing clones.

Mutation to 6-thioguanine Resistance. Cells were cultured in FM10 for 7 days after treatment, the cell density being maintained between 1 and 5×10^5 cells/ml. After 7 days the cell density was adjusted to 5×10^4/ml and 0.4 ml removed for determination of viability. 120 ml of the cell suspension was adjusted to 1.8×10^{-4} M with 6-thioguanine and 0.2-ml aliquots added to each well of two 96-well "microtitre" plates giving approximately 1×10^4 cells/well. Plates were incubated for 2 weeks at 37 °C and the number of cells containing clones counted.

Control Compounds. Benzo(a)pyrene at a concentration of 1.5×10^{-5} M was used as the positive control compound plus liver activation and 4-nitroquinoline-1-oxide (1×10^{-6} M) used as the positive control compound without liver.

Preparation of Liver S9 Mix. Aroclor-1254 induced rat liver S9 was prepared as previously described (Booth et al., 1980). S9 mix contained 1.0 ml glucose 6-phosphate (180 mg/ml), 1.0 ml NADP (25 mg/ml), 1.0 ml 150 mM potassium chloride and 2.0 ml rat-liver S9. 1.0 ml of the above was used per 20 ml of mouse lymphoma cell suspension.

Treatment of results. (a) Survival percentage was calculated by counting the number of wells with clones from the treated cell population and comparing the value with that for the solvent control. (b) Mutation frequency was calculated

according to the method of Furth et al. (1981) by the Poissonian distribution of wells containing cells. Mutation frequency =

$$-\ln\frac{(x_s/n_s)}{(x_o/n_0)} \times \text{dilution factor}$$

where x_s, number of wells with no colonies in selective medium, n_s, total number of wells, x_0, number of wells with no colonies in nonselective medium, n_0, total number of wells.

(c) Criteria for a positive result: Since the number of cells plated in the selective agent was the same whether or not the original cells had been treated with the test chemical, a chi^2-test was performed between solvent controls and treated cells. A positive result was one in which the p-value was less than 0.05 for at least two concentrations.

Results

For each of the 10 test substances a preliminary experiment was carried out to determine the toxicity of the chemical to mouse lymphoma L5178Y cells after a 2-h exposure. This experiment was carried out in the absence of liver S9. Once a suitable dose range had been identified each chemical was tested at 5 doubling concentrations. The dose range used for each of the test chemicals is detailed in Table 1. For each compound only one expression time was used, namely 48 h for ouabain and 7 days for 6-thioguanine. Only data from experiments in which the positive control compounds were active have been used in the evaluation. While it is our normal practice to use 4 plates per dose to test compounds, due to lack of resources for this study we have only used 2 plates per dose. This means that approximately 8×10^6 cells were plated to measure ouabain resistance and 2×10^6 cells for 6-thioguanine resistance (the spontaneous mutation frequency ranged from 0 to 2×10^6 for ouabain and $5–62 \times 10^6$ for 6-thioguanine). The two positive control compounds, 4-nitroquinoline-1-oxide and benzo[a]pyrene, were mutagenic usually at both of the loci scored. Mutations to 4NQO (0.19 μg/ml) gave ouabain-resistant mutants at a frequency between 0.6 and 4.0×10^6 and 6-TG mutants at a range between 16 and 441×10^6. For benzo[a]pyrene (BP) (3.75 μg/ml) the ranges were between 1.8 and 42.1×10^6 for ouabain and between 75 and 569×10^6 for 6-thioguanine. Of the test chemicals, none were potent genotoxic chemicals in comparison with BP and 4NQO. Table 1 shows the maximum mutation frequency induced by each compound, whether or not activity was seen at more than one concentration, and if the result was regarded as being positive. No compound gave what we regard as a clear-cut positive response, probably due to the limited number of cells plated. Experiments were not repeated to confirm results.

TABLE 1

SUMMARY RESULTS OF COMPOUND TESTING

Compound	Dose range (μg/ml)	Oua^R Maximum MF $\times 10^6$		6GT^R Maximum MF $\times 10^6$		Overall conclusion
		+S9	−S9	+S9	−S9	
HMPA	12.5 –200	0.12(0)	0.89(0.17)	85.80(62)	65.47(57)	−
TOL	12.5 –200	0.96(0)	0.19(0)	[a] 79.92(62)	57.97(57)	+
BEN	12.5 –200	2.13(0)	0.53(0)	40.23(19)	[a] 33.65(18)	+
SAF	6.25–100	0.69(0)	0.67(0)	21.69(19)	[a] 25.98(8)	+
CAP	12.5 –200	0.78(0)	1.34(0)	14.71(6)	7.25(6)	−
ACN	12.5 –200	0.19(0)	0.80(0)	[a] 32.26(19)	[a] 34.6 (18)	+
DEHP	12.5 –200	0.48(0)	0.38(0)	7.04(11)	21.79(5)	−
ZOIN	12.5 –200	0.18(0)	0.16(0)	20.44(15)	20.12(16)	−
DES	3.13– 50	0.78(0.22)	0 (0)	[a] 27.65(11)	[a] 44.86(9)	+
PB	12.5 –200	0.64(0)	0.50(0)	13.78(15)	37.85(16)	−

() Numbers indicate negative control values. [a] Significant using chi^2 for at least two concentrations. MF, mutation frequency.

Discussion

Mammalian cell mutation tests have been proposed for inclusion in a battery of short-term tests for chemical screening. While there are a number of attractions of such tests, including their greater relevance to man, the fact is that if they cannot augment the Salmonella/microsome assay there is little point in carrying them out. Unfortunately the results from our laboratory do little to shed any further light on this problem. This we feel is a reflection of the compounds selected for the study rather than an unsatisfactory assay. A variety of cell lines have been used for mutation studies including V79, CHO (hamster), C3H10T1/2, L5178Y (mouse) and ARL6 (rat) cells. We used L5178Y cells because of their ease of handling, rapidity of growth, their high plating efficiency, and the fact that they can be cultured in horse serum. L5178Y cells have been used by a number of laboratories and mutations have normally been screened at the thymidine kinase locus. A survey of mammalian mutation tests carried out by a committee of the US-EPA Gene-Tox Program (Clive et al., 1983) came out strongly in favour of this particular system on the grounds that little published data was available in other systems. We have, on the other hand, studied mutation to both ouabain and 6-thioguanine resistance. Only agents inducing missense mutations will mutate at the ouabain locus while frame-shift, base-substitution, and deletion mutagens are active in the latter. In our laboratories we have found a number of compounds to be active in the mouse lymphoma system including nitroquinoline-1-oxide, aflatoxin B_1, benzo[*a*]pyrene, 2-acetylaminofluorene, 3,3′-dichlorobenzidine, various nitrated polycyclic aromatic hydrocarbons, some diesel exhaust subfractions, etc. We feel, therefore, that the procedure we use is validated and reliable.

Turning to the results we obtained with the 10 study chemicals, it should be stated that none of the chemicals could be regarded as being strong mammalian cell mutagens in comparison with the positive control compounds. Of the 3 carcinogens that could be said to be genotoxic by virtue of their reaction with DNA, hexamethylphosphoramide, *o*-toluidine and safrole, the latter two were active under the conditions of the assay. For safrole, the positive response was obtained in the absence of liver and some doubt therefore is cast on the meaning of this result in relation to the known hepatocarcinogenicity of this chemical. Diethylstilboestrol appeared active both in the presence and absence of liver activation. Various reports have appears on this compound inducing in vivo transformation but not mutation (Barrett et al., 1982). However, in our tests, diethylstilboestrol was the most potent of the 10 chemicals tested. The positive result for benzene was somewhat surprising, since this compound has not been shown to be genotoxic previously. Benzene is a potent clastogen, which has been previously reported to be nonmutagenic (IARC, 1982). These results would require confirmation to determine if they are real. The remaining compound that appeared to have some activity was acrylonitrile; liver metabolism was not a prerequisite for the biological activity of this presumptive human carcinogen. The failure to detect the genotoxic activity of HMPA probably relates to a failure to test at a high enough concentration.

The results we obtained raise some general questions relating to mammalian cell mutation tests and over the selection of chemicals in this study. There was sufficient evidence in the literature that the majority of the selected test chemicals would be inactive in mammalian cell mutation systems. While we have tested thousands of chemicals in Salmonella, it is true to say that only a few have been tested in L5178Y cells. Nevertheless it is exceptional for a compound to be active in the mammalian cell test and inactive in Salmonella. It is our opinion that mammalian cell assays are more useful in confirming positive Salmonella data than testing false negatives. Of the ten test chemicals only two, benzene and acrylonitrile, are thought to be human carcinogens. It is likely that many of the animal results obtained for compounds such as phenobarbitone, diethylhexylphthalate, and so on have very little relevance to human cancer. On the other hand a compound that is a strong mammalian cell mutagen is likely to be at the very least an animal carcinogen. It is the relationship between in vitro tests, animal carcinogenicity, and risk to man that remains to be elucidated. The results of testing these 10 chemicals unfortunately sheds no new light on this problem.

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Assays for the induction of gene mutations at the thymidine kinase locus and the hypoxanthine guanine phosphoribosyltransferase locus in L5178Y mouse lymphoma cells in culture

Ada G.A.C. Knaap and Peter B. Langebroek

Laboratory for Carcinogenesis and Mutagenesis, National Institute of Public Health and Environmental Hygiene, Bilthoven (The Netherlands)

Summary

5 chemicals were tested for mutagenic activity at both the thymidine kinase locus and the hypoxanthine guanine phosphoribosyltransferase locus in L5178Y mouse lymphoma cells in vitro. Several systems for metabolic activation were applied, i.e. an Aroclor 1254-induced rat-liver homogenate preparation (S9), primary rat hepatocytes, primary chick-embryo hepatocytes and Syrian hamster-embryo cells. Hexamethylphosphoramide was shown to be mutagenic in the presence of each of the metabolic activation systems. An indication for a mutagenic effect was obtained with safrole. This compound should be tested further before a definite conclusion can be drawn. Negative results were obtained with *o*-toluidine, benzene and caprolactam.

Within the framework of the Collaborative Study on Short-Term Tests (CSSTT) of the International Program on Chemical Safety, we determined the mutagenic activity of 5 chemicals in an in vitro mammalian cell system, using L5178Y mouse lymphoma cells heterozygous at the thymidine kinase (TK) locus. The use of this cell line allows the measurement of the effects of chemicals on the forward mutation frequency at multiple gene loci, namely the TK locus ($TK^{+/-} \rightarrow TK^{-/-}$) and the hypoxanthine guanine phosphoribosyltransferase locus ($HPRT^{+} \rightarrow HPRT^{-}$). Each compound was tested in the absence and in the presence of various systems for metabolic activation.

Materials and methods

Cells

The experiments were carried out with L5178Y mouse lymphoma cells, heterozygous for thymidine kinase, $TK^{+/-}$, which grow in suspension culture with a doubling time of about 12 h in a humidified CO_2 incubator at 37 °C. Cultures were diluted when the cell density exceeded $8 \cdot 10^5$ cells/ml. For an experiment one ampule, containing $3-5 \cdot 10^6$ cells was taken from a large stock of cells, stored at −95 °C.

Media

Ham's F10 medium (Flow Laboratories) modified by the omission of hypoxanthine and thymidine and supplemented with 15% newborn calf serum (Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (50 μg/ml) and folic acid (9 μg/ml) was used as standard medium. For cloning the standard medium was supplemented with Noble agar (Difco), the final agar concentration being 0.4%. For the selection of TK-deficient ($TK^{-/-}$) cells, BrdUrd (Sigma, 50 μg/ml) is added to the agar medium. To select for cells with the HPRT-deficient phenotype, the agar medium is supplemented with the purine analog 6-thioguanine (TG) (Fluka, 5 μg/ml).

Compounds

Test chemicals were received from J. Ashby in

purified form and stored at 4°C until use. Immediately before each assay the compounds were dissolved or diluted in DMSO (Merck). This stock solution, or when necessary dilutions of it, was added to the cells in the appropriate amount to obtain the desired concentration. The final concentration of DMSO in the cell suspensions was 1%.

Metabolic activation

Several types of metabolic activation were applied.

Rat-liver homogenate (S9). Liver enzymes were induced by giving adult male SPF Wistar rats an injection of Aroclor 1254 (500 mg/kg) 5 days before sacrifice. The S9 was made under sterile conditions according to the general procedure and stored at −95°C. The cofactor solution was freshly prepared for each assay. The final concentrations in the incubation medium were 5.4 mM KCl, 1.3 mM $MgCl_2$, 20 mM Hepes buffer (pH 7.3), 1 mM NADP, 1.25 mM glucose 6-phosphate and 5% S9.

Rat hepatocytes. Liver cells from male SPF Wistar rats (6 weeks old) were obtained by perfusion. Before perfusion the rats were injected with 2500 i.e. heparin; livers were perfused with saline containing 0.5 mM EGTA (10 min), followed by 0.2% collagenase (10 min). The cells were filtered, centrifuged and suspended in Williams medium E with 10% fetal calf serum. Viability of the cells was 80–90% as determined by trypan blue exclusion. The primary hepatocytes were then seeded at a density of $5 \cdot 10^6$ viable cells/75-cm^2 flask, and allowed to attach. After 4 h, the flasks were replenished with 10 ml of medium and the target cells, at a density of $2 \cdot 10^5$/ml, and the substance under-test was added.

Primary chick embryo hepatocytes. A suspension of primary chick embryo hepatocytes was prepared by perfusing livers of 15-day-old embryos with Hank's EDTA, followed by a dispase treatment, as described by van der Hoeven et al. (1984). The hepatocytes were then seeded at a density of $2.7 \cdot 10^6$ viable cells/75-cm^2 flask in Williams E medium with 15% newborn calf serum, and allowed to attach. After 4 h the flasks were replenished with 7.5 ml of medium and the target cells, at a density of $2 \cdot 10^5$/ml, and the substance under-test was added.

Syrian Hamster Embryo Cells. The cells were derived from Syrian hamster embryos according to the procedure described by Pienta et al. (1977). Secondary cultures were used for metabolic activation by seeding $1 \cdot 10^6$ cells/75-cm^2 flask in MEM medium with 10% fetal calf serum. After 24 h, the flasks were replenished with 7.5 ml of medium and the target cells, at a density of $2 \cdot 10^5$/ml, and the substance under-test was added.

Treatment

Cells, in the absence or presence of S9, were treated in glass tubes in a total volume of 10 ml, the final cell density being 10^6 cells/ml. The order of addition to the tubes was: cells, standard medium without serum, S9 and cofactors where appropriate, chemical under-test. The tubes were gassed with 5% CO_2 in air, caps were tightened, and the material was mixed and incubated for 2 h at 37°C.

In the cocultivation experiments, 1.5×10^6 lymphoma cells were seeded at a density of $2 \cdot 10^5$/ml in flasks, in which the metabolically active cells were already attached. After addition of the test chemical, the cultures were incubated for 20 h at 37°C.

After treatment the lymphoma cells were collected, washed free of the test substance, and processes as described under mutagenesis assay.

Cytotoxicity tests

Cytotoxicity tests of the chemicals were performed both in the presence and in the absence of the S9 mixture using a number of 1:3 serial dilutions. After treatment, a small aliquot of cells from each experimental group was seeded in agar medium to determine the viability (cloning efficiency): 150 cells per 15 ml of agar medium per 9-cm dish (P94, Greiner), 5 dishes per group. The survival of each treated group was calculated by correcting the observed cloning efficiency of that group with the cloning efficiency of the control. The results of the cytotoxicity test were then used to select at least 4 concentrations of the test chemical to be used in the mutagenesis assay, with the lowest concentration showing no toxicity and the highest concentration giving a survival of about 15% immediately after treatment.

Controls

A negative (solvent) control was included in each experiment. The negative control received 1% DMSO. For assays with metabolic activation, the activation system was also added to the negative control. In most experiments positive controls were included in the protocol according to the suggestion of preferred positive controls for this international study.

Mutagenesis assays

Mutant cells were detected and quantitated through colony formation in agar medium supplemented with one of the selective agents, i.e. BrdUrd or TG.

After treatment, cells of each experimental group were (a) seeded for survival, 150 cells per 15 ml of agar medium per P94 (5 dishes per group) and (b) propagated for several days in standard medium to allow the expression of induced mutants that had resulted from the treatment; approximately 10^7 cells were propagated. 3 days was chosen as the near-optimal expression time for BrdUrd-resistant mutants and 7 days for TG-resistant mutants. During the expression time, cell counts were made daily or every other day, to keep record of cell growth and according to these, the cultures were diluted. At the end of the expression periods cells from each group were seeded for (a) cloning efficiency, 150 cells per 15 ml of agar medium per P94 (5 dishes per group) and (b) selection of mutants. The conditions for selection of mutants with BrdUrd-resistance or TG-resistance respectively, were 1.5×10^5 cells per 15 ml of BrdUrd containing medium per P94 (20 dishes per group) and 3×10^5 per 15 ml of TG containing medium per P94 (10 dishes per group).

Between 12 and 14 days after the cells were seeded for cloning efficiency and selection, the colonies were counted manually. The mutant frequency for each concentration and for each marker was calculated by dividing the observed number of colonies by the number of cells seeded, corrected for the corresponding cloning efficiency. The induced mutant frequency was then obtained by subtracting the control mutant frequency from the frequency of the treated population.

Results and discussion

5 compounds were tested for their ability to induce gene mutations at 2 loci in L5178Y mouse lymphoma cells in vitro, under different experimental conditions. The concentrations used in the mutation assays were based on preceding toxicity tests (data not shown); in general when no toxicity was observed, the highest concentration tested was 1%. The results obtained with hexamethylphos-

TABLE 1

MUTANT FREQUENCIES FOR TK-DEFICIENCY AND HPRT-DEFICIENCY AFTER TREATMENT WITH HEXAMETHYLPHOSPHORAMIDE

Concentration (μl/ml)	Survival after treatment (%)	Mutant frequency ($\times 10^{-5}$)	
		$TK^{-/-}$	$HPRT^-$
No activation, 2 h			
0	100.0	3.4 (119) [a]	0.5 (17)
0.5	99.9	2.5 (96)	0.3 (11)
1.0	63.3	3.7 (125)	1.3 (43)
2.5	81.3	2.7 (99)	1.1 (38)
5.0	87.3	2.7 (99)	0.5 (15)
10.0	99.0	2.4 (82)	0.6 (21)
EMS, 124 μg/ml	101.0	11.5 (278)	5.3 (91)
Activation with rat-liver S9, 2 h			
0	100.0	3.2 (109)	0.3 (11)
3.0	97.9	4.6 (137)	0.3 (8)
10.0	95.1	4.2 (132)	0.6 (15)
30.0	17.7	4.3 (115)	-
B(a)P, 5 μg/ml	66.6	12.8 (381)	4.7 (127)
Cocultivation with primary rat hepatocytes, 20 h			
0	100.0	4.0 (123)	0.7 (27)
0.3	89.7	3.2 (100)	1.0 (31)
1.0	81.7	3.0 (87)	1.2 (37)
3.0	69.9	3.9 (107)	1.0 (31)
10.0	46.9	6.5 (166)	2.9 (81)
DEN, 10 μg/ml	46.3	18.9 (470)	14.1 (411)
Cocultivation with Syrian hamster-embryo cells, 20 h			
0	100.0	2.2 (71)	0.5 (16)
3.0	75.1	3.6 (93)	0.8 (22)
10.0	27.7	5.7 (141)	0.6 (18)
B(a)P, 1 μg/ml	41.0	13.8 (331)	2.8 (89)
B(a)P, 5 μg/ml	33.2	14.6 (330)	5.3 (156)
Cocultivation with primary chick-embryo hepatocytes, 20 h			
0	100.0	2.9 (82)	0.9 (25)
3.0	95.0	5.3 (140)	1.5 (40)
10.0	45.7	7.0 (187)	2.3 (62)
B(a)P, 1 μg/ml	86.3	15.0 (347)	8.7 (201)

[a] Number within brackets is number of mutant clones observed.

TABLE 2

MUTANT FREQUENCIES FOR TK-DEFICIENCY AND HPRT-DEFICIENCY AFTER TREATMENT WITH *o*-TOLUIDINE

Concentration (μl/ml)	Survival after treatment (%)	Mutant frequency ($\times 10^{-5}$)	
		$TK^{-/-}$	$HPRT^{-}$
No activation, 2 h			
0	100.0	2.6 (78)[a]	0.6 (17)
0.1	74.5	3.1 (76)	0.5 (14)
0.3	53.9	2.9 (83)	0.9 (25)
1.0	49.7	3.1 (86)	0.4 (11)
3.0	0.2	not done	
10.0	toxic	not done	
MNNG, 0.015 μg/ml	47.0	11.7 (287)	2.0 (55)
Activation with rat-liver S9, 2 h			
0	100.0	4.4 (131)	0.5 (10)
0.1	67.3	4.4 (128)	0.2 (4)
0.3	68.2	3.8 (114)	0.5 (12)
1.0	47.8	4.5 (131)	0.3 (9)
1.3	46.0	4.5 (131)	0.2 (6)
1.6	9.1	4.8 (141)	0.7 (16)
2.0	0.7	not done	
B(a)P, 5 μg/ml	59.8	14.0 (386)	7.3 (184)
Cocultivation with Syrian hamster embryo cells, 20 h			
0	100.0	2.2 (71)	0.5 (16)
0.1	67.0	3.3 (98)	0.4 (10)
0.3	59.0	2.7 (79)	0.6 (16)
B(a)P, 1 μg/ml	41.0	13.8 (331)	2.8 (89)
B(a)P, 5 μg/ml	33.2	14.6 (330)	5.3 (156)
Cocultivation with primary chick-embryo hepatocytes, 20 h			
0	100.0	2.9 (82)	0.9 (25)
0.1	99.3	2.7 (72)	0.8 (23)
0.3	79.0	3.2 (79)	1.7 (42)
B(a)P, 1 μg/ml	86.3	15.9 (347)	8.7 (201)

[a] Number within brackets is number of mutant clones observed.

TABLE 3

MUTANT FREQUENCIES FOR TK-DEFICIENCY AND HPRT-DEFICIENCY AFTER TREATMENT WITH BENZENE

Concentration (μl/ml)	Survival after treatment (%)	Mutant frequency ($\times 10^{-5}$)	
		$TK^{-/-}$	$HPRT^{-}$
No activation, 2h			
0	100.0	2.8 (101)[a]	1.2 (33)
0.1	86.4	2.6 (80)	1.2 (36)
0.2	77.4	2.8 (86)	0.6 (17)
0.4	66.6	3.2 (99)	1.0 (25)
0.6	13.5	2.7 (75)	0.4 (13)
0.8	0	not done	
ENU, 117 μg/ml	26.9	98.6 (1748)	46.2 (1375)
Activation with rat-liver S9, 2 h			
0	100.0	1.8 (50	1.4 (38)
0.1	67.3	2.4 (67)	1.5 (43)
0.2	44.5	2.5 (69)	1.6 (45)
0.4	1.1	1.9 (61)	2.7 (88)
0.6	0	not done	
0.8	0.3	not done	
DEN, 20 μg/ml	91.3	22.2 (654)	11.3 (369)
Cocultivation with Syrian hamster-embryo cells, 20 h			
0	100.0	2.8 (82)	1.1 (34)
0.075	119.3	3.4 (69)	1.1 (28)
0.150	99.9	2.9 (75)	1.2 (33)
B(a)P, 2 μg/ml	34.6	11.9 (250)	3.6 (110)
Cocultivation with primary chick-embryo hepatocytes, 20 h			
0	100.0	2.7 (68)	1.3 (37)
0.075	93.7	2.7 (81)	0.5 (14)
0.150	95.9	2.4 (65)	1.0 (27)
B(a)P, 2 μg/ml	14.3	17.0 (272)	–

[a] Number within brackets is number of mutant clones observed.

phoramide, *o*-toluidine, benzene, safrole and caprolactam are shown in Tables 1–5 respectively. Since it was known that most of the compounds were difficult to evaluate with respect to their activity in short-term tests, we have tried to enhance the sensitivity of our system by applying different types of metabolic activation. Apart from the rat-liver S9 fraction, also whole cells from different origin, which are known for their metabolic capacity, were used in cocultivation experiments. Moreover, since there might be differences in the mutability of different genes, the induction of mutations was determined at 2 loci.

Hexamethylphosphoramide (Table 1)

This compound showed no cytotoxicity or mutagenicity in the absence of S9. However, in the presence of the metabolic activation systems, toxicity as well as mutagenic activity was observed. In each of the activation experiments an increase of mutations at the TK-locus and the HPRT-locus was found at a concentration of 10 μl/ml and with the primary chick embryo hepatocytes also at 3 μl/ml. In comparison with

TABLE 4

MUTANT FREQUENCIES FOR TK-DEFICIENCY AND HPRT-DEFICIENCY AFTER TREATMENT WITH SAFROLE

Concentration (μl/ml)	Survival after treatment (%)	Mutant frequency ($\times 10^{-5}$)	
		$TK^{-/-}$	$HPRT^{-}$
No activation, 2h			
0	100.0	2.9 (85) [a]	0.6 (20)
0.030	91.3	2.8 (88)	0.7 (22)
0.045	69.4	3.4 (102)	0.6 (21)
0.060	16.7	3.1 (110)	1.0 (32)
0.075	toxic	not done	
EMS, 124 μg/ml	101.0	11.5 (278)	5.3 (91)
Activation with rat-liver S9, 2 h			
0	100.0	4.0 (98)	1.0 (26)
0.03	58.1	not done	
0.04	46.1	not done	
0.05	64.1	5.6 (128)	1.5 (44)
0.06	49.7	6.6 (136)	0.4 (11)
0.07	67.7	5.4 (123)	0.5 (15)
0.08	8.7	5.2 (109)	1.6 (45)
B(a)P, 5 μg/ml	62.8	12.5 (382)	6.0 (201)
Cocultivation with Syrian hamster-embryo cells, 20 h			
0	100.0	2.8 (82)	1.1 (34)
0.025	97.2	3.0 (82)	1.2 (33)
0.050	124.2	2.7 (59)	1.1 (25)
B(a)P, 2 μg/ml	34.6	11.9 (272)	3.6 (110)
Cocultivation with primary chick-embryo hepatocytes, 20 h			
0	100.0	2.7 (60)	1.3 (37)
0.025	100.2	2.2 (61)	0.7 (20)
0.050	110.4	2.8 (78)	0.4 (12)
B(a)P, 2 μg/ml	14.3	17.9 (272)	–

[a] Number within brackets is number of mutant clones observed.

TABLE 5

MUTANT FREQUENCIES FOR TK-DEFICIENCY AND HPRT-DEFICIENCY AFTER TREATMENT WITH CAPROLACTAM

Concentration (μg/ml)	Survival after treatment (%)	Mutant frequency ($\times 10^{-5}$)	
		$TK^{-/-}$	$HPRT^{-}$
No activation, 2 h			
0	100.0	3.2 (95) [a]	0.8 (25)
500	96.6	2.5 (84)	0.4 (15)
1000	100.1	3.0 (101)	0.4 (12)
2500	100.8	3.7 (105)	0.2 (6)
5000	90.9	3.2 (97)	0.3 (12)
10000	96.3	3.0 (99)	0.8 (23)
ENU, 117 μg/ml	26.9	98.6 (1748)	46.3 (1375)
Activation with rat-liver S9, 2 h			
0	100.0	3.2 (71)	1.0 (32)
5000	105.3	3.6 (79)	–
10000	99.2	5.0 (81)	0.9 (27)
12500	113.6	3.9 (74)	2.1 (58)
15000	122.5	4.7 (90)	1.1 (33)
DEN, 10 μg/ml	106.7	9.4	7.3
Cocultivation with Syrian hamster-embryo cells, 20 h			
0	100.0	2.8 (82)	1.1 (34)
1000	84.5	2.5 (57)	0.7 (20)
B(a)P, 2 μg/ml	34.6	11.9 (250)	3.6 (110)
Cocultivation with primary chick-embryo hepatocytes, 20 h			
0	100.0	2.7 (68)	1.3 (37)
1000	64.5	5.2 (105)	1.3 (40)
B(a)P, 2 μg/ml	14.3	17.0 (272)	–

[a] Number within brackets is number of mutant clones observed.

the positive controls and their concentrations the increase in mutant frequency, however, was not strong: 2–3 times the control frequency at a concentration of 10 μl/ml (10270 μg/ml).

o-Toluidine (Table 2)

This compound, at a concentration of 2 μl/ml (2000 μg/ml) or higher for 2 h, was almost completely toxic to the cells. No mutagenic effect of *o*-toluidine was observed under any of the test conditions.

Benzene (Table 3)

This compound appeared to be more toxic in the presence of S9 than in its absence. A 2-h treatment with 0.4 μl/ml (352 μg/ml) resulted in a survival of 66.6% and 1% respectively. A mutagenic effect of benzene could not be detected, either in the short exposure experiments or after prolonged exposure in the cocultivation experiments.

Safrole (Table 4)

This compound appeared to be toxic at relatively low concentrations; in the absence of S9 no survival was observed at 0.075 μl/ml (82 μg/ml). In the presence of S9 an increase in the mutant frequency was observed for the TK locus at all dose levels and at the HPRT locus at 0.05 and 0.08 μl/ml. However, since the spontaneous mutant

TABLE 6

SUMMARY OF MUTAGENIC ACTIVITY OF THE 5 COMPOUNDS AT 2 LOCI IN L5178Y MOUSE LYMPHOMA CELLS

Chemical	$TK^{-/-}$		$HPRT^{-}$	
	− Activation	+ Activation	− Activation	+ Activation
Hexamethylphosphoramide	−	+	−	+
o-Toluidine	−	−	−	−
Benzene	−	−	−	−
Safrole	−	inconclusive	−	inconclusive
Caprolactam	−	−	−	−

frequency in this particular experiment was quite high, no definite conclusion with respect to its mutagenic effect could be drawn at the moment.

Caprolactam (Table 5)

This compound is a very special one. No toxicity could be reached although massive doses were applied, up to 15000 μg/ml in one experiment. No indication of a mutagenic effect of this compound was found in any of the experiments.

Results for all compounds tested are presented in Table 6.

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Assays for the induction of mutations to 6-thioguanine resistance in Chinese hamster V79 cells in culture

Y. Kuroda, A. Yokoiyama [1] and T. Kada [1]

Departments of Morphological Genetics and [1] Induced Mutation, National Institute of Genetics, Mishima (Japan)

Summary

5 carcinogens (hexamethylphosphoramide, *o*-toluidine, benzene, safrole and phenobarbital) and 2 noncarcinogens (caprolactam and benzoin) were tested at various concentrations for the induction of 6-thioguanine-resistant mutations in Chinese hamster V79 cells in culture. Hexamethylphosphoramide, *o*-toluidine, benzene and caprolactam were slightly cytotoxic, whereas safrole, benzoin and phenobarbital had strong or moderate cytotoxic effects.

All these carcinogens tested had weak or moderate mutagenic activities in Chinese hamster V79 cells. The addition of S9 mix showed no increase in the induced mutation frequency of cells treated with these carcinogens. *o*-Toluidine without S9 mix had the strongest mutagenic activity among chemicals tested in the present assay. Among 2 noncarcinogens, caprolactam showed no significant mutagenic activity, but benzoin had a weak mutagenic activity in Chinese hamster V79 cells.

The samples of 5 carcinogens (hexamethylphosphoramide, *o*-toluidine, benzene, safrole, and phenobarbital) and 2 noncarcinogens (caprolactam and benzoin) were tested for mutagenic activity in cultured Chinese hamster cells. Chinese hamster V79 cells were inoculated on petri dishes and exposed to the chemicals at various concentrations for 3 h. Then cells were cultured in normal medium to determine the colony forming activity. Hexamethylphosphoramide (HMPA), *o*-toluidine, benzene and caprolactam were slightly cytotoxic, whereas safrole, benzoin and phenobarbital had strong or moderate cytotoxic effects.

Cells exposed to these chemicals were cultured in normal medium for 6 days. Then cells were dissociated from petri dishes, replated in new dishes, and cultured in 6-thioguanine(6TG)-containing medium to determine the induced 6TG-resistant mutant colonies. All 5 carcinogens tested had weak or moderate mutagenic activities in Chinese hamster V79 cells. The addition of S9 mix produced no increase in the induced mutation frequency of cells treated with these chemicals. Of the 2 noncarcinogens tested, caprolactam showed no significant mutagenic activity but benzoin had a weak mutagenic activity in Chinese hamster V79 cells. The results of these experiments are summarized in Table 1.

Materials and methods

Chemicals tested

The chemicals tested 5 carcinogens and 2 noncarcinogens were supplied by J. Ashby, Imperial Chemical Industries, Alderlay Park, Cheshire (England). The positive control chemical was ethyl methanesulfonate (EMS). Chemicals were dissolved in dimethyl sulfoxide (DMSO) before use, and diluted with Hanks' solution to obtain the appropriate concentrations for treatment of cells.

Cells and culture

The cell line used was the V79 strain of Chinese hamster lung cells, isolated by Ford and Yerganian (1958) from a normal young adult male Chinese hamster. The cells were grown in large mass, dis-

TABLE 1

MUTAGENIC ACTIVITY OF 5 CARCINOGENS AND 2 NONCARCINOGENS IN CHINESE HAMSTER V79 CELLS

Chemical	S9	Concentrations tested (μg/ml)	Treatment time (h)	Mutagenicity
Carcinogens				
HMPA	−	0, 100, 250, 500	3	Positive
o-Toluidine	−	0, 1, 10, 50, 100, 500, 1000	3	Positive
	+	0, 1, 10, 50, 100, 500	3	Negative
Benzene	−	0, 1, 10, 50, 100, 500, 1000	3	Positive
	+	0, 1, 10, 50, 100, 500, 1000	3	Negative
Safrole	−	0, 100, 200, 300	3	Positive
Phenobarbital	−	0, 100, 250, 500	3	Positive
Noncarcinogens				
Caprolactam	−	0, 1, 10, 50, 100, 500, 1000	3	Negative
	+	0, 1, 10, 50, 100, 500, 1000	3	Negative
Benzoin	−	0, 100, 200, 300	3	Negative
	+	0, 100, 200, 400	3	Positive

tributed in many small ampules, and frozen at −80°C. Before use, cells were thawed at 38°C, suspended in fresh medium and cultured in GHAT medium (3×10^{-6} M glycine, 10^{-4} M hypoxanthine, 4×10^{-7} M amethopterine and 1.6×10^{-5} M thymidine) at 38°C for 24 h, to remove the preexisting 6TG-resistant cells in the cell population. In further subcultivation in normal medium, the appearance of spontaneous 6TG-resistant mutations was occasionally noted. For this reason, stock cultures were discarded at the 10th subculture and replaced from frozen stocks. The culture medium was Eagle's minimal essential medium (Nissui Seiyaku Co., Tokyo) supplemented with 10% fetal bovine serum (Filtron PTY Ltd., Australia). The cells were cultured in 60-mm plastic contour petri dishes (Lux Sci. Coop., U.S.A.; catalog No. 5216) in 5 ml of medium under 5% CO_2 and 95% air at 38°C.

Cytotoxicity assay

Cells were dissociated from monolayer cultures by treatment with 0.25% trypsin (Difco, 1:250) solution for 10 min at 38°C. Duplicate or triplicate inocula of 10^2 cells in 5 ml of normal medium in 60-mm petri dishes were incubated for 20 h at 38°C, during which time most cells became attached to the surface of the dishes. Then cells were treated with test chemicals at concentrations of 0, 50, 100 and 250 μg/ml for HMPA; 0, 1, 10, 50, 100, and 500 μg/ml for *o*-toluidine, benzene and caprolactam; 0, 10, 30, 100 and 400 μg/ml for safrole,; and 0, 30, 100, 300 and 500 μg/ml for benzoin and phenobarbital in Hanks' solution at 38°C for 3 h.

After treatment of cells with chemicals, the cells were rinsed twice with Hanks' solution and incubated in normal medium for 7 days at 38°C. The cell colonies formed in petri dishes were fixed with methanol, and stained with May–Grünwald–Giemsa solution. Colonies containing 50 cells or more were scored under a binocular microscope, and the colony-forming activity was calculated as the average number of colonies with at least 50 cells as a percentage of the number of cells initially inoculated. The effects of test chemicals on cell survival are expressed as fractions (surviving fractions) of the colony-forming activity of untreated control cultures.

Mutagenicity assay

The mutagenic activity of the chemicals was determined by the 'replating' method (Fox, 1975; Sugiura et al., 1978). The inocula of 2×10^5 cells in 10 ml of normal medium in 100-mm plastic petri dishes (Lux Sci. Corp., U.S.A.; catalog No. 5211) were incubated in normal medium for 20 h at 38°C and then treated with test chemicals at almost the same concentrations as those used for the cytotoxicity assay for 3 h, except for slight

changes in concentrations of safrole, benzoin and phenobarbital. A highest concentration of 1000 μg/ml was also tested for *o*-toluidine, benzene and caprolactam. Then the cells were rinsed twice with Hanks' solution and incubated in normal medium for a mutation expression time of 6 days. The cells were then treated with trypsin, and 2×10^5 cells were replated each in five 100-mm petri dishes in medium containing 6TG (5 μg/ml) (Sigma Chemical Company). After incubation for 14 days, the cell colonies formed were fixed and stained and the number of 6TG-resistant colonies was scored.

TABLE 2

CYTOTOXIC EFFECTS OF CHEMICALS ON CHINESE HAMSTER V79 CELLS

Chemical	Concentration (μg/ml)	Treatment time (h)	Number of cells inoculated	Number of colonies formed	Colony-forming activity (%)	Surviving fraction
HMPA	0	3	10^2	101, 112, 93	102	1.00
	50	3	10^2	93, 87	90	0.88
	100	3	10^2	88, 89, 85	87	0.85
	250	3	10^2	87, 92, 84	88	0.86
	500	3	10^2	70, 71, 74	72	0.70
o-Toluidine	0	3	10^2	108, 100	104	1.00
	1	3	10^2	87, 103	95	0.91
	10	3	10^2	91, 86	89	0.85
	50	3	10^2	103, 87	95	0.91
	100	3	10^2	102, 104	103	0.99
	500	3	10^2	67, 66	67	0.64
Benzene	0	3	10^2	108, 100	104	1.00
	1	3	10^2	121, 97	109	1.05
	10	3	10^2	101, 107	104	1.00
	50	3	10^2	105, 97	101	0.97
	100	3	10^2	92, 84	88	0.85
	500	3	10^2	83, 98	91	0.87
Safrole	0	3	10^2	74, 98, 86	86	1.00
	10	3	10^2	24, 35, 22	27	0.31
	30	3	10^2	22, 21, 25	23	0.27
	100	3	10^2	7, 6, 10	7	0.09
	300	3	10^2	0, 0, 0	0	0
Phenobarbital	0	3	10^2	74, 98, 86	86	1.00
	30	3	10^2	28, 29, 22	28	0.36
	100	3	10^2	22, 29, 25	25	0.29
	300	3	10^2	20, 33, 29	24	0.28
	500	3	10^2	34, 31, 20	27	0.31
Caprolactam	0	3	10^2	102, 100	101	1.00
	1	3	10^2	109, 110	110	1.08
	10	3	10^2	112, 101	107	1.05
	50	3	10^2	97, 90	94	0.93
	100	3	10^2	105, 85	95	0.94
	500	3	10^2	96, 71	84	0.83
Benzoin	0	3	10^2	74, 98, 86	86	1.00
	30	3	10^2	65, 51, 64	60	0.70
	100	3	10^2	35, 25, 38	33	0.38
	300	3	10^2	27, 19, 27	25	0.29
	500	3	10^2	0, 0, 0	0	0

TABLE 3

MUTAGENIC ACTIVITY OF CHEMICALS IN CHINESE HAMSTER V79 CELLS

Chemical	Concentration (μg/ml)	S9 mix	Number of cells inoculated	Colony-forming activity (%)	Number of colonies formed	Average number of colonies	Corrected number of colonies	Number of induced mutants	Induced mutation frequency per 10^5 survivors
HMPA	0	–	2×10^5	71	0, 0, 1, 0, 1	0.4	0.6	0	0
	100	–	2×10^5	96	0, 0, 0, 0, 0	0	0	0	0
	250	–	2×10^5	80	1, 1, 0, 0, 1	0.6	0.8	0.2	0.1
	500	–	2×10^5	91	4, 3, 1, 3, 1	2.4	2.6	2.0	1.0
o-Toluidine	0	–	2×10^5	70	3, 2, 1, 0, 0	1.4	2.0	0	0
		+	2×10^5	122	0, 3, 3, 6, 1	2.6	2.1	0	0
	1	–	2×10^5	20	3, 0, 0, 0, 0	0.6	3.0	1.0	0.5
		+	2×10^5	107	0, 0, 0, 0, 0	0	0	0	0
	10	–	2×10^5	21	0, 0, 0, 0, 0	0	0	0	0
		+	2×10^5	52	1, 2, 0, 1, 0	0.8	1.6	0	0
	50	–	2×10^5	16	1, 2, 1, 2, 0	1.2	7.5	5.5	2.8
		+	2×10^5	53	1, 1, 3, 0, 0	1.0	1.9	0	0
	100	–	2×10^5	48	1, 0, 1, 1, 0	1.0	2.1	0.1	0.1
		+	2×10^5	83	0, 0, 1, 1, 0	0.4	0.5	0	0
	500	–	2×10^5	14	1, 2, 1, 2, 3	1.8	12.9	10.9	5.5
		+	2×10^5	92	2, 2, 5, 7, 5	4.2	4.6	0	0
	1000	–	2×10^5	23	1, 0, 1, 3, 0	1.0	4.3	2.3	1.1
Benzene	0	–	2×10^5	60	5, 6, 4, 2, 2	3.8	6.3	0	0
		+	2×10^5	122	0, 3, 3, 6, 1	2.6	2.1	0	0
	1	–	2×10^5	83	0, 1, 4, 0, 0	1.0	1.2	0	0
		+	2×10^5	71	0, 0, 0, 0, 0	0	0	0	0
	10	–	2×10^5	75	1, 0, 1, 1, 5	1.6	2.1	0	0
		+	2×10^5	102	0, 0, 0, 0, 0	0	0	0	0
	50	–	2×10^5	32	2, 3, 3, 3, 2	2.6	8.1	1.8	0.9
		+	2×10^5	45	1, 0, 0, 0, 0	0.2	0.4	0	0
	100	–	2×10^5	47	5, 0, 1, 6, 4	3.2	6.8	0.5	0.3
		+	2×10^5	50	0, 0, 0, 0, 0	0	0	0	0

	500	–	2×10^5	96	0, 0, 0, 0, 0	0	0	0	0
		+	2×10^5	53	0, 1, 1, 3, 0	1.0	1.9	0	0
	1000	–	2×10^5	65	2, 1, 1, 1, 0	1.0	1.6	0	0
		+	2×10^5	68	1, 0, 0, 0, 0	0.2	0.3	0	0
Safrole	0	–	2×10^5	161	0, 0, 0, 0, 0	0	0	0	0
	100	–	2×10^5	127	0, 1, 0, 0, 1	0.4	0.3	0.3	0.2
	200	–	2×10^5	112	4, 2, 5, 1, 2	2.8	2.5	2.5	1.3
	300	–	2×10^5	115	3, 5, 3, 1, 1	2.6	2.3	2.3	1.2
Phenobarbital	0	–	2×10^5	101	0, 0, 0, 0, 0	0	0	0	0
	100	–	2×10^5	79	0, 0, 0, 1, 2	0.6	0.8	0.8	0.4
	250	–	2×10^5	83	1, 1, 3, 0, 3	1.6	1.9	1.9	1.0
	500	–	2×10^5	49	2, 2, 2, 1, 2	1.8	3.7	3.7	1.9
Caprolactam	0	–	2×10^5	90	0, 1, 0, 0, 0	0.2	1.1	0	0
		+	2×10^5	122	0, 3, 3, 6, 1	2.6	2.1	0	0
	1	–	2×10^5	83	0, 0, 0, 0, 0	0	0	0	0
		+	2×10^5	46	0, 0, 1, 0, 2	0.6	1.3	0	0
	10	–	2×10^5	55	0, 0, 0, 0, 0	0	0	0	0
		+	2×10^5	52	1, 2, 0, 0, 3	1.2	2.3	0.2	0.1
	50	–	2×10^5	94	0, 0, 0, 0, 0	0	0	0	0
		+	2×10^5	32	0, 0, 0, 0, 0	0	0	0	0
	100	–	2×10^5	72	0, 0, 0, 0, 0	0	0	0	0
		+	2×10^5	33	0, 0, 0, 0, 0	0	0	0	0
	500	–	2×10^5	56	0, 0, 0, 0, 0	0	0	0	0
		+	2×10^5	34	0, 0, 0, 0, 0	0	0	0	0
	1000	–	2×10^5	43	0, 0, 0, 0, 0	0	0	0	0
		+	2×10^5	24	0, 0, 0, 0, 0	0	0	0	0
Benzoin	0	–	2×10^5	71	0, 0, 1, 0, 1	0.4	0.6	0	0
		+	2×10^5	106	0, 0, 0, 0, 1	0.2	0.2	0	0
	100	–	2×10^5	107	1, 0, 1, 0, 1	0.4	0.4	0	0
		+	2×10^5	108	1, 1, 3, 0, 0	1.0	0.9	0.3	0.2
	200	–	2×10^5	110	0, 0, 0, 0, 0	0	0	0	0
		+	2×10^5	131	1, 0, 3, 1, 2	1.4	1.1	0.5	0.3
	300	–	2×10^5	99	0, 1, 0, 0, 0	0.2	0.2	0	0
	400	+	2×10^5	103	1, 3, 1, 5, 5	3.0	2.9	2.3	1.2

In parallel experiments, duplicate inocula of 10^2 cells in 60-mm petri dishes were incubated in normal medium for 7 days. The colony-forming activity of replated cells was determined as described above. The observed number of 6TG-resistant mutant colonies induced by chemicals was corrected for the decrease in the colony-forming activity of cells in normal medium due to the cytotoxic action of the chemicals. The number of induced mutants was calculated by subtracting the number of colonies in untreated control cultures from those in treated cultures. The number of colonies in control cultures included colonies of mutants present among cells plated initially, as well as those formed during the treatment. The induced mutation frequency was expressed as the number of induced mutants per 10^5 colony-forming cells.

Metabolic activation

The rat-liver 9000 *g* (S9) fraction with cofactors (Oriental Yeast Co., Ltd., Tokyo) were used (as S9 mix) for testing the activity of chemicals after metabolic activation. The S9 fraction had been obtained from 7-week-old male Sprague–Dawley rats (Charles River Japan Inc.) that had been intraperitoneally injected with phenobarbital and 5,6-benzoflavone. The S9 fractions and cofactors were stored at $-80\,^{\circ}C$. Before use, they were thawed and mixed together. The cells were treated with chemicals for 3 h at $38\,^{\circ}C$ in the presence and in the absence of the S9 fraction with cofactors containing 8×10^{-6} M $MgCl_2$, 3×10^{-5} M KCl, 5×10^{-6} M glucose-6-phosphate, 4×10^{-6} M NADPH and 4×10^{-6} M NADH in 1×10^{-4} M sodium phosphate buffer solution. The procedure for obtaining the induced mutation frequency was the same as that described above.

Results and discussion

Cytotoxicity assay

The cytotoxic effects of chemicals on Chinese hamster V79 cells are shown in Table 2. HMPA, *o*-toluidine, benzene and caprolactam were not too highly cytotoxic, but cells treated with these chemicals at the concentration of 500 μg/ml were slightly reduced in their colony-forming activity. On the other hand, safrole, benzoin and phenobarbital had strong or moderate cytotoxic activities on Chinese hamster cells, showing marked reductions in the surviving fraction of cells treated with these chemicals at concentrations more than 100 μg/ml.

Mutagenicity assay

The activities of chemicals that induced 6TG-resistant mutations in Chinese hamster V79 cells are shown in Table 3. HMPA had a weak mutagenic activity at the concentration of 500 μg/ml. *o*-Toluidine without S9 mix had the strongest mutagenic activity among chemicals tested in the present experiment. The addition of S9 mix diminished the frequency of mutations induced by *o*-toluidine, because the treatment with S9 mix alone induced many mutations in control cultures.

Benzene induced slight mutations at concentrations of 50 and 100 μg/ml in the absence of S9 mix. In the presence of S9 mix, no mutations were induced by benzene at any of the concentrations tested. Safrole and phenobarbital also induced mutations at relatively high frequency at concentrations more than 200 μg/ml. Since these chemicals had positive mutagenic activity in the absence of S9 mix, further assay was not done with S9 mix. Of the 2 noncarcinogens tested, caprolactam produced no significant increase in induced mutation frequency, but benzoin did produce slight induced mutations in Chinese hamster V79 cells with S9 mix. These results indicate that all 5 carcinogens induced 6TG-resistant mutations in Chinese hamster V79 cells.

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Assays for the induction of mutations to ouabain resistance in V79 Chinese hamster cells in culture with cell- or microsome-mediated metabolic activation

Toshio Kuroki and Kimie Munakata

Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Tokyo (Japan)

Summary

V79 Chinese hamster cells were used for the assay of mutagenic activity of 5 chemicals: safrole, *o*-toluidine, benzoin, caprolactam and hexamethylphosphoramide, all of which were supplied by the International Program on Chemical Safety. These chemicals showed little or no toxicity to the cells. Mutation was assayed in the direct, cell-mediated and microsome-mediated assays. Although marginal increase in mutation at the Na^+/K^+-dependent ATPase locus was observed with *o*-toluidine and hexamethylphosphoramide, there was no dose–response relationship. Safrole, benzoin and caprolactam were not mutagenic in this system.

Several systems for mammalian cell mutagenesis have been developed and are now widely used in the detection of environmental mutagens (Kuroki et al., 1980). Isolation of drug-resistant mutation such as ouabain resistance in V79 Chinese hamster cells is one of the best methods available for this purpose because of its high sensitivity and the ease of the isolation procedure.

The mutagenic/carcinogenic effect of many chemicals is dependent on the formation of reactive electrophilic intermediates, mainly by the activity of microsomal mixed-function oxidases. Most of the cell lines currently used for mutagenesis studies, e.g. V79 Chinese hamster cells, are not capable of activating such chemicals, and inclusion of metabolic activation system in a test system is therefore essential. Activation of chemicals in the presence of a microsomal fraction has been used extensively in mammalian cell mutagenesis assays. This system is useful in detection of a wide spectrum of chemicals. Cocultivation of target cells with metabolically competent cells is also used for activation of chemicals. This system, often referred as cell-mediated system, appears to reflect more accurately the metabolic pathway that occurs in vivo.

Under the collaborative studies conducted by the International Program on Chemical Safety (IPCS), we have examined the mutagenicity of 5 chemicals: safrole, *o*-toluidine, benzoin, caprolactam and hexamethylphosphoramide (HMPA) in V79 Chinese hamster cells. These 5 chemicals are not or only marginally mutagenic in Na^+/K^+-dependent ATPase locus of V79 Chinese hamster cells in the cell- and microsome-mediated assays.

Materials and methods

Mutagenesis assays

Mutations were assayed by induction of ouabain-resistant clones in V79 Chinese hamster cells, as reported elsewhere (Kuroki et al., 1977; Kuroki and Drevon, 1978). In brief, V79 Chinese hamster cells, grown in Eagle's MEM plus 10% FCS, were treated with test compounds at 3 concentrations in the presence and absence of metabolic activation. Test compounds were dissolved in DMSO which was added to cultures at a final

TABLE 1

MUTAGENICITY OF SAFROLE, BENZOIN, *o*-TOLUIDINE, CAPROLACTAM AND HMPA IN OUABAIN-RESISTANT LOCUS OF V79 CHINESE HAMSTER CELLS BY CELL- AND MICROSOME-MEDIATED ASSAY

Compound	Concentration (mM)	Metabolic activation		
		Absence	Cell	Microsome
DMSO	0.3%	0	0–0.25	0
BP	0.02	–	20.2	–
DMN	20.0	–	–	2.5
Safrole	0.01	0	0	0
	0.1	0.125	0.125	0
	1.0	0	0	0
Benzoin	0.01	0	0	0
	0.1	0	0	0
	1.0	0	0.125	0
o-Toluidine	0.1	0.375	–	0
	1.0	0.125	–	0.625
	5.0	0.875	–	0.125
Caprolactam	0.01	–	0.52	0.31
	0.1	–	0.91	0.32
	1.0	–	0	0
HMPA	0.01	–	0.94	0
	0.1	–	0.22	0.16
	1.0	–	0.34	0

concentration of 0.5%. After expression time of 3–5 days, the cells were replated for determination of the cytotoxicity and mutagenicity induced. Cytotoxicity was determined by plating 100 cells/60-mm dish (4 dishes per point) and cultured for 7 days. For mutagenesis, 10^5 cells were plated in the medium containing 1 mM ouabain and cultured for 12–14 days (8 dishes per point). The medium containing ouabain was changed once, 7 days later. Mutation frequency was calculated per 10^5 survivors, taking into account the number of cells plated and the plating efficiency. Among two or three expression periods, the highest mutation frequencies are listed in Table 1.

Cell-mediated mutagenesis assay

The experimental protocol adopted from that of Huberman and Sacks (1974) was reported elsewhere (Kuroki and Drevon, 1978). In brief, secondary cultures of hamster embryo cells were lethally irradiated at 5000 rad and plated at 10^6 cells per 60-mm dish and cultured overnight. V79 cells (3×10^5 cells) were plated on top of hamster embryo cells; 3 h later, test compounds were added for 48 h. After cultivation for another 48 h, they were replated for determination of cytotoxicity and mutagenicity as described above. For positive control, benzo[*a*]pyrene (BP) was added at 20 μM (5.0 μg/ml).

Microsome-mediated mutagenesis assay

The protocol established by Kuroki et al. (1977) was used with a slight modification. V79 cells were plated at 5×10^5 cells per 60-mm dish and cultured overnight. They were then incubated for 3 h at 37 °C in the reaction mixture containing:

S15 of rat liver	2%
G6P	5 μmoles/ml
NADP	0.8 μmoles/ml
NADPH	0.8 μmoles/ml
Mg^{2+}	1.5 μmoles/ml
PO_4^{2-}	20 μmoles/ml
Test compound	0.01–5 mM

For the rat liver, Sprague–Dawley rats were injected i.p. with phenobarbital at 30 mg/kg 4 days, at 60 mg/kg 1, 2 and 3 days before killing and with 5,6-benzoflavone at 80 mg/kg 2 days before killing (Matsushima et al., 1976).

The cells were then washed with PBS and culture for 3–4 days followed by determination of cytotoxicity and mutagenicity induced. Dimethylnitrosamine (DMN) was used as a positive control at 20 mM, although 2% S15 fraction was not optimum for DMN (Kuroki et al., 1979).

Results and discussion

Among the 5 chemicals tested, safrole, *o*-toluidine and HMPA are carcinogenic but negative in the standard Salmonella mutation assays. The other 2 chemicals, benzoin and caprolactam are reported neither carcinogenic nor mutagenic. These chemicals showed little or no toxicity to V79 Chinese hamster cells (data not included). A decrease in plating efficiency by 20–30% was observed with safrole and HMPA at a concentration of 1 mM in the presence of rat S15 fraction or irradiated Syrian hamster cells, while the others were not toxic to the cells at a concentration of 1 mM or more.

Table 1 summarizes mutation frequency of the 5 chemicals in direct, microsome-mediated and cell-mediated assays. Dimethylnitrosamine (DMN) and benzo[*a*]pyrene (BP) were included as positive controls for the microsome- and cell-mediated assays, respectively. Although marginal increase in mutation frequency (0.5–0.9 per 10^6 survivors) was observed with *o*-toluidine and HMPA, there was no dose–response relationship. Other chemicals produced little or no mutation (0–0.5 per 10^6 survivors). We therefore concluded that these chemicals are negative in inducing gene mutation at the Na^+/K^+-dependent ATPase locus of V79 Chinese hamster cells. Thus, so far as these 5 compounds are concerned, this assay has no advantages over the Salmonella assay.

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The induction of gene mutations in the mouse lymphoma L5178Y/TK$^{+/-}$ assay and the Chinese hamster V79/HGPRT assay

C.G. Lee and T.D. Webber

Chemical Defence Establishment, Porton Down, Salisbury, Wilts, (Great Britain)

Summary

The 10 test compounds were tested for the induction of gene mutations in mammalian cells in culture in both the mouse lymphoma L5178Y/TK$^{+/-}$ assay and the Chinese Hamster V79/HGPRT assay. Benzoin was positive in the V79 assay, but only in the presence of a microsome-cofactor mix. Acrylonitrile was the only compound that gave a positive result in the L5178Y assay, and it was mutagenic both in the presence and absence of extrinsic metabolic activation.

The V79/HPRT and L5178Y/TK$^{+/-}$ mammalian cell, forward-mutation assays have been used to test 10 compounds selected for the IPCS Collaborative Study on Short-Term Tests. Mutation at the HPRT locus was detected by mutant selection with 8-azaguanine and mutation at the TK locus was detected by selection in medium containing a mixture of TFT and BrdUrd. Both cell lines were exposed to the test compounds while suspended in serum-reduced medium, with and without the addition of a microsome-cofactor mix as an extrinsic metabolic activation system. The test protocols were of a replating format, the cells being transferred to the selective medium after expression times of 3 or 4 days (L5178Y and V79 respectively).

Materials and methods

Cell culture

V79 cells were grown from cloned, frozen stocks derived from an original flask culture obtained from the Institute of Cancer Research. Eagle's minimum essential medium supplemented with 10% foetal bovine serum (FBS10-EMEM90) was used for both routine cell culture and mutation experiments, and the cells were grown at 37°C in a humidified incubator gassed with a mixture of 5% CO_2 in air. The cultures were subjected to a weekly cycle of exposure to antibiotic-free medium containing HAT and HT (Littlefield, 1964) to prevent accumulation of spontaneous azaguanine-resistant mutants in the serially subcultured, stock cell line. Cells for mutation assays were grown to 90% confluence in plastic flasks, the cultures receiving a complete change of medium 24 h before cell harvesting.

L5178Y cells, heterozygous at the TK locus (TK$^{+/-}$), were grown from frozen stocks derived from an original culture obtained from the Central Toxicology Laboratory, ICI. Eagle's minimum essential medium supplemented with 10% horse serum (HS10-EMEM90) and containing sodium pyruvate (200 μg/ml) was used for both routine cell culture and mutation experiments. Cells were cultured in 1 oz. polystyrene universal bottles (20 ml medium per bottle) at 37°C with intermittent agitation and a gas phase of 5% CO_2 in air. As recommended by Clive and Spector (1975), the L5178Y cells were subjected to a regular cycle of HAT and HT treatment to purge the cultures of accumulated TK$^{-/-}$ mutants. The cell seeding densities, and hence maximum cell densities that were obtained during the HAT/HT cycle were found to be critical to the production of healthy, purged cultures for the mutation assays. The cells

were maintained in antibiotic-free medium and seeded at 1×10^5 cells per bottle into HAT medium to yield approximately 10×10^6 cells per bottle 3 days later. The recovery period in HT medium was 4 days, an initial inoculum of 5×10^4 HAT-treated cells per bottle yielding approximately 15×10^6 cells. Cells for mutation assays were removed from HT medium and grown for 24 h in HS10-EMEM90 (inoculum 5×10^6, yield approximately 12×10^6).

The media used in the mutation assay cultures, for both V79 and L5178Y cells, contained penicillin (100 units/ml), streptomycin (100 μg/ml) and amophotericin B(2.5 μg/ml).

Chemicals and media

The IPCS-CSSTT series of test compounds were provided by the Central Toxicology Laboratory, ICI. Benzo[*a*]pyrene, 8-azaguanine, trifluorothymidine, 5-bromodeoxyuridine, NADP and glucose 6-phosphate were obtained from Sigma London Ltd.; MNNG from Koch-Light Laboratories Ltd.; EMEM, FBS, HEPES buffer, trypsin–EDTA, sodium pyruvate and antibiotics from Flow Laboratories; Aroclor 1254 from Monsanto chemicals; DMSO ("Analar") from BDH Ltd.; Agar Noble from Difco Laboratories.

The test compounds, benzo[*a*]pyrene and MNNG were prepared as 100× concentrated stock solutions in DMSO.

Microsome-cofactor mix (S9)

Microsomes were prepared as a 9000-*g* homogenate supernatant from the livers of male, Porton strain rats. Each animal received an i.p. injection of Aroclor 1254 (100 mg in 0.5 ml corn oil) 5 days prior to sacrifice. Livers were homogenized at 25% w/v in 0.15 M KCl in a glass tissue grinder fitted with a teflon pestle. All preparations were handled at 4°C and the microsomes were stored under liquid nitrogen. The microsome–cofactor mix (metabolic activation system) was formulated as follows: microsomes, 500 μl; NADP (0.1 M), 200 μl; glucose 6-phosphate (0.1 M), 254 μl; phosphate buffer (0.1 M, pH 7.4), 4000 μl.

Mutation assays

V79/HPRT assay. Azaguanine-sensitive V79 cells were used in a forward mutation, replating type of assay as previously described (Lee and Webber, 1982), mutant cells being identified by their ability to grow in medium containing 30 μg/ml 8-azaguanine. Cells in subconfluent flask cultures were detached with trypsin–EDTA, washed in EMEM–HEPES and resuspended in fresh FBS3–EMEM97 (also HEPES buffered) at 5×10^5 cells/ml. The cells were treated in suspension, in polystyrene centrifuge tubes, each 1.0 ml aliquot of cells receiving 10 μl of test compound or MNNG or benzo[*a*]pyrene or DMSO (solvent control). If extrinsic metabolic activation was required, 100 μl of microsome–cofactor mix was added to each tube. The cell suspensions were incubated for 2 h at 37°C on an orbital shaker at 150 rev/min, amplitude 10 mm, and treatment was terminated by centrifuging and resuspending each tube of cells in 25 ml FBS10–EMEM90. Each 25 ml of cell suspension, representing a single tube of treated cells, was seeded into a 90-mm dish and incubated for 4 days. The 4-day expression period was terminated by trypsinization of the cell monolayers, resuspension of the cells in FBS10–EMEM90 containing 30 μg/ml 8-azaguanine and replating at 5×10^5 cells per 90-mm dish; 5 dishes per 1 ml original treated cell suspension. The replated cells were grown in the selective medium for 8 days, the cultures were fixed in formol–saline and the mutant colonies stained with Giemsa.

Cells from the 1-ml suspensions were plated after the 2-h treatment period into duplicate 50-mm dishes containing 5 ml FBS10–EMEM90 at a nominal 400 cells per dish. After 6 days incubation, the cultures were fixed, stained and the colonies counted. Cytotoxicity was expressed as percentage survival relative to 100% survival in the DMSO-treated cultures.

Cell samples were also taken from the suspensions used to seed the mutation dishes at the replating step, the cells being removed before the addition of 8-azaguanine to the medium. 50-mm dishes were seeded, in duplicate, with a nominal 200 cells per dish; media, incubation period and processing were as for the cytotoxicity assay, and the mean colony counts were used as the basis for calculating the absolute numbers of cells seeded into the mutation plates.

L5178Y/TK$^{+/-}$ assay. The L5178Y/TK$^{+/-}$ assay was based on the method of Clive and

Spector (1975) with our own modifications to reduce the physical bulk of the assay, to parallel the V79 protocol, and to produce mutant colonies consistently amenable to counting with an automatic colony counter.

Cells that had been grown for 24 h in HS10–EMEM90 (see "Cell Culture") were resuspended at 10^6 cells/ml in HS3–EMEM97(HEPES) and treated in suspension, in polystyrene centrifuge tubes, as described in the V79 assay above. Treatment was terminated by centrifuging and resuspending each tube of cells in 25 ml HS10–EMEM90 in a Universal bottle and incubating the cultures for 2 days at 37 °C. The cultures were examined by inverted microscope at the end of the first growth period and those cultures covering the required toxicity range were selected for further treatment. The cultures were diluted, as necessary, with fresh medium to maintain optimal cell densities and to avoid cell overgrowth. At the end of a further 24 h incubation, giving a total expression time of 3 days, 10^6 cells from each culture were seeded into 25 ml HS20–EMEM80 containing TFT (2.0 μg/ml) and BrdUrd (50 μg/ml), to which was added 1.7 ml 5% molten agar (Difco Noble). The dilute-agar cell suspensions were poured into 90-mm plastic dishes (1 dish per 1 ml original treated cell suspension) and allowed to gel for 15 min on an ice-cooled tray. The cultures were then incubated for 10 days at 37 °C in a humidified incubator gassed with 5% CO_2 in air.

Cells were removed from the 25-ml suspensions prior to the addition of TFT and BrdUrd and plated out in dilute-agar medium into duplicate 50-mm dishes (nominal 600 cells per dish). After 10 days incubation, the colonies were counted and the mean counts were used to calculate the absolute numbers of cells seeded into the mutation dishes for each treatment (nominal 10^6 cells per dish).

Cytotoxicity (cell survival) was expressed as a percentage relative to the cell yield in the solvent controls (100%). The total cell yield for each culture at 3 days post-treatment was calculated from the interim dilution step and corrected for absolute numbers on the basis of the plating assay.

Cell colony counts in both mutation dishes and plating assay dishes were determined using an automated electronic colony counter (System III Image Analyser, Micromeasurements Ltd.).

TABLE 1

SUMMARY OF RESULTS OBTAINED WITH THE IPCS-CSSTT SERIES OF COMPOUNDS IN THE V79/HPRT AND L5178Y/TK$^{+/-}$ ASSAYS [a]

Compound	V79/HPRT		L5178Y/TK$^{+/-}$	
	−S9 [b]	+S9	−S9	+S9
HMPA	−	−	−	−
SAF	−	−	−	−
TOL	−	−	−	−
CAP	−	−	−	−
ZOIN	−	+	−	−
BEN	−	−	−	−
DEHP	−	−	−	−
ACN	−	−	+	+
PB	−	−	−	−
DES	−	−	−	−

−, negative result

+, positive result.

[a] Each compound was tested in a minimum of two separate experiments in each mutagenicity assay.

[b] Microsome-cofactor mix present (+) or absent (−)

Results and conclusion

The results obtained with the ten test compounds in both the V79/HPRT and L5178Y/TK$^{+/-}$ assays are summarised in Table 1; and the detailed cytotoxicity and mutagenicity data for benzoin and acrylonitrile, from both assays, are given in Tables 2–5.

Benzoin was positive in the V79 assay, but only in the presence of a microsome–cofactor mix, while acrylonitrile was the only compound that gave a positive result in the L5178Y assay — it was mutagenic both in the presence and absence of extrinsic metabolic activation.

TABLE 2

INDUCTION OF 8-AZAGUANINE RESISTANCE IN V79 CELLS BY BENZOIN (ZOIN)

Compound	Concentration (μg/ml)	Cytotoxicity [a]	S9 [b]	Mutation frequency [c]
Expt. 1				
DMSO	(1%)	100	−, + [d]	8.3(±1.2) [e]
MNNG	0.3	78.9	−	411.3
	0.2	85.8	−	386.3
Benzo[*a*]pyrene	15.0	77.0	+	87.2
	15.0	59.9	+	148.4
ZOIN	1000	48.6	−	3.4
	500	54.9	−	14.0
	250	95.3	−	7.3
	1000	61.2	+	57.6
	500	87.1	+	40.0
	250	93.4	+	42.5
Expt. 2				
DMSO	(1%)	100	−, +	8.0(±1.2)
MNNG	0.4	74.6	−	247.5
	0.3	93.8	−	176.5
Benzo[*a*]pyrene	15.0	76.6	+	133.8
	15.0	85.2	+	136.0
ZOIN	2000	68.9	−	14.3
	1260	69.9	−	8.1
	794	66.0	−	8.2
	500	82.3	−	7.6
	2000	84.2	+	43.1
	1260	83.3	+	28.9
	794	95.7	+	3.5
	500	95.7	+	16.4
Expt. 3				
DMSO	(1%)	100	−, +	6.4(±1.3)
MNNG	0.4	85.9	−	231.1
	0.4	91.9	−	303.3
Benzo[*a*]pyrene	15.0	81.9	+	119.3
	15.0	75.2	+	149.5
ZOIN	3000	94.6	−	5.5
	2080	67.8	−	4.3
	1440	63.1	−	6.5
	1000	55.7	−	5.2
	690	56.4	−	6.7
	3000	69.1	+	33.6
	2080	50.3	+	75.3
	1440	51.7	+	58.7
	1000	52.3	+	48.4
	690	58.4	+	46.4

[a] Percentage cell survival. Mean survival of solvent controls = 100%.
[b] Microsome–cofactor mix present (+) or absent (−).
[c] Number of mutant colonies/10^6 viable plated cells. Total of 2.5×10^6 cells replated.
[d] − and + S9 control data pooled.
[e] Mean ± SE.
Number of controls, 6.

TABLE 3

INDUCTION OF TK $^{+/-}$ MUTANTS IN L5178Y CELLS BY BENZOIN (ZOIN)

Data from 1 of 3 replicate experiments.

Compound	Concentration (μg/ml)	Cytotoxicity [a]	S9 [b]	Mutation frequency [c]
DMSO	(1%)	100	−, + [d]	36.2($\pm$5.0) [e]
MNNG	0.08	34.5	−	523
	0.05	66.0	−	224
Benzo[*a*]pyrene	3.0	50.0	+	307
	3.0	41.5	+	254
ZOIN	900	57.5	−	51
	900	71.5	−	25
	684	97.5	−	31
	684	86.5	−	19
	519	77.0	−	25
	519	90.0	−	22
	394	85.0	−	30
	394	86.5	−	27
	300	70.0	−	27
	300	91.0	−	41
	900	7.5	+	30
	900	7.0	+	45
	684	9.0	+	67
	684	13.0	+	91
	519	33.5	+	46
	519	37.0	+	55
	394	36.5	+	25
	394	29.5	+	62
	300	27.0	+	20
	300	22.5	+	64

For explanation of superscripts see Table 2.
Number of controls, 10.

TABLE 4

INDUCTION OF 8-AZAGUANINE RESISTANCE IN V79 CELLS BY ACRYLONITRILE (ACN)

Compound	Concentration (μg/ml)	Cytotoxicity [a]	S9 [b]	Mutation frequency [c]
Expt. 1				
DMSO	(1%)	100	−, + [d]	7.7(±1.5) [e]
MNNG	0.3	78.9	−	411.3
	0.2	85.8	−	386.3
Benzo[*a*]pyrene	15.0	77.0	+	87.2
	15.0	59.9	+	148.4
ACN	200	6.3	−	1.2
	100	55.5	−	10.2
	50	92.1	−	11.8
	200	17.0	+	20.1
	100	70.7	+	7.7
	50	97.2	+	6.4
Expt. 2				
DMSO	(1%)	100	−, +	8.0(±1.2)
MNNG	0.4	74.6	−	247.5
	0.3	93.8	−	176.5
Benzo[*a*]pyrene	15.0	76.6	+	133.8
	15.0	85.2	+	136.0
ACN	200	40.2	−	16.9
	148	89.0	−	7.3
	110	109.1	−	5.4
	200	55.0	+	4.2
	148	80.4	+	3.4
	110	82.3	+	3.6
Expt. 3				
DMSO	(1%)	100	−, +	6.4(±1.3)
MNNG	0.4	85.9	−	231.1
	0.4	91.9	−	303.3
Benzo[*a*]pyrene	15.0	81.9	+	119.3
	15.0	75.2	+	149.5
ACN	210	36.9	−	7.2
	177	43.6	−	22.7
	149	54.4	−	10.1
	125	43.6	−	16.8
	210	42.3	+	13.4
	177	51.0	+	28.0
	149	63.8	+	11.1
	125	60.4	+	6.1

For explanation of superscripts see table 2.
Number of controls, 6.

TABLE 5

INDUCTION OF TK$^{-/-}$ MUTANTS IN L5178Y CELLS BY ACRYLONITRILE (ACN)

Compound	Concentration (μg/ml)	Cytotoxicity [a]	S9 [b]	Mutation frequency [c]
Expt. 1				
DMSO	(1%)	100	−, + [d]	31.9(±4.3) [e]
MNNG	0.3	2.2	−	1700
	0.15	6.0	−	975
Benzo[*a*]pyrene	3.0	47.8	+	371
	3.0	43.6	+	224
ACN	225	18.3	−	86
	169	21.3	−	122
	127	70.1	−	88
	95	79.1	−	63
	71	90.1	−	30
	225	27.5	+	118
	169	29.6	+	146
	127	60.3	+	92
	95	82.6	+	47
	71	105.2	+	37
Expt. 2				
DMSO	(1%)	100	−, +	25.5(±3.3)
MNNG	0.10	24.7	−	571
	0.08	40.0	−	558
Benzo[*a*]pyrene	3.0	61.7	+	332
	3.0	58.8	+	286
ACN	184	10.4	−	49
	184	10.9	−	65
	136	23.2	−	85
	136	16.3	−	81
	100	51.4	−	44
	100	44.4	−	70
	184	8.9	+	133
	184	10.4	+	98
	136	28.1	+	85
	136	24.2	+	137
	100	45.4	+	65
	100	44.0	+	109
Expt. 3				
DMSO	(1%)	100	−, +	31.0(±2.7)
MNNG	0.10	17.0	−	798
	0.08	38.6	−	408
Benzo[*a*]pyrene	3.0	39.9	+	330
	3.0	37.7	+	229
ACN	200	9.4	−	131
	200	13.9	−	86
	147	25.6	−	85
	147	26.0	−	84
	109	43.5	−	72
	109	42.6	−	72
	80	39.9	−	90
	80	40.4	−	35

TABLE 5 (continued)

Compound	Concentration (μg/ml)	Cytotoxicity [a]	S9 [b]	Mutation frequency [c]
	200	10.3	+	102
	200	10.8	+	59
	147	30.5	+	124
	147	27.4	+	58
	109	39.0	+	69
	109	44.4	+	83
	80	60.1	+	93
	80	49.8	+	74

For explanation of superscripts see table 2.
Number of controls, 10.

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Assays for the induction of gene mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells in culture

Brian Myhr, Linda Bowers and William J. Caspary [1]

Department of Molecular Toxicology, Litton Bionetics, Inc., Kensington, MD (U.S.A.) and [1] Cellular and Genetic Toxicology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, NC (U.S.A.)

Summary

8 organic carcinogens and 2 established noncarcinogens, benzoin and caprolactam, were tested for mutagenic activity at the thymidine kinase locus in L5178Y mouse lymphoma cells. The following 6 carcinogens were evaluated as mutagenic: acrylonitrile, diethylstilbestrol, hexamethylphosphoramide, phenobarbital, safrole, and *o*-toluidine. The noncarcinogen, benzoin, was also found to be mutagenic. Mutagenic activity was not obtained for the two carcinogens, benzene and diethylhexylphthalate, as well as the noncarcinogen, caprolactam.

Mammalian cell mutagenesis assays are commonly used to confirm or extend mutagenicity data obtained with microbial systems. In particular, the in vitro system using L5178Y mouse lymphoma cells heterozygous at the thymidine kinase (TK) locus has been used extensively in many testing laboratories. The 10 chemicals in the IPCS collaborative study were provided by John Ashby and were sent to the NTP Chemical Respository. These chemicals were coded prior to being sent to Litton Bionetics, Inc., for testing in the mouse lymphoma assay. The test system was similar to that described by Clive et al. (1979). All chemicals were tested without metabolic activation, and if a clear response was not obtained, the chemical was retested in the presence of Aroclor 1254-induced rat-liver homogenate (S9). The code was broken after the evaluation of the chemical mutagenic activity was completed.

Materials and methods

Cells and culture

L5178Y mouse lymphoma cells, Clone 3.7.2C, were obtained from D. Clive, Burroughs Wellcome Co., and were free of mycoplasma as determined by culturing techniques. Laboratory cultures were grown as suspension cultures in Fischer's medium at 37 °C and routinely had doubling times near 10 h. The medium was supplemented with 2 mM L-glutamine, 110 μg/ml sodium pyruvate, 0.05% pluronic F68, antibiotics, and heat-inactivated horse serum (10% by volume). To reduce the spontaneous level of mutants, subcultures intended for mutation experiments were exposed once to medium containing THMG (3 μg/ml thymidine, 5 μg/ml hypoxanthine, 0.1 μg/ml methotrexate, and 7.5 μg/ml glycine) for 1 day, to THG medium for 1 day, and to normal medium for 3–5 days. For cloning the cells and selecting mutants, the horse serum content of the medium was increased to 20% and 0.35% Noble agar was added. The mutant selection agent was 3 μg/ml trifluorothymidine (TFT).

S9 activation conditions

S9 fractions were prepared from the livers of both noninduced and induced Fischer 344 male rats weighing approximately 200 g. The induced rats were injected i.p. with Aroclor 1254 in corn oil (500 mg/kg body weight) at 5 days prior to kill. The livers were homogenized in cold 0.15 M KCl

(3 ml/g liver weight), and the homogenate was centrifuged at 9000 *g* for 20 min at 4°C. The supernatant (S9) was stored at −80°C.

An S9 activation mix was prepared just prior to use in an activation experiment and held on ice. After addition to the cell cultures, the concentrations of the components were 1 mM NADP, 5 mM isocitrate and 50–60 μl/ml of S9.

Test chemicals

All 10 chemicals were received as coded substances and were stored at 4°C. Just prior to each experiment, an aliquot was placed into an appropriate solvent (deionized water, absolute ethanol, acetone or dimethyl sulfoxide), and serial dilutions were performed with the solvent. The treatments were initiated by making dilutions of these solutions into the cell suspensions (1 : 10 for aqueous solutions and 1 : 100 for organic solvents).

Controls

Replicate positive controls were included with each experiment. Under nonactivation conditions, the treatments were 250 nl/ml ethyl methanesulfonate (EMS) or 5–10 nl/ml methyl methanesulfonate (MMS). 3-Methylcholanthrene (MCA) requires activation by S9 to become mutagenic to L5178Y cells, and activation experiments included treatments with 2.5 μg/ml (induced S9) and 3.5 μg/ml) (non-induced S9). Solvent (negative) controls were performed in quadruplicate and contained 1% organic solvent or 10% water. For activation experiments, the solvent controls also contained the S9 mix.

Mutation experiments

Each culture consisted of 6×10^6 cells suspended in 10 ml of medium (with 5% horse serum) in a 50-ml tube. The volume included the S9 mix for activation experiments. Replicate cultures (usually 3) were used for each treatment. The tubes were closed and placed on a roller drum for 4 h at 37°C for the treatment period. After removal of the chemical, the cells were resuspended in 20 ml medium and returned to the roller drum for a 2-day expression and growth period. The cell densities were determined each day, and if the density exceeded 4×10^5 cells/ml after the first day, the culture was diluted to 3×10^5 cells/ml (20 ml total volume).

After the expression period, appropriate cultures were seeded into soft agar medium. A sample of 3×10^6 cells from each culture was distributed into three 100-mm dishes such that each dish contained approximately 1×10^6 cells in 33 ml of cloning medium containing 3 μg/ml TFT. The cloning efficiency was determined by seeding 600 cells among another three 100-mm dishes without using TFT. The dishes were incubated at 37°C with 5% CO_2/humidified air for 11–12 days. The colonies were counted by an Artek 880 electronic counter set to detect both small and large colonies.

The mutant frequency (MF) was calculated by dividing the total number of colonies in the set of

TABLE 1

MINIMUM CRITERIA FOR EXPERIMENT ACCEPTABILITY

Solvent Control Cultures

1. The cloning efficiency must be in the 50–115% range.
2. At least two acceptable cultures must be available.
3. The *average* mutant frequency for all acceptable cultures must be between 15×10^{-6} and 110×10^{-6} for negative evaluations. For clearly mutagenic test chemicals, the range is extended to 10×10^{-6} to 150×10^{-6}.
4. A Chi-square test for consistency among the mutant frequencies of the acceptable cultures must be significant at $p \leq 0.05$

Positive Control Cultures

1. The cloning efficiency must be in the 10–115% range.
2. The relative total growth must not be less than 1%.
3. At least one acceptable culture must be available.
4. The *average* mutant frequency for all acceptable cultures must be within the historical range.

Test Chemical Cultures

1. The cloning efficiency must be in the 10–115% range.
2. The relative total growth must not be less than 1%.
3. The minimum cell density at the end of the expression period is 3×10^5 cells/ml.
4. The test chemical must remain soluble during treatment.
5. The maximum dose is 5 mg/ml for solids and 5 μl/ml for liquids.
6. Each dose level must have two or more acceptable cultures.
7. A Chi-square test for consistency among the mutant frequencies of the acceptable cultures must be significant at $p \leq 0.05$.
8. At least three acceptable dose sets must be available, except when no response is obtained and sets are rejected due to precipitation.

3 mutant selection dishes by the total count in the set of 3 nonselective dishes and multiplying this ratio by 2×10^{-4}. The measurement of the toxicity of each treatment was a parameter called the relative total growth (RTG). The RTG for a treated culture was calculated as a growth ratio for the expression period (the increase in cell number divided by the average increase in cell number for the solvent control cultures) multiplied by a cloning efficiency ratio (cloning efficiency divided by the average cloning efficiency of the solvent controls).

Evaluation of data

Each experiment was evaluated for compliance with the quality control criteria (Table 1) before it was accepted for evaluation of the response. 5 response categories were defined for the evaluation of each acceptable experiment (Table 2). These categories were objective and based on a statistical model developed by NIEHS for the mouse lymphoma assay. Evaluations of individual experiments are occasionally overruled by consensus interpretation of the data, but judgment was introduced primarily at the test level, where the results of two or more experiments were considered for an evaluation of the mutagenic activity. The 3 response categories for the nonactivation and activation test conditions are defined in Table 3. Finally, the evaluation of a chemical in the mouse lymphoma assay was considered to depend on the test condition yielding the greatest response. The chemical was decoded when this evaluation process was considered completed.

TABLE 2

RESPONSE CATEGORIES FOR EXPERIMENTS

The mean response for each dose set is evaluated statistically for significance ($p \leq 0.05$). However, if a significant increase in mutant frequency is not supported by an increase in the mutant count for a treatment causing relative total growth between 1% and 5% and/or a cloning efficiency between 10% and 20%, the statistical call is overruled.

1. *Positive* (+)
   Significant response for at least 1 of the 3 highest dose sets and a significant trend ($p \leq 0.05$).
2. *Questionable* (?)
   I. Significant response for 1 of the 3 highest dose sets but no significant trend, or
   II. Significant trend but no significant dose set.
3. *Inconclusive* (i)
   I. Significant response for a dose set other than 1 of the 3 highest but no significant trend, or
   II. No significant responses or trend, but the relative total growth is greater than 30% and higher toxicity can be attained (see 5).
4. *No response* (=)
   No significant responses or trend, and the relative total growth is greater than 30% under conditions where a 1.5-fold increase in dose causes precipitation or where the 5 mg/ml (or 5 µl/ml) concentration limit is attained.
5. *Negative* (−)
   No significant responses or trend, and either the relative total growth is less than 30% or excessive toxicity occurs for a 1.5-fold higher dose.

TABLE 3

RESPONSE CATEGORIES FOR TEST CONDITIONS

A chemical is evaluated for a test condition (−S9, +S9, +NS9) only if two or more acceptable experiments are available.

1. *Positive* (+)
   I. Replicate experiments are positive
   II. Questionable experiments are reproducible.
2. *Questionable* (?)
   I. Replicate experiments yield results that just meet or just fail the tests for significance.
   II. Replicate experiments are evaluated as positive and not positive (= or −) and no reason exists to subordinate either evaluation.
3. *Negative* (−)
   Replicate experiments are not positive (= or −).

Results and discussion

Acrylonitrile (ACN)

Of the 10 chemicals studied, ACN induced the largest mutagenic response in the mouse lymphoma assay (as high as 13 times the solvent control mutant frequency). S9 activation was not required and was not used. Two nonactivation experiments (Table 6, 1A-1B) clearly showed mutagenic activity, although the response in the second trial was about one-half that in the first trial. ACN was evaluated as mutagenic at concentrations of 30 nl/ml or less; only ZOIN was mutagenic at a lower weight concentration.

Benzene (BEN)

Benzene was considered to be nonmutagenic. 6 trials with or without S9 activation (Table 6, 3A-3F) gave no hint of a mutagenic response even though high toxicity was achieved. The addition of S9 mix appeared to have no influence on the toxicity caused by BEN. BEN was evaluated with both induced and noninduced S9 because earlier, unacceptable experiments (not shown) suggested weak responses with noninduced S9. Significant responses were not obtained for the two acceptable experiments shown. The two induced S9 experiments were formally evaluated as inconclusive because high toxicity was not obtained within a 1.5-fold increase in dose. However, BEN was lethal at 1500 nl/ml in unreported experiments, and a negative test evaluation was supported by the lack of any significant increases in mutant frequency. A precipitate occurred at dosing at 500 nl/ml but appeared to redissolve for concentrations up to 1500 nl/ml. The solubility limit may actually have been exceeded for the highest assayed doses. Since BEN is known to be clastogenic in other mammalian cells, the possibility of obtaining a response at the TK locus in L5178Y cells may depend on the use of longer treatment times and/or different S9 activation conditions.

Benzoin (ZOIN)

Without S9 activation, ZOIN (Table 6, 2A–2D) was evaluated as not mutagenic, although treatment with 125 μg/ml in the first trial induced a significant, 1.6-fold increase in mutant frequency. This response occurred primarily in only 1 of the 3 treated cultures. Small increases in mutant frequency that did not quite achieve statistical significance also occurred randomly in the second trial within the 25–200 μg/ml concentration range. It is possible that careful, repetitive experiments could resolve some mutagenic activity without the addition of S9. However, S9 activation markedly increased ZOIN toxicity, allowing only one-tenth the concentrations used without S9, and resulted in consistent, positive responses in two experiments. The magnitude of the mutagenic response was less than that obtained for ACN but greater than for TOL or PB. ZOIN was mutagenic at the lowest concentration (5–10 μg/ml) for the set of 10 chemicals. Noninduced S9 was not tested and may not be as effective in producing mutagenic intermediates (see other investigator reports). It is possible that benzoyl groups are formed in vitro, since benzophenone is mutagenic with S9 in the L5178Y system (Lebowitz et al., 1982).

Caprolactam (CAP)

CAP was not toxic, or only weakly so, for concentrations up to the testing limit of 5000 μg/ml. 5 experiments performed with or without S9 activation gave no responses that could be interpreted as evidence for mutagenesis (Table 6, 4A–4E). The largest observed increase in mutant

TABLE 4

EVALUATION SUMMARY

Chemical	Acronym	Evaluation			
		Assay	−S9	+S9	+NS9
Acrylonitrile	ACN	+	+[+,+]		
Benzene	BEN	−	−[−,−]	−[i,i]	−[−,−]
Benzoin	ZOIN	+	−[?,−]	+[+,+]	
Caprolactam	CAP	−	−[=,=]	−[i,=]	[=]
Diethylhexylphthalate	DEHP	−	−[−,−]	−[−,=]	[=]
Diethylstilbestrol	DES	+	+[+,+]		
Hexamethylphosphoramide	HMPA	+	−[=,=]	+[+,+]	
Phenobarbital	PB	+	+[i,+,+]	[−]	
Safrole	SAF	+	−[−,−]	+[+,−,−,+]	[+]
o-Toluidine	TOL	+	+[+,+]		

The symbols for the evaluations are defined in Tables 1 and 2. Individual experimental evaluations are given in the brackets under each test condition. +NS9, noninduced rat-liver S9.

TABLE 5

SUMMARY OF MUTAGENIC ACTIVITIES

Chemical	Test	LMC or [HTC]	RTG at LMC or [HTC]	Maximum observed MF (*n*-fold) [a]	Evaluation
ACN	−S9	12.5–30 nl/ml	44–57%	5.7–12.7	+
BEN	−S9	[1000 nl/ml]	[14-25%]	1.1–1.3	
	+S9	[1000 nl/ml]	[35%]	1.5	−
	+NS9	[1000 nl/ml]	[34–47%]	1.0–1.4	
ZOIN	+S9	5–10 μg/ml	31–40%	3.7–3.8	+
CAP	−S9	[5000 μg/ml]	[79–95%]	1.3	
	+S9	[5000 μg/ml]	[92%]	1.7	−
	+NS9 [b]	[5000 μg/ml]	[96%]	1.3	
DEHP [c]	−S9	[5000 nl/ml]	[13–16%]	1.2–1.9	
	+S9	[5000 nl/ml]	[18%]	2.0 [d]	−
	+NS9 [b]	[3000 nl/ml]	[31%]	1.4	
DES	−S9	25 μg/ml	11–57%	2.4–2.7	+
HMPA	+S9	250–2000 nl/ml	66–75%	1.8–6.8	+
PB	−S9	1000–1200 μg/ml	34–47%	2.3–3.3	+
SAF	−S9	[80 nl/ml]	[47%]	1.3	
	+S9	20 nl/ml	31–53%	1.9–3.5	+
	+NS9 [b]	80 nl/ml	61%	2.0	
TOL	−S9	125–300 nl/ml	19–26%	2.0–3.0	+

LMC, lowest mutagenic concentration.
RTG, relative total growth.
NS9, non-induced S9.
HTC, highest tested concentration.
MF, mutant frequency.
[a] Highest average mutant frequency (times spontaneous) observed for any treatment set.
[b] 1 Expt. was performed.
[c] Precipitation occurred at all tested doses.
[d] Occurred only at low dose of 125 nl/ml and high dose of 5000 nl/ml.

frequency, 1.7-fold for the 3000 μg/ml treatment in Expt. 4C, was not statistically significant, and the low mutant colony counts obtained at the higher, weakly toxic doses did not provide evidence for mutagenesis. Thus, CAP fell into the insufficient toxicity subcategory of negative evaluations in the mouse lymphoma assay.

Diethylhexylphthalate (DEHP)

DEHP was evaluated as negative in the mouse lymphoma assay with or without the S9 activation system (Table 6, 5A–5E), although significant increases in mutant frequency appeared to occur. All of the tested doses were insoluble because the dispersion characteristics of DEHP were not realized until after the mutation studies. DEHP initially dispersed in the medium and appeared to dissolve at concentrations less than approximately 2000 nl/ml. However, oil droplets soon formed which were not easily observable in suspension cultures in translucent tubes. After 4 h incubation at 37 °C, a suspension of droplets was clearly observable at 100 nl/ml in the collected medium, and microscopic examinations indicated that the solubility limit was approximately 25 nl/ml. Since the lowest tested dose was 125 nl/ml, it is not surprising that the mutant frequencies appeared to fluctuate randomly as a function of dose. A 1.9-fold increase in mutant frequency was observed for the 1000 nl/ml treatment without S9 in one trial (5B) but not in the other trial or at higher doses. 2-fold increases in mutant frequency occurred for the low

TABLE 6

RESULTS FROM INDIVIDUAL MUTATION EXPERIMENTS WITH L5178Y CELLS

1A. ACRYLONITRILE (ACN)

NONACTIVATION TRIAL 1	TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)	58.0	85.2	100.0	22.7
SOLVENT CONTROL (E)	91.0	69.2	100.0	43.8
SOLVENT CONTROL (E)	55.0	72.2	100.0	25.4
SOLVENT CONTROL (E)	88.0	76.7	100.0	38.3
EMS .25 UL/ML	682.0	54.0	49.7	421.0
EMS .25 UL/ML	686.0	49.3	41.9	463.5
EMS .25 UL/ML	684.0	49.3	50.3	462.2
ACN		RELATIVE TO SOLV CONTROL (%)		
3.13 NL/ML	53.0	81.8	68.5	28.5
3.13 NL/ML	78.0	104.2	52.5	32.9
3.13 NL/ML	64.0	88.8	52.7	31.7
6.25 NL/ML	65.0	69.0	68.0	41.4
6.25 NL/ML	61.0	76.5	70.3	35.0
6.25 NL/ML	74.0	92.8	62.4	35.1
P 12.5 NL/ML	C	67.3	53.0	C
12.5 NL/ML	186.0	93.4	47.1	87.5
12.5 NL/ML	138.0	113.5	71.2	53.5
P 25.0 NL/ML	395.0	52.1	11.8	333.3
25.0 NL/ML	337.0	95.0	30.5	156.0
25.0 NL/ML	346.0	103.8	31.0	146.6
P 50.0 NL/ML	588.0	60.5	2.8	427.6
50.0 NL/ML	500.0	65.5	3.1	335.6
50.0 NL/ML	491.0	45.1	3.2	479.0

C CONTAMINATED PLATES
E ETHANOL (1%)
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT p<0.05
TREND: SIGNIFICANT AT p<0.05

2A. BENZOIN (ZOIN)

NONACTIVATION TRIAL 1	TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (D)	59.0	73.3	100.0	26.8
SOLVENT CONTROL (D)	69.0	83.5	100.0	27.5
SOLVENT CONTROL (D)	47.0	65.7	100.0	23.8
SOLVENT CONTROL (D)	76.0	75.7	100.0	33.5
EMS .25 UL/ML	695.0	41.5	30.0	558.2
EMS .25 UL/ML	727.0	37.0	30.0	654.9
EMS .25 UL/ML	704.0	42.5	29.4	552.1
ZOIN		RELATIVE TO SOLV CONTROL (%)		
15.6 UG/ML	64.0	80.0	69.8	35.7
15.6 UG/ML	74.0	98.8	62.3	33.5
15.6 UG/ML	64.0	91.2	68.5	31.4
31.3 UG/ML	92.0	94.5	46.7	43.5
31.3 UG/ML	55.0	87.4	67.4	28.1
31.3 UG/ML	74.0	110.1	76.7	30.0
62.5 UG/ML	50.0	62.8	37.1	35.6
62.5 UG/ML	51.0	75.7	43.1	30.1
62.5 UG/ML	57.0	58.1	35.1	43.8
P125.0 UG/ML	81.0	93.2	16.4	38.8
125.0 UG/ML	89.0	92.1	14.0	43.2
125.0 UG/ML	125.0	101.4	15.8	55.1
250.0 UG/ML	TOO TOXIC TO CLONE			
250.0 UG/ML	TOO TOXIC TO CLONE			
250.0 UG/ML	TOO TOXIC TO CLONE			

D DIMETHYLSULFOXIDE (1%)
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT p<0.05
TREND: SIGNIFICANT AT p<0.05

2C. BENZOIN (ZOIN)

INDUCED ACTIVATION TRIAL 1	TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (D)	86.0	86.0	100.0	33.3
SOLVENT CONTROL (D)	69.0	C	C	C
SOLVENT CONTROL (D)	74.0	97.8	100.0	25.2
SOLVENT CONTROL (D)	69.0	81.3	100.0	28.3
MCA 2.5 UG/ML	539.0	89.5	54.6	200.7
MCA 2.5 UG/ML	C	75.7	68.6	C
MCA 2.5 UG/ML	515.0	76.0	57.7	225.9
ZOIN		RELATIVE TO SOLV CONTROL (%)		
1.25 UG/ML	67.0	98.8	110.1	25.6
1.25 UG/ML	59.0	99.0	76.3	22.5
1.25 UG/ML	C	93.5	103.8	C
2.5 UG/ML	88.0	120.5	108.5	27.5
2.5 UG/ML	71.0	88.8	94.9	30.1
2.5 UG/ML	C	93.5	77.2	C
5.0 UG/ML	116.0	100.9	53.7	43.4
5.0 UG/ML	105.0	109.9	57.1	36.0
5.0 UG/ML	120.0	104.4	61.2	45.5
P 10.0 UG/ML	192.0	103.1	26.7	70.2
10.0 UG/ML	120.0	85.8	33.5	52.7
10.0 UG/ML	C	87.9	32.1	C
P 15.0 UG/ML	177.0	82.2	16.8	81.2
15.0 UG/ML	163.0	73.2	21.9	84.0
15.0 UG/ML	C	76.7	11.3	C
P 20.0 UG/ML	228.0	87.3	8.0	98.5
20.0 UG/ML	229.0	95.0	14.2	90.9
20.0 UG/ML	276.0	81.6	7.3	127.5
30.0 UG/ML	TOO TOXIC TO CLONE			
30.0 UG/ML	TOO TOXIC TO CLONE			
30.0 UG/ML	TOO TOXIC TO CLONE			

C CONTAMINATED PLATES
D DIMETHYLSULFOXIDE (1%)
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT p<0.05
TREND: SIGNIFICANT AT p<0.05

1B. ACRYLONITRILE (ACN)

NONACTIVATION TRIAL 2	TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)	C	85.3	100.0	C
SOLVENT CONTROL (E)	90.0	68.3	100.0	43.9
SOLVENT CONTROL (E)	83.0	97.0	100.0	28.5
SOLVENT CONTROL (E)	69.0	77.8	100.0	29.5
EMS .25 UL/ML	820.0	58.8	36.9	464.6
MMS 10 NL/ML	439.0	C	C	C
MMS 10 NL/ML	447.0	26.5	6.0	562.3
ACN		RELATIVE TO SOLV CONTROL (%)		
5.0 NL/ML	45.0	83.8	65.5	21.8
5.0 NL/ML	C	76.1	67.1	C
5.0 NL/ML	35.0	54.8	50.6	25.9
10.0 NL/ML	59.0	78.0	68.6	30.7
10.0 NL/ML	52.0	102.1	70.6	20.7
10.0 NL/ML	27.0	63.1	60.1	17.4
20.0 NL/ML	95.0	78.4	55.9	49.2
20.0 NL/ML	69.0	67.0	56.1	41.8
20.0 NL/ML	142.0	100.7	50.7	57.2
P 30.0 NL/ML	155.0	94.6	45.1	66.5
30.0 NL/ML	226.0	121.0	45.3	75.8
30.0 NL/ML	164.0	90.3	40.9	73.7
P 40.0 NL/ML	226.0	82.8	29.1	110.8
40.0 NL/ML	381.0	89.5	23.4	172.8
40.0 NL/ML	316.0	C	C	C
P 50.0 NL/ML	C	72.9	9.4	C
50.0 NL/ML	407.0	106.8	22.1	154.7
50.0 NL/ML	365.0	64.6	7.4	229.5

C CONTAMINATED PLATES
E ETHANOL (1%)
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT p<0.05
TREND: SIGNIFICANT AT p<0.05

2B. BENZOIN (ZOIN)

NONACTIVATION TRIAL 2	TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (D)	39.0	65.5	100.0	19.8
SOLVENT CONTROL (D)	59.0	74.2	100.0	26.5
EMS .25 UL/ML	489.0	53.5	61.2	304.7
EMS .25 UL/ML	445.0	54.3	68.5	273.0
ZOIN		RELATIVE TO SOLV CONTROL (%)		
25.0 UG/ML	49.0	71.0	76.1	38.6
25.0 UG/ML	65.0	91.2	100.7	39.9
25.0 UG/ML	C	68.5	75.0	C
50.0 UG/ML	68.0	81.9	67.9	46.4
50.0 UG/ML	25.0	100.1	59.5	14.0
50.0 UG/ML	33.0	93.1	89.1	19.8
100.0 UG/ML	29.0	83.6	52.7	19.4
100.0 UG/ML	57.0	55.9	29.7	57.0
100.0 UG/ML	43.0	78.9	33.6	30.5
125.0 UG/ML	52.0	87.5	32.7	33.2
125.0 UG/ML	38.0	85.9	46.9	24.7
125.0 UG/ML	54.0	103.7	27.8	29.1
150.0 UG/ML	57.0	131.2	16.8	24.3
150.0 UG/ML	49.0	124.7	22.7	22.0
150.0 UG/ML	53.0	95.4	18.1	31.1
200.0 UG/ML	51.0	97.6	10.6	29.2
200.0 UG/ML	62.0	111.3	5.6	31.1
200.0 UG/ML	83.0	114.6	13.3	40.5

C CONTAMINATED PLATES
D DIMETHYLSULFOXIDE (1%)

2D. BENZOIN (ZOIN)

INDUCED ACTIVATION TRIAL 2	TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (D)	81.0	C	C	C
SOLVENT CONTROL (D)	84.0	88.0	100.0	31.8
SOLVENT CONTROL (D)	47.0	75.3	100.0	20.8
SOLVENT CONTROL (D)	80.0	99.0	100.0	26.9
MCA 2.5 UG/ML	C	80.7	79.1	C
MCA 2.5 UG/ML	505.0	76.5	66.9	220.0
ZOIN		RELATIVE TO SOLV CONTROL (%)		
1.25 UG/ML	70.0	88.7	80.9	30.1
1.25 UG/ML	56.0	91.0	89.4	23.5
1.25 UG/ML	46.0	77.8	71.5	22.5
2.5 UG/ML	62.0	88.7	84.4	26.7
2.5 UG/ML	C	96.9	75.7	C
2.5 UG/ML	61.0	110.0	86.0	21.1
P 5.0 UG/ML	102.0	73.6	42.0	52.8
5.0 UG/ML	114.0	81.8	37.4	53.1
P 10.0 UG/ML	170.0	72.5	16.3	89.5
10.0 UG/ML	158.0	51.9	15.5	101.5
10.0 UG/ML	200.0	68.3	15.4	111.7
15.0 UG/ML	205.0	69.0	8.5	112.1
15.0 UG/ML	160.0	C	C	C
15.0 UG/ML	TOO TOXIC TO CLONE			
20.0 UG/ML	TOO TOXIC TO CLONE			
20.0 UG/ML	TOO TOXIC TO CLONE			
20.0 UG/ML	TOO TOXIC TO CLONE			

C CONTAMINATED PLATES
D DIMETHYLSULFOXIDE (1%)
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT p<0.05
TREND: SIGNIFICANT AT p<0.05

3A. BENZENE (BEN)

NONACTIVATION TRIAL 1		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)		77.0	73.8	100.0	34.8
SOLVENT CONTROL (E)		83.0	80.0	100.0	34.6
SOLVENT CONTROL (E)		120.0	90.5	100.0	44.2
SOLVENT CONTROL (E)		91.0	88.8	100.0	34.1
EMS .25 UL/ML		931.0	81.7	44.1	380.0
EMS .25 UL/ML		843.0	71.2	44.9	394.8
EMS .25 UL/ML		740.0	69.7	49.3	354.1
BEN			RELATIVE TO SOLV CONTROL (%)		
62.5	NL/ML	66.0	71.8	72.2	36.8
62.5	NL/ML	81.0	84.2	73.6	38.5
62.5	NL/ML	63.0	60.6	61.7	41.6
125.0	NL/ML	88.0	86.0	66.0	40.9
125.0	NL/ML	75.0	87.2	65.2	34.4
125.0	NL/ML	64.0	96.0	65.1	26.7
250.0	NL/ML	90.0	76.6	44.0	47.0
250.0	NL/ML	88.0	88.2	62.0	39.9
250.0	NL/ML	68.0	86.8	58.3	31.3
500.0	NL/ML	C	92.8	54.8	C
500.0	NL/ML	113.0	104.4	31.9	43.3
500.0	NL/ML	C	100.6	23.9	C
1000.0	NL/ML	100.0	88.2	11.8	45.3
1000.0	NL/ML	121.0	90.0	13.7	53.8
1000.0	NL/ML	108.0	94.0	16.2	45.9

C CONTAMINATED PLATES
E ETHANOL (1%)

3B. BENZENE (BEN)

NONACTIVATION TRIAL 2		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)		111.0	C	C	C
SOLVENT CONTROL (E)		106.0	113.3	100.0	31.2
SOLVENT CONTROL (E)		144.0	90.7	100.0	52.9
SOLVENT CONTROL (E)		151.0	100.2	100.0	50.2
EMS .25 UL/ML		789.0	58.3	47.6	450.8
EMS .25 UL/ML		912.0	83.0	49.1	366.3
EMS .25 UL/ML		859.0	79.5	50.5	360.2
BEN			RELATIVE TO SOLV CONTROL (%)		
31.3	NL/ML	88.0	89.9	89.0	32.2
31.3	NL/ML	85.0	C	C	C
31.3	NL/ML	126.0	108.0	92.2	38.3
62.5	NL/ML	88.0	104.2	99.2	27.8
62.5	NL/ML	101.0	99.6	103.9	33.3
62.5	NL/ML	69.0	93.9	104.1	24.2
125.0	NL/ML	81.0	92.7	97.3	28.7
125.0	NL/ML	111.0	105.0	104.0	34.7
125.0	NL/ML	91.0	101.7	100.1	29.4
250.0	NL/ML	102.0	90.2	92.8	37.1
250.0	NL/ML	91.0	101.7	102.0	29.4
250.0	NL/ML	107.0	98.6	82.4	35.7
500.0	NL/ML	80.0	75.8	62.3	34.7
500.0	NL/ML	105.0	110.6	83.1	31.2
500.0	NL/ML	68.0	113.9R	73.7	19.6R
1000.0	NL/ML	96.0	82.8	25.8	38.1
1000.0	NL/ML	141.0	78.7	23.5	58.9
1000.0	NL/ML	TOO TOXIC TO CLONE			

C CONTAMINATED PLATES
E ETHANOL (1%)
R REJECTED DUE TO HIGH OR LOW VIABLE COLONY COUNT

3C. BENZENE (BEN)

INDUCED ACTIVATION TRIAL 1		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)		146.0	79.5	100.0	61.2
SOLVENT CONTROL (E)		153.0	89.7	100.0	56.9
SOLVENT CONTROL (E)		139.0	82.2	100.0	56.4
SOLVENT CONTROL (E)		142.0	100.5	100.0	47.1
MCA 2.5 UG/ML		639.0	92.2	74.3	231.1
MCA 2.5 UG/ML		501.0	76.7	60.4	217.8
MCA 2.5 UG/ML		C	72.7	53.1	C
BEN			RELATIVE TO SOLV CONTROL (%)		
62.5	NL/ML	128.0	81.6	117.2	59.4
62.5	NL/ML	130.0	107.6	107.4	45.8
62.5	NL/ML	131.0	87.7	122.1	56.6
125.0	NL/ML	110.0	82.6	83.8	50.4
125.0	NL/ML	145.0	93.2	116.4	58.9
125.0	NL/ML	183.0	101.3	78.8	68.4
250.0	NL/ML	106.0	75.0	114.2	53.5
250.0	NL/ML	115.0	80.9	119.9	53.9
250.0	NL/ML	127.0	74.6	84.7	64.5
500.0	NL/ML	145.0	97.0	92.1	56.6
500.0	NL/ML	101.0	92.2	73.3	41.5
500.0	NL/ML	169.0	116.1	45.9	55.1
1000.0	NL/ML	193.0	83.1	32.2	87.9
1000.0	NL/ML	179.0	84.3	38.2	80.4
1000.0	NL/ML	TOO TOXIC TO CLONE			
2000.0	NL/ML I	TOO TOXIC TO CLONE			
2000.0	NL/ML	TOO TOXIC TO CLONE			
2000.0	NL/ML	TOO TOXIC TO CLONE			

I INSOLUBLE TEST MATERIAL WAS OBSERVED AT THIS AND HIGHER DOSE LEVELS
C CONTAMINATED PLATES
E ETHANOL (1%)

3D. BENZENE (BEN)

INDUCED ACTIVATION TRIAL 2		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)		162.0	110.3	100.0	48.9
SOLVENT CONTROL (E)		148.0	117.7R	100.0	41.9R
SOLVENT CONTROL (E)		186.0	99.3	100.0	62.4
SOLVENT CONTROL (E)		141.0	99.0	100.0	47.5
MCA 2.5 UG/ML		501.0	94.8	75.3	176.1
MCA 2.5 UG/ML		457.0W	80.5	68.3	189.2
MCA 2.5 UG/ML		508.0	86.7	72.0	195.4
BEN				RELATIVE TO SOLV CONTROL (%)	
62.5	NL/ML	124.0	79.3	96.3	48.9
62.5	NL/ML	111.0	81.9	87.6	42.4
62.5	NL/ML	149.0	61.8	88.1	75.4
125.0	NL/ML	96.0	79.9	89.7	37.6
125.0	NL/ML	103.0	70.7	82.5	45.6
125.0	NL/ML	120.0	71.3	92.0	52.6
250.0	NL/ML	122.0	79.9	93.1	47.7
250.0	NL/ML	160.0	68.0	78.1	73.6
250.0	NL/ML	152.0	76.5	77.1	62.2
500.0	NL/ML	151.0	108.8	71.6	43.4
500.0	NL/ML	165.0	86.9	55.8	59.3
500.0	NL/ML	168.0	94.3	69.9	55.7
1000.0	NL/ML	TOO TOXIC TO CLONE			
1000.0	NL/ML	TOO TOXIC TO CLONE			
1000.0	NL/ML	LETHAL			

R REJECTED DUE TO HIGH OR LOW VIABLE COLONY COUNT
E ETHANOL (1%)

3E. BENZENE (BEN)

NON-INDUCED ACTIVATION TRIAL 1		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)		133.0	80.2	100.0	55.3
SOLVENT CONTROL (E)		109.0	80.8	100.0	44.9
SOLVENT CONTROL (E)		179.0	78.7	100.0	75.8
SOLVENT CONTROL (E)		91.0	76.8	100.0	39.5
MCA 3.5 UG/ML		523.0	101.7	68.7	171.5
MCA 3.5 UG/ML		608.0	99.2	70.6	204.4
MCA 3.5 UG/ML		538.0	86.5	63.1	207.3
BEN			RELATIVE TO SOLV CONTROL (%)		
125.0	NL/ML	132.0	97.8	57.2	56.9
125.0	NL/ML	111.0	111.5	77.8	42.0
125.0	NL/ML	107.0	87.2	68.5	51.7
250.0	NL/ML	89.0	76.1	61.4	49.3
250.0	NL/ML	86.0	73.7	67.0	49.1
250.0	NL/ML	92.0	69.9	58.0	55.4
500.0	NL/ML	140.0	76.3	61.1	77.3
500.0	NL/ML	78.0	73.5	64.5	44.7
500.0	NL/ML	72.0	78.4	61.5	38.7
750.0	NL/ML	135.0	80.5	44.8	70.7
750.0	NL/ML	61.0	82.4	52.8	31.2
750.0	NL/ML	135.0	85.3	24.0	66.7
1000.0	NL/ML	113.0	90.4	45.3	52.7
1000.0	NL/ML	C	104.9	38.3	C
1000.0	NL/ML	74.0	62.2	18.8	50.2
1500.0	NL/ML	130.0	118.0	28.7	46.4
1500.0	NL/ML	LETHAL			
1500.0	NL/ML	LETHAL			

C CONTAMINATED PLATES
E ETHANOL (1%)

3F. BENZENE (BEN)

NON-INDUCED ACTIVATION TRIAL 2		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)		58.0	66.8	100.0	28.9
SOLVENT CONTROL (E)		88.0	71.0	100.0	41.3
SOLVENT CONTROL (E)		84.0	64.7	100.0	43.3
SOLVENT CONTROL (E)		74.0	67.5	100.0	36.5
MCA 3.5 UG/ML		332.0	86.5	92.7	127.9
MCA 3.5 UG/ML		378.0	93.5	87.5	134.7
MCA 3.5 UG/ML		349.0	87.2	77.8	133.5
BEN			RELATIVE TO SOLV CONTROL (%)		
125.0	NL/ML	113.0	106.9	67.8	52.2
125.0	NL/ML	86.0	92.6	70.3	45.9
125.0	NL/ML	57.0	92.8	64.0	30.3
250.0	NL/ML	68.0	99.5	69.2	33.7
250.0	NL/ML	80.0	90.1	61.0	43.8
250.0	NL/ML	94.0	111.9	87.1	41.5
500.0	NL/ML	105.0	95.6	53.7	54.3
500.0	NL/ML	90.0	111.4	61.5	39.9
500.0	NL/ML	100.0	93.1	66.8	53.0
750.0	NL/ML	134.0	106.4	46.8	62.2
750.0	NL/ML	88.0	86.2	70.7	50.4
750.0	NL/ML	76.0	92.8	15.7	40.4
1000.0	NL/ML	71.0	105.7	71.7	33.2
1000.0	NL/ML	84.0	97.5	46.2	42.5
1000.0	NL/ML	100.0	115.3	22.1	42.8
1500.0	NL/ML	111.0	95.3	6.2	57.5
1500.0	NL/ML	TOO TOXIC TO CLONE			
1500.0	NL/ML	LETHAL			

E ETHANOL (1%)

4A. CAPROLACTAM (CAP)

NONACTIVATION TRIAL 1		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (W)		47.0	86.2	100.0	18.2
SOLVENT CONTROL (W)		70.0	80.0	100.0	29.2
SOLVENT CONTROL (W)		114.0	95.8	100.0	39.6
SOLVENT CONTROL (W)		100.0	C	C	C
EMS .25 UL/ML		718.0	81.8	77.5	292.5
EMS .25 UL/ML		817.0	75.5	78.7	360.7
EMS .25 UL/ML		C	90.7	71.6	C
CAP			RELATIVE TO SOLV CONTROL (%)		
500.0	UG/ML	79.0	121.6	126.5	24.8
500.0	UG/ML	92.0	106.7	119.7	32.9
500.0	UG/ML	102.0	106.5	114.6	36.5
1000.0	UG/ML	92.0	97.4	92.4	36.1
1000.0	UG/ML	70.0	76.2	93.3	35.1
1000.0	UG/ML	84.0	92.6	112.1	34.6
2000.0	UG/ML	77.0	92.0	89.2	31.9
2000.0	UG/ML	104.0	87.8	90.9	45.2
2000.0	UG/ML	124.0	124.1	94.2	38.1
3000.0	UG/ML	118.0	101.6	85.9	44.4
3000.0	UG/ML	91.0	87.6	76.9	39.6
3000.0	UG/ML	99.0	114.5	86.0	33.0
4000.0	UG/ML	104.0	122.6	102.0	32.4
4000.0	UG/ML	105.0	126.2	120.6	31.8
4000.0	UG/ML	82.0	124.9	93.3	25.1
5000.0	UG/ML	76.0	97.2	83.7	29.9
5000.0	UG/ML	112.0	116.3	91.5	36.8
5000.0	UG/ML	89.0	98.3	109.9	34.6

C CONTAMINATED PLATES
W WATER (10%)

4B. CAPROLACTAM (CAP)

NONACTIVATION TRIAL 2		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (W)		61.0	49.8	100.0	40.8
SOLVENT CONTROL (W)		79.0	77.2	100.0	34.1
SOLVENT CONTROL (W)		62.0	97.8	100.0	21.1
SOLVENT CONTROL (W)		81.0	88.3	100.0	30.6
MMS 10 NL/ML		380.0	16.5	6.8	767.7
MMS 10 NL/ML		515.0	20.7	9.0	830.6
MMS 5 NL/ML		611.0	55.7	44.0	365.9
MMS 5 NL/ML		570.0	55.8	46.8	340.3
CAP			RELATIVE TO SOLV CONTROL (%)		
500.0	UG/ML	50.0	96.8	94.8	22.0
500.0	UG/ML	82.0	87.9	136.0	39.7
500.0	UG/ML	73.0	106.6	112.8	29.1
1000.0	UG/ML	66.0	84.1	129.9	33.4
1000.0	UG/ML	42.0	129.6	117.0	13.8
1000.0	UG/ML	74.0	86.2	102.8	36.5
2000.0	UG/ML	52.0	106.9	67.1	20.7
2000.0	UG/ML	75.0	104.1	141.5	30.7
2000.0	UG/ML	62.0	74.9	81.0	35.2
3000.0	UG/ML	43.0	82.4	81.9	22.2
3000.0	UG/ML	53.0	79.8	65.0	28.3
3000.0	UG/ML	71.0	76.6	70.7	39.4
4000.0	UG/ML	109.0	113.7	92.7	40.8
4000.0	UG/ML	101.0	85.8	74.9	50.1
4000.0	UG/ML	90.0	110.3	82.7	34.7
5000.0	UG/ML	73.0	75.6	90.0	41.1
5000.0	UG/ML	82.0	89.6	71.8	38.9
5000.0	UG/ML	88.0	97.7	74.6	38.3

W WATER (10%)

4C. CAPROLACTAM (CAP)

INDUCED ACTIVATION TRIAL 1		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (W)		34.0	55.0	100.0	20.6
SOLVENT CONTROL (W)		C	72.8	100.0	C
SOLVENT CONTROL (W)		35.0	68.7	100.0	17.0
SOLVENT CONTROL (W)		29.0	72.3	100.0	13.4
MCA 2.5 UG/ML		353.0	89.0	60.8	132.2
CAP			RELATIVE TO SOLV CONTROL (%)		
500.0	UG/ML	38.0	78.6	69.2	24.0
500.0	UG/ML	34.0	93.0	89.6	18.1
500.0	UG/ML	31.0	82.3	83.0	18.7
1000.0	UG/ML	44.0	92.0	79.0	23.7
1000.0	UG/ML	39.0	93.3	109.7	20.7
1000.0	UG/ML	33.0	102.4	84.8	16.0
2000.0	UG/ML	63.0	101.2	53.6	30.9
3000.0	UG/ML	53.0	76.6	72.2	34.3
3000.0	UG/ML	48.0	108.6	76.2	21.9
4000.0	UG/ML	C	79.4	43.4	C
4000.0	UG/ML	C	125.0	75.8	C
4000.0	UG/ML	C	97.7	93.6	C
5000.0	UG/ML	20.0	78.4	75.7	12.6
5000.0	UG/ML	36.0	C	C	C
5000.0	UG/ML	34.0	C	C	C

C CONTAMINATED PLATES
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT $p<0.05$
W WATER (10%)

4D. CAPROLACTAM (CAP)

INDUCED ACTIVATION TRIAL 2		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (W)		79.0	95.2	100.0	27.7
SOLVENT CONTROL (W)		139.0	83.8	100.0	55.3
SOLVENT CONTROL (W)		154.0	106.2	100.0	48.3
SOLVENT CONTROL (W)		151.0	109.2	100.0	46.1
MCA 2.5 UG/ML		691.0	83.7	56.0	275.3
MCA 2.5 UG/ML		679.0	79.5	48.5	284.7
MCA 2.5 UG/ML		682.0	57.2	17.4	397.7
CAP			RELATIVE TO SOLV CONTROL (%)		
500.0	UG/ML	118.0	110.0	69.1	36.2
500.0	UG/ML	122.0	81.3	71.0	50.7
500.0	UG/ML	127.0	77.8	76.6	55.2
1000.0	UG/ML	111.0	67.3	61.3	55.8
1000.0	UG/ML	106.0	75.4	63.8	47.5
1000.0	UG/ML	100.0	98.9	63.4	34.2
2000.0	UG/ML	96.0	85.2	63.4	38.1
2000.0	UG/ML	151.0	80.1	57.4	63.7
2000.0	UG/ML	91.0	78.6	70.4	39.1
3000.0	UG/ML	161.0	106.8	57.4	50.9
3000.0	UG/ML	155.0	85.9	56.7	61.0
3000.0	UG/ML	151.0	102.9	74.2	49.6
4000.0	UG/ML	147.0	85.4	62.4	58.2
4000.0	UG/ML	175.0	107.2	59.1	55.2
4000.0	UG/ML	163.0	104.6	91.4	52.7
5000.0	UG/ML	148.0	116.6	94.6	42.9
5000.0	UG/ML	199.0	107.7	102.4	62.5
5000.0	UG/ML	150.0	117.3	79.5	43.2

W WATER (10%)

4E. CAPROLACTAM (CAP)

NON-INDUCED ACTIVATION TRIAL 1		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (W)		71.0	102.7	100.0	23.0
SOLVENT CONTROL (W)		93.0	114.2	100.0	27.1
SOLVENT CONTROL (W)		126.0	89.8	100.0	46.7
SOLVENT CONTROL (W)		98.0	100.7	100.0	32.4
MCA 3.5 UG/ML		365.0	86.2	59.0	141.2
MCA 3.5 UG/ML		352.0	75.7	55.8	155.1
MCA 3.5 UG/ML		487.0	118.3R	57.7	137.2R
CAP			RELATIVE TO SOLV CONTROL (%)		
500.0	UG/ML	62.0	76.2	70.7	26.6
500.0	UG/ML	49.0	100.3	102.5	16.0
500.0	UG/ML	89.0	121.9R	78.9	23.9R
1000.0	UG/ML	85.0	83.4	79.1	33.3
1000.0	UG/ML	67.0	103.7	84.6	21.1
1000.0	UG/ML	104.0	118.9R	92.6	28.6R
2000.0	UG/ML	87.0	101.7	90.4	28.0
2000.0	UG/ML	123.0	104.2	113.1	38.6
2000.0	UG/ML	96.0	96.7	85.1	32.5
3000.0	UG/ML	87.0	88.0	94.3	32.3
3000.0	UG/ML	102.0	84.6	94.7	39.4
3000.0	UG/ML	163.0	92.7	111.3	57.5
4000.0	UG/ML	85.0	77.0	84.5	36.1
4000.0	UG/ML	99.0	105.0	86.0	30.8
4000.0	UG/ML	108.0	111.4	91.3	31.7
5000.0	UG/ML	64.0	88.6	110.5	23.6
5000.0	UG/ML	94.0	95.4	81.7	32.2

R REJECTED DUE TO HIGH OR LOW VIABLE COLONY COUNT
W WATER (10%)

5A. DIETHYLHEXYLPHTHALATE (DEHP)

NONACTIVATION TRIAL 1			TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			101.0	43.5	100.0	77.4
SOLVENT CONTROL (E)			121.0	75.7	100.0	53.3
SOLVENT CONTROL (E)			88.0	53.0	100.0	55.3
SOLVENT CONTROL (E)			113.0	53.2	100.0	70.8
MMS 10 NL/ML			626.0	33.7	30.4	619.8
MMS 10 NL/ML			627.0	31.3	32.6	667.0
MMS 10 NL/ML			622.0	33.7	18.7	615.8
DEHP				RELATIVE TO SOLV CONTROL (%)		
250.0	NL/ML	I	70.0	152.2	125.0	27.2
250.0	NL/ML		114.0	156.5	94.1	43.1
250.0	NL/ML		85.0	145.1	115.8	54.6
500.0	NL/ML		98.0	176.4	54.9	52.8
500.0	NL/ML		86.0	101.4	76.2	50.1
500.0	NL/ML		94.0	138.3	95.7	40.2
1000.0	NL/ML		99.0	106.4	38.8	55.0
1000.0	NL/ML		87.0	117.6	58.3	43.7
1000.0	NL/ML		C	125.9	31.5	C
2000.0	NL/ML		C	92.2	19.1	C
2000.0	NL/ML		C	130.3	26.6	C
2000.0	NL/ML		82.0	114.9	40.9	42.1
3000.0	NL/ML		C	91.6	14.4	C
3000.0	NL/ML		103.0	100.5	29.2	60.6
3000.0	NL/ML		137.0	99.3	21.1	81.5
5000.0	NL/ML		114.0	73.6	17.8	91.6
5000.0	NL/ML		103.0	89.0	20.4	68.4
5000.0	NL/ML		100.0	74.2	9.3	79.7

C CONTAMINATED PLATES
E ETHANOL (1%)
I INSOLUBLE TEST MATERIAL WAS OBSERVED AT THIS AND HIGHER DOSE LEVELS

5B. DIETHYLHEXYLPHTHALATE (DEHP)

NONACTIVATION TRIAL 2			TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			96.0	106.8	100.0	29.9
SOLVENT CONTROL (E)			78.0	91.0	100.0	28.6
SOLVENT CONTROL (E)			96.0	106.3	100.0	30.1
MMS 10 NL/ML			664.0	51.7	24.2	428.4
MMS 10 NL/ML			575.0	43.0	22.2	444.2
MMS 5 NL/ML			519.0	69.8	51.9	247.7
MMS 5 NL/ML			515.0	C	C	C
DEHP				RELATIVE TO SOLV CONTROL (%)		
125.0	NL/ML	I	99.0	67.9	26.5	46.0
125.0	NL/ML		C	78.2	28.3	C
125.0	NL/ML		100.0	62.9	20.8	50.2
250.0	NL/ML		91.0	59.6	14.8	48.3
250.0	NL/ML		84.0	58.1	15.0	45.6
250.0	NL/ML		79.0	64.1	17.2	38.9
500.0	NL/ML		90.0	57.2	23.3	49.7
500.0	NL/ML		105.0	74.7	25.6	44.4
500.0	NL/ML		76.0	62.7	18.1	38.3
P 1000.0	NL/ML		122.0	69.4	16.2	55.6
1000.0	NL/ML		111.0	74.2	15.2	47.2
1000.0	NL/ML		109.0	56.4	5.4	61.1
5000.0	NL/ML		88.0	58.1	13.0	47.8
5000.0	NL/ML		74.0	70.9	12.7	35.0
5000.0	NL/ML		57.0	44.1	10.5	40.9
5000.0	NL/ML		71.0	56.4	11.4	39.8
5000.0	NL/ML		57.0	51.2	18.4	35.2
5000.0	NL/ML		77.0	59.6	9.8	40.8

C CONTAMINATED PLATES
E ETHANOL (1%)
I INSOLUBLE TEST MATERIAL WAS OBSERVED AT THIS AND HIGHER DOSE LEVELS
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT p<0.05

5C. DIETHYLHEXYLPHTHALATE (DEHP)

INDUCED ACTIVATION TRIAL 1			TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			79.0	70.5	100.0	57.4
SOLVENT CONTROL (E)			114.0	108.0	100.0	55.2
SOLVENT CONTROL (E)			86.0	88.8	100.0	52.3
SOLVENT CONTROL (E)			124.0	95.2	100.0	43.4
MCA 2.5 UG/ML			653.0	97.5	65.7	225.2
MCA 2.5 UG/ML			429.0	86.5	71.1	165.3
MCA 2.5 UG/ML			C	99.2	60.1	C
DEHP				RELATIVE TO SOLV CONTROL (%)		
P 125.0	NL/ML	I	106.0	55.5	20.1	109.8
125.0	NL/ML		134.0	65.8	37.5	77.2
125.0	NL/ML		91.0	94.2	42.8	55.5
250.0	NL/ML		88.0	67.5	61.8	47.9
250.0	NL/ML		101.0	55.9	56.1	66.4
250.0	NL/ML		85.0	59.6	55.7	52.5
500.0	NL/ML		130.0	96.9	48.2	49.3
500.0	NL/ML		89.0	64.4	39.5	50.8
500.0	NL/ML		112.0	85.5	41.6	48.2
1000.0	NL/ML		90.0	71.7	36.4	46.1
1000.0	NL/ML		104.0	77.6	27.4	49.5
1000.0	NL/ML		120.0	78.2	47.2	56.5
3000.0	NL/ML		73.0	58.1	34.5	46.2
3000.0	NL/ML		75.0	60.3	32.6	45.7
3000.0	NL/ML		82.0	47.1	22.2	64.1
P 5000.0	NL/ML		94.0	56.2	15.5	95.4
5000.0	NL/ML		113.0	71.2	21.4	58.4
5000.0	NL/ML		100.0	53.9	17.4	68.2

C CONTAMINATED PLATES
E ETHANOL (1%)
I INSOLUBLE TEST MATERIAL WAS OBSERVED AT THIS AND HIGHER DOSE LEVELS
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT p<0.05

5D. DIETHYLHEXYLPHTHALATE (DEHP)

INDUCED ACTIVATION TRIAL 2			TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			137.0	86.7	100.0	52.7
SOLVENT CONTROL (E)			116.0	72.2	100.0	53.6
SOLVENT CONTROL (E)			140.0	83.7	100.0	55.8
SOLVENT CONTROL (E)			93.0	71.8	100.0	43.1
MCA 2.5 UG/ML			557.0	66.2	48.6	280.6
MCA 2.5 UG/ML			666.0	82.8	66.1	268.0
MCA 2.5 UG/ML			563.0	72.0	59.3	260.6
DEHP				RELATIVE TO SOLV CONTROL (%)		
250.0	NL/ML	I	76.0	67.6	61.0	47.6
250.0	NL/ML		112.0	81.6	54.3	58.2
250.0	NL/ML		115.0	116.0	110.5	42.0
500.0	NL/ML		125.0	94.6	79.5	56.0
500.0	NL/ML		77.0	80.8	66.3	40.4
500.0	NL/ML		89.0	88.2	59.1	42.8
750.0	NL/ML		71.0	67.4	55.8	44.6
750.0	NL/ML		C	80.2	53.7	C
750.0	NL/ML		112.0	100.7	62.6	47.1
1000.0	NL/ML		76.0	67.0	49.2	48.1
1000.0	NL/ML		87.0	93.7	61.7	39.4
1000.0	NL/ML		142.0	92.5	72.3	65.1
2000.0	NL/ML		142.0	101.4	66.4	59.4
2000.0	NL/ML		118.0	104.1	86.1	48.1
2000.0	NL/ML		128.0	122.1	88.4	44.4
3000.0	NL/ML		124.0	86.9	51.2	60.5
3000.0	NL/ML		98.0	88.2	64.0	47.1
3000.0	NL/ML		97.0	126.8	72.1	32.4

C CONTAMINATED PLATES
E ETHANOL (1%)
I INSOLUBLE TEST MATERIAL WAS OBSERVED AT THIS AND HIGHER DOSE LEVELS

5E. DIETHYLHEXYLPHTHALATE (DEHP)

NON-INDUCED ACTIVATION TRIAL 1			TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			116.0	99.0	100.0	39.0
SOLVENT CONTROL (E)			63.0	98.7	100.0	21.3
SOLVENT CONTROL (E)			152.0	120.8R	100.0	41.9R
SOLVENT CONTROL (E)			143.0	115.2	100.0	41.4
MCA 3.5 UG/ML			407.0	98.5	86.4	137.7
MCA 3.5 UG/ML			417.0	116.3R	97.2	119.5R
MCA 3.5 UG/ML			400.0	88.5	86.3	150.6
DEHP				RELATIVE TO SOLV CONTROL (%)		
500.0	NL/ML		97.0	69.8	27.0	42.7
500.0	NL/ML		90.0	66.6	36.8	41.6
500.0	NL/ML		96.0	55.4	27.6	53.3
750.0	NL/ML		94.0	86.4	42.6	33.4
750.0	NL/ML		111.0	68.0	41.5	50.2
750.0	NL/ML		103.0	77.8	33.0	40.7
1000.0	NL/ML		78.0	72.9	23.3	32.9
1000.0	NL/ML		114.0	106.2	52.0	33.0
1000.0	NL/ML		158.0	97.6	34.1	49.8
1500.0	NL/ML		137.0	101.3	39.6	41.6
1500.0	NL/ML		119.0	94.1	33.5	38.9
1500.0	NL/ML		149.0	99.9	28.8	45.8
2000.0	NL/ML		106.0	60.0	16.6	54.3
2000.0	NL/ML		85.0	99.9	37.3	26.1
2000.0	NL/ML		66.0	84.9	40.8	23.9
3000.0	NL/ML		116.0	81.5	33.2	45.8
3000.0	NL/ML		120.0	92.6	31.8	39.9
3000.0	NL/ML		77.0	65.8	26.8	36.0

R REJECTED DUE TO HIGH OR LOW VIABLE COLONY COUNTS
E ETHANOL (1%)
I INSOLUBLE TEST MATERIAL WAS OBSERVED AT THIS AND HIGHER DOSE LEVELS

6G. SAFROLE (SAF)

NON-INDUCED ACTIVATION TRIAL 1			TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			150.0	103.3	100.0	48.4
SOLVENT CONTROL (E)			172.0	97.7	100.0	58.7
SOLVENT CONTROL (E)			139.0	85.5	100.0	54.2
SOLVENT CONTROL (E)			122.0	68.7	100.0	59.2
MCA 3.5 UG/ML			580.0	88.0	78.5	219.7
MCA 3.5 UG/ML			535.0	74.3	40.3	239.9
MCA 3.5 UG/ML			534.0	89.3	75.3	199.2
SAF				RELATIVE TO SOLV CONTROL (%)		
P 20.0	NL/ML		222.0	105.3	74.1	79.1
20.0	NL/ML		302.0	118.8	71.6	95.4
20.0	NL/ML		193.0	97.6	81.1	74.2
40.0	NL/ML		197.0	114.1	92.1	64.8
40.0	NL/ML		182.0	85.0	68.0	80.3
40.0	NL/ML		138.0	83.9	72.2	61.7
60.0	NL/ML		201.0	91.0	44.8	82.9
60.0	NL/ML		185.0	94.2	73.9	73.7
60.0	NL/ML		182.0	97.2	79.0	70.3
P 80.0	NL/ML		298.0	113.5	64.9	98.5
80.0	NL/ML		252.0	100.2	58.7	94.4
80.0	NL/ML		290.0	111.3	59.1	97.8
P 100.0	NL/ML		188.0	94.8	46.1	74.4
100.0	NL/ML		269.0	89.5	40.7	112.8
100.0	NL/ML		253.0	95.9	34.3	99.0
P 120.0	NL/ML		293.0	89.9	22.7	122.3
120.0	NL/ML		260.0	95.9	34.0	101.8
120.0	NL/ML		LETHAL			

E ETHANOL (1%)
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT p<0.05
TREND: SIGNIFICANT AT p<0.05

6A. SAFROLE (SAF)

NONACTIVATION TRIAL 1			TOTAL MUTANT COLONIES	CLONING EFFICIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			C	112.2	100.0	C
SOLVENT CONTROL (E)			78.0	99.3	100.0	26.2
SOLVENT CONTROL (E)			92.0	100.8	100.0	30.4
SOLVENT CONTROL (E)			C	103.5	100.0	C
EMS .25 UL/ML			773.0	75.3	65.0	351.4
MMS 10 NL/ML			601.0	59.5	32.8	336.7
MMS 10 NL/ML			C	52.3	23.3	C
SAF				RELATIVE TO SOLV CONTROL (%)		
	10.0	NL/ML	86.0	85.6	84.3	52.2
	10.0	NL/ML	116.0	87.5	82.0	42.5
	10.0	NL/ML	54.0	88.6	85.2	19.5
	20.0	NL/ML	47.0	78.8	75.5	19.1
	20.0	NL/ML	65.0	81.6	84.6	25.5
	20.0	NL/ML	C	108.3	78.2	C
	40.0	NL/ML	84.0	79.5	58.9	33.9
	40.0	NL/ML	45.0	81.4	64.4	17.7
	40.0	NL/ML	75.0	84.9	55.6	28.3
	50.0	NL/ML	77.0	83.7	64.7	29.5
	50.0	NL/ML	89.0	88.1	66.3	32.4
	50.0	NL/ML	C	104.8	50.0	C
	60.0	NL/ML	91.0	96.0	39.9	30.4
	60.0	NL/ML	75.0	75.8	43.6	31.7
	60.0	NL/ML	C	81.7	9.2	C
	80.0	NL/ML	138.0	84.0	14.9	52.7
	80.0	NL/ML	LETHAL			
	80.0	NL/ML	LETHAL			

C CONTAMINATED PLATES
E ETHANOL (1%)

6B. SAFROLE (SAF)

NONACTIVATION TRIAL 2			TOTAL MUTANT COLONIES	CLONING EFFICIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			48.0	62.8	100.0	25.5
SOLVENT CONTROL (E)			78.0	66.8	100.0	38.9
SOLVENT CONTROL (E)			85.0	95.0	100.0	29.8
SOLVENT CONTROL (E)			58.0	65.2	100.0	29.7
EMS .25 UL/ML			474.0	89.2	96.7	158.5
EMS .25 UL/ML			516.0	64.7	95.4	162.9
EMS .25 UL/ML			387.0	52.0	62.8	248.1
SAF				RELATIVE TO SOLV CONTROL (%)		
	30.0	NL/ML	39.0	93.5	76.7	19.2
	30.0	NL/ML	61.0	109.0	68.6	25.7
	30.0	NL/ML	64.0	101.1	90.8	29.1
	40.0	NL/ML	66.0	91.0	80.0	33.3
	40.0	NL/ML	64.0	114.0	102.3	25.8
	40.0	NL/ML	65.0	116.1	81.7	25.7
	50.0	NL/ML	68.0	115.2	68.3	27.1
	50.0	NL/ML	73.0	75.4	81.7	44.5
	50.0	NL/ML	77.0	100.0	82.6	35.4
	60.0	NL/ML	105.0	145.3	59.9	33.2
	60.0	NL/ML	91.0	117.5	61.8	35.6
	60.0	NL/ML	81.0	87.8	82.4	42.4
	80.0	NL/ML	101.0	86.4	32.2	53.7
	80.0	NL/ML	67.0	103.4	61.7	29.8
	100.0	NL/ML	63.0	73.6	33.0	39.4
	100.0	NL/ML	TOO TOXIC TO CLONE			
	100.0	NL/ML	TOO TOXIC TO CLONE			

E ETHANOL (1%)

6C. SAFROLE (SAF)

INDUCED ACTIVATION TRIAL 1			TOTAL MUTANT COLONIES	CLONING EFFICIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			130.0	110.8	100.0	39.1
SOLVENT CONTROL (E)			113.0	109.2	100.0	34.5
MCA 2.5 UG/ML			639.0	92.2	46.8	231.1
MCA 2.5 UG/ML			501.0	76.7	38.1	217.8
SAF				RELATIVE TO SOLV CONTROL (%)		
P	20.0	NL/ML	164.0	77.4	31.5	64.2
	20.0	NL/ML	150.0	77.1	29.9	58.9
	20.0	NL/ML	162.0	145.3R	49.3	33.8R
P	40.0	NL/ML	208.0	87.0	25.3	72.5
	40.0	NL/ML	182.0	88.9	28.0	62.0
	40.0	NL/ML	166.0	94.4	23.4	53.3
	60.0	NL/ML	163.0	99.1	22.4	49.8
	60.0	NL/ML	166.0	97.7	27.2	51.5
P	80.0	NL/ML	240.0	99.8	24.8	72.8
	80.0	NL/ML	367.0	144.2R	20.3	77.1R
	80.0	NL/ML	207.0	96.7	19.0	64.9
P	100.0	NL/ML	230.0	103.6	15.5	67.2
	100.0	NL/ML	191.0	100.8	12.6	57.4
	100.0	NL/ML	173.0	83.2	7.9	63.0
	125.0	NL/ML	TOO TOXIC TO CLONE			
	125.0	NL/ML	TOO TOXIC TO CLONE			
	125.0	NL/ML	TOO TOXIC TO CLONE			

E ETHANOL (1%)
R REJECTED DUE TO HIGH OR LOW VIABLE COLONY COUNTS
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT $p<0.05$
TREND: SIGNIFICANT AT $p<0.05$

6D. SAFROLE (SAF)

INDUCED ACTIVATION TRIAL 2			TOTAL MUTANT COLONIES	CLONING EFFICIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			100.0	91.2	100.0	36.6
SOLVENT CONTROL (E)			111.0	79.7	100.0	46.4
SOLVENT CONTROL (E)			120.0	62.7	100.0	63.8
SOLVENT CONTROL (E)			106.0	81.8	100.0	43.2
MCA 2.5 UG/ML			480.0	89.7	77.2	178.4
MCA 2.5 UG/ML			C	110.5	112.2	C
MCA 2.5 UG/ML			394.0	88.8	93.9	147.8
SAF				RELATIVE TO SOLV CONTROL (%)		
	20.0	NL/ML	109.0	111.3	65.1	41.4
	20.0	NL/ML	105.0	87.5	57.8	50.7
	40.0	NL/ML	121.0	103.3	47.1	49.5
	40.0	NL/ML	135.0	103.1	53.4	54.5
	60.0	NL/ML	113.0	96.7	42.9	49.3
	60.0	NL/ML	136.0	97.4	32.9	59.0
	60.0	NL/ML	152.0	118.9	49.7	54.0
	80.0	NL/ML	168.0	141.3	48.5	50.2
	80.0	NL/ML	135.0	112.4	38.3	50.7
	80.0	NL/ML	131.0	87.9	32.6	63.0
	100.0	NL/ML	129.0	110.7	10.8	49.2
	100.0	NL/ML	142.0	108.6	11.7	55.2
	100.0	NL/ML	130.0	115.8	38.3	47.4
	125.0	NL/ML	125.0	92.9	10.5	56.8
	125.0	NL/ML	LETHAL			
	125.0	NL/ML	LETHAL			

C CONTAMINATED PLATES
E ETHANOL (1%)

6E. SAFROLE (SAF)

INDUCED ACTIVATION TRIAL 3			TOTAL MUTANT COLONIES	CLONING EFFICIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			143.0	74.5	100.0	64.0
SOLVENT CONTROL (E)			133.0	54.2	100.0	81.8
SOLVENT CONTROL (E)			143.0	82.2	100.0	58.0
SOLVENT CONTROL (E)			90.0	72.2	100.0	41.6
MCA 2.5 UG/ML			484.0	58.8	48.8	274.2
MCA 2.5 UG/ML			623.0	90.8	77.1	228.6
MCA 2.5 UG/ML			551.0	81.2	55.1	226.3
SAF				RELATIVE TO SOLV CONTROL (%)		
	20.0	NL/ML	56.0	102.9	77.8	25.6
	20.0	NL/ML	71.0	108.1	76.9	30.9
	20.0	NL/ML	79.0	75.3	62.9	49.4
	40.0	NL/ML	79.0	89.4	67.2	41.6
	40.0	NL/ML	73.0	84.5	74.3	40.7
	40.0	NL/ML	54.0	73.4	78.4	34.6
	60.0	NL/ML	99.0	88.0	54.2	52.9
	60.0	NL/ML	93.0	68.5	47.7	63.9
	100.0	NL/ML	50.0	65.4	37.8	36.0
	100.0	NL/ML	107.0	57.4	36.5	87.7
	100.0	NL/ML	133.0	68.5	22.9	91.4
	125.0	NL/ML	96.0	62.1	9.9	72.7
	125.0	NL/ML	150.0	64.5	28.8	109.5
	125.0	NL/ML	TOO TOXIC TO CLONE			

E ETHANOL (1%)

6F. SAFROLE (SAF)

INDUCED ACTIVATION TRIAL 4			TOTAL MUTANT COLONIES	CLONING EFFICIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			288.0	98.5	100.0	97.5
SOLVENT CONTROL (E)			324.0	126.5R	100.0	85.4R
SOLVENT CONTROL (E)			216.0	99.0	100.0	72.7
SOLVENT CONTROL (E)			197.0	84.2	100.0	78.0
MCA 2.5 UG/ML			616.0	29.2	5.9	704.0
MCA 2.5 UG/ML			769.0	49.5	23.1	517.8
SAF				RELATIVE TO SOLV CONTROL (%)		
P	20.0	NL/ML	370.0	71.7	47.1	168.6
	20.0	NL/ML	212.0	54.4	46.2	127.3
	20.0	NL/ML	310.0	81.8	64.6	123.7
P	40.0	NL/ML	457.0	98.0	20.9	257.5
	40.0	NL/ML	434.0	60.2	24.7	235.2
	40.0	NL/ML	444.0	72.6	42.3	199.5
P	60.0	NL/ML	453.0	64.3	19.3	229.9
	60.0	NL/ML	405.0	52.2	20.6	253.1
	60.0	NL/ML	462.0	61.5	24.9	245.1
P	80.0	NL/ML	620.0	68.4	20.6	295.9
	80.0	NL/ML	612.0	82.8	20.6	241.4
	80.0	NL/ML	482.0	66.6	23.0	236.3
P	100.0	NL/ML	651.0	86.4	15.3	246.1
	100.0	NL/ML	577.0	64.5	16.3	292.1
	100.0	NL/ML	513.0	75.9	20.9	220.6
P	125.0	NL/ML	383.0	58.8	14.3	212.8
	125.0	NL/ML	605.0	47.7	8.0	414.4
	125.0	NL/ML	530.0	71.2	20.3	243.1

E ETHANOL (1%)
R REJECTED DUE TO HIGH OR LOW VIABLE COLONY COUNTS
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT $p<0.05$
TREND: SIGNIFICANT AT $p<0.05$

7A. DIETHYLSTILBESTROL (DES)

NONACTIVATION TRIAL 1			TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			77.0	39.5R	100.0	65.0R
SOLVENT CONTROL (E)			65.0	56.7	100.0	38.2
SOLVENT CONTROL (E)			53.0	62.0	100.0	28.5
SOLVENT CONTROL (E)			67.0	45.0R	100.0	49.6R
EMS .25 UL/ML			260.0	9.2	2.5	945.4
EMS .25 UL/ML			261.0	8.7	3.0	1003.8
EMS .25 UL/ML			222.0	7.0	1.9	1057.1
DES				RELATIVE TO SOLV CONTROL (%)		
12.5	UG/ML		64.0	107.6	52.5	39.0
12.5	UG/ML		112.0	121.4	82.2	60.5
12.5	UG/ML		77.0	93.5	79.2	54.0
P 25.0	UG/ML		87.0	92.2	61.7	61.9
25.0	UG/ML		84.0	81.0	51.5	68.0
P 50.0	UG/ML		116.0	102.0	7.5	74.6
50.0	UG/ML		190.0	146.0	7.9	85.4
50.0	UG/ML		199.0	112.9	11.6	115.7
P 100.0	UG/ML		66.0	87.6	22.3	49.4
100.0	UG/ML		71.0	119.4	33.8	39.0
100.0	UG/ML		113.0	78.4	8.1	94.6
P 200.0	UG/ML	I	125.0	106.6	20.7	76.9
200.0	UG/ML		159.0	118.8	10.9	87.8
200.0	UG/ML		173.0	116.1	7.3	97.7
300.0	UG/ML		TOO TOXIC TO CLONE			
300.0	UG/ML		TOO TOXIC TO CLONE			
300.0	UG/ML		TOO TOXIC TO CLONE			

E ETHANOL (1%)
R REJECTED DUE TO HIGH OR LOW VIABLE COLONY COUNTS
I INSOLUBLE TEST MATERIAL WAS OBSERVED AT THIS AND HIGHER DOSE LEVELS
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT $p \leq 0.05$
TREND: SIGNIFICANT AT $p \leq 0.05$

7B. DIETHYLSTILBESTROL (DES)

NONACTIVATION TRIAL 2			TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)			80.0	83.3	100.0	32.0
SOLVENT CONTROL (E)			83.0	83.3	100.0	33.2
SOLVENT CONTROL (E)			87.0	120.0R	100.0	24.2R
MMS 5 NL/ML			374.0	62.0	43.8	201.1
MMS 5 NL/ML			529.0	70.5	37.2	250.1
MMS 5 NL/ML			379.0	55.5	40.4	227.6
DES				RELATIVE TO SOLV CONTROL (%)		
12.5	UG/ML		310.0	230.7R	88.1	46.9R
12.5	UG/ML		123.0	97.6	52.9	44.0
12.5	UG/ML		125.0	78.4	32.1	55.7
P 25.0	UG/ML		245.0	82.2	9.6	104.0
25.0	UG/ML		141.0	90.1	12.3	54.6
P 50.0	UG/ML		178.0	84.3	13.5	73.7
50.0	UG/ML		167.0	100.3	20.3	58.1
50.0	UG/ML		217.0	100.5	6.3	75.3
P 75.0	UG/ML		165.0	98.3	13.8	58.6
75.0	UG/ML		149.0	96.0	11.4	54.2
75.0	UG/ML		TOO TOXIC TO CLONE			
P 100.0	UG/ML		152.0	94.4	11.4	56.2
100.0	UG/ML		185.0	81.3	4.4	79.4
100.0	UG/ML		TOO TOXIC TO CLONE			
150.0	UG/ML	I	TOO TOXIC TO CLONE			
150.0	UG/ML		TOO TOXIC TO CLONE			
150.0	UG/ML		TOO TOXIC TO CLONE			

R REJECTED DUE TO HIGH OR LOW VIABLE COLONY COUNTS
I INSOLUBLE TEST MATERIAL WAS OBSERVED AT THIS AND HIGHER DOSE LEVELS
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT $p \leq 0.05$
TREND: SIGNIFICANT AT $p \leq 0.05$

8A. HEXAMETHYLPHOSPHORAMIDE (HMPA)

NONACTIVATION TRIAL 1		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (W)		90.0	81.3	100.0	36.9
SOLVENT CONTROL (W)		68.0	107.3	100.0	21.1
SOLVENT CONTROL (W)		48.0	86.0	100.0	18.6
SOLVENT CONTROL (W)		72.0	96.7	100.0	24.8
EMS .25 UL/ML		747.0	68.7	37.7	362.6
EMS .25 UL/ML		724.0	66.2	46.4	364.7
EMS .25 UL/ML		1019.0	79.7	38.2	426.3
HMPA			RELATIVE TO SOLV CONTROL (%)		
125.0	NL/ML	54.0	87.1	100.9	22.5
125.0	NL/ML	64.0	122.0	93.2	18.8
125.0	NL/ML	45.0	125.9R	110.0	12.8R
250.0	NL/ML	58.0	105.6	92.4	19.7
250.0	NL/ML	38.0	76.0	88.5	18.0
250.0	NL/ML	71.0	102.6	147.5	24.9
500.0	NL/ML	46.0	96.1	81.8	17.2
500.0	NL/ML	61.0	84.8	101.9	25.8
500.0	NL/ML	68.0	99.0	86.5	24.7
1000.0	NL/ML	87.0	100.6	87.6	31.1
1000.0	NL/ML	54.0	80.6	75.3	24.0
1000.0	NL/ML	55.0	112.1	104.6	17.6
3000.0	NL/ML	88.0	87.8	100.7	36.0
3000.0	NL/ML	71.0	102.4	88.8	24.9
3000.0	NL/ML	75.0	85.5	95.9	31.5
5000.0	NL/ML	74.0	102.9	71.4	25.8
5000.0	NL/ML	59.0	89.3	89.8	23.7
5000.0	NL/ML	41.0	87.3	72.0	16.9

R REJECTED DUE TO HIGH OR LOW VIABLE COLONY COUNTS
W WATER (10%)

8B. HEXAMETHYLPHOSPHORAMIDE (HMPA)

NONACTIVATION TRIAL 2		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (W)		103.0	105.0	100.0	32.7
SOLVENT CONTROL (W)		129.0	83.7	100.0	51.4
SOLVENT CONTROL (W)		72.0	74.0	100.0	32.4
SOLVENT CONTROL (W)		82.0	85.3	100.0	32.0
MMS 10 NL/ML		512.0	41.0	20.0	416.3
MMS 10 NL/ML		539.0	50.2	20.7	358.1
MMS 10 NL/ML		529.0	44.5	22.2	396.2
HMPA			RELATIVE TO SOLV CONTROL (%)		
125.0	NL/ML	89.0	93.3	88.5	36.5
125.0	NL/ML	86.0	104.4	93.4	31.5
125.0	NL/ML	97.0	116.9	91.8	31.8
250.0	NL/ML	63.0	83.5	92.9	28.9
250.0	NL/ML	71.0	86.2	97.8	31.5
250.0	NL/ML	48.0	73.6	74.5	25.0
500.0	NL/ML	69.0	87.6	82.6	30.2
500.0	NL/ML	73.0	92.7	86.0	30.2
500.0	NL/ML	68.0	104.4	95.0	24.9
1000.0	NL/ML	89.0	87.6	76.4	38.9
1000.0	NL/ML	74.0	102.5	102.3	27.7
1000.0	NL/ML	74.0	83.1	77.7	34.1
3000.0	NL/ML	99.0	87.6	88.3	43.3
3000.0	NL/ML	99.0	103.4	81.1	36.7
3000.0	NL/ML	130.0	108.4	102.0	45.9
5000.0	NL/ML	83.0	87.6	76.6	36.3
5000.0	NL/ML	75.0	91.2	84.7	31.5
5000.0	NL/ML	89.0	90.6	71.9	37.6

W WATER (10%)

8C. HEXAMETHYLPHOSPHORAMIDE (HMPA)

INDUCED ACTIVATION TRIAL 1		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (W)		77.0	87.7	100.0	29.3
SOLVENT CONTROL (W)		113.0	99.0	100.0	38.0
SOLVENT CONTROL (W)		65.0	76.2	100.0	27.6
MCA 2.5 UG/ML		480.0	61.7	58.2	259.4
MCA 2.5 UG/ML		574.0	90.2	61.9	212.2
MCA 2.5 UG/ML		645.0	75.2	27.9	286.0
HMPA			RELATIVE TO SOLV CONTROL (%)		
250.0	NL/ML	99.0	87.1	85.0	43.2
250.0	NL/ML	47.0	59.7	73.2	29.9
250.0	NL/ML	67.0	76.3	104.8	33.4
500.0	NL/ML	56.0	72.1	78.4	29.5
500.0	NL/ML	65.0	83.9	77.4	29.5
500.0	NL/ML	71.0	70.6	68.3	38.3
1500.0	NL/ML	71.0	64.1	83.5	42.1
1500.0	NL/ML	68.0	75.5	74.1	34.2
1500.0	NL/ML	152.0	114.3	82.0	43.9
P 2000.0	NL/ML	125.0	91.9	80.7	51.7
2000.0	NL/ML	125.0	91.3	68.6	52.1
2000.0	NL/ML	C	78.6	68.3	C
P 3000.0	NL/ML	108.0	103.9	118.1	39.6
3000.0	NL/ML	139.0	91.1	71.6	58.0
3000.0	NL/ML	134.0	96.5	74.1	52.8
P 5000.0	NL/ML	147.0	84.5	82.1	66.2
5000.0	NL/ML	129.0	92.3	74.1	55.2
5000.0	NL/ML	110.0	79.5	59.5	52.6

W WATER (10%)
C CONTAMINATED PLATES
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT $p \leq 0.05$
TREND: SIGNIFICANT AT $p \leq 0.05$

8D. HEXAMETHYLPHOSPHORAMIDE (HMPA)

INDUCED ACTIVATION TRIAL 2		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (W)		179.0	66.2	100.0	90.2
SOLVENT CONTROL (W)		196.0	77.5	100.0	84.3
SOLVENT CONTROL (W)		166.0	57.8	100.0	95.7
SOLVENT CONTROL (W)		196.0	102.2	100.0	65.9
MCA 2.5 UG/ML		761.0	38.3	16.2	661.7
MCA 2.5 UG/ML		517.0	24.5	6.5	703.4
MCA 2.5 UG/ML		664.0	36.0	17.3	614.8
HMPA			RELATIVE TO SOLV CONTROL (%)		
P 250.0	NL/ML	411.0	88.7	82.0	203.5
250.0	NL/ML	444.0	73.1	75.1	266.7
250.0	NL/ML	447.0	71.6	41.9	274.2
P 500.0	NL/ML	445.0	69.6	65.7	280.7
500.0	NL/ML	405.0	75.8	93.5	234.8
500.0	NL/ML	469.0	81.3	77.6	253.5
P 1500.0	NL/ML	657.0	67.4	20.9	428.0
1500.0	NL/ML	587.0	78.0	25.5	330.7
1500.0	NL/ML	591.0	60.6	12.2	428.3
P 2000.0	NL/ML	651.0	55.8	28.0	531.4
2000.0	NL/ML	756.0	79.3	30.0	418.8
2000.0	NL/ML	727.0	98.4	36.5	324.5
P 3000.0	NL/ML	610.0	70.5	38.6	380.1
3000.0	NL/ML	671.0	54.5	17.6	541.1
3000.0	NL/ML	689.0	58.9	26.3	514.2
P 5000.0	NL/ML	734.0	62.1	26.2	518.7
5000.0	NL/ML	831.0	78.0	25.2	468.2
5000.0	NL/ML	741.0	45.5	10.7	715.9

W WATER (10%)
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT $p \leq 0.05$
TREND: SIGNIFICANT AT $p \leq 0.05$

9A. PHENOBARBITAL (PB)

NONACTIVATION TRIAL 1		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (A)		103.0	93.0	100.0	36.9
SOLVENT CONTROL (A)		92.0	95.0	100.0	32.3
SOLVENT CONTROL (A)		116.0	104.7	100.0	36.9
EMS .25 UL/ML		910.0	74.2	37.2	409.0
PB			RELATIVE TO SOLV CONTROL (%)		
31.3	UG/ML	72.0	80.4	89.1	30.6
62.5	UG/ML	94.0	93.4	74.3	34.4
62.5	UG/ML	57.0	60.8	67.4	32.0
62.5	UG/ML	73.0	81.5	78.4	30.6
125.0	UG/ML	81.0	59.9	61.4	46.1
125.0	UG/ML	52.0	68.6	72.7	25.9
250.0	UG/ML	74.0	91.9	93.4	27.5
250.0	UG/ML	122.0	95.8	74.5	43.5
250.0	UG/ML	71.0	73.8	67.9	32.9
500.0	UG/ML	109.0	80.9	73.9	46.0
500.0	UG/ML	78.0	77.5	69.5	34.4
500.0	UG/ML	89.0	87.3	61.4	34.8
1000.0	UG/ML	92.0	89.1	43.9	35.2
1000.0	UG/ML	C	99.7	57.1	C
1000.0	UG/ML	91.0	71.2	60.7	43.6
2000.0	UG/ML	LETHAL			
2000.0	UG/ML	LETHAL			
2000.0	UG/ML	NOT CLONED			

C CONTAMINATED PLATES
A ACETONE (1%)

9B. PHENOBARBITOL (PB)

NONACTIVATION TRIAL 2		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (A)		43.0	61.5	100.0	23.3
SOLVENT CONTROL (A)		66.0	65.7	100.0	33.5
SOLVENT CONTROL (A)		54.0	62.3	100.0	28.9
SOLVENT CONTROL (A)		63.0	70.3	100.0	29.8
EMS .25 UL/ML		655.0	78.5	68.6	278.1
MMS 10 NL/ML		483.0	48.8	36.1	329.7
MMS 10 NL/ML		392.0	27.5	22.9	475.1
PB			RELATIVE TO SOLV CONTROL (%)		
250.0	UG/ML	25.0	77.7	64.9	16.5
250.0	UG/ML	19.0	73.8	64.4	13.2
250.0	UG/ML	20.0	74.9	59.2	13.7
500.0	UG/ML	43.0	72.6	80.1	30.4
500.0	UG/ML	51.0	82.6	72.5	31.7
500.0	UG/ML	51.0	86.4	82.3	30.3
750.0	UG/ML	65.0	90.8	42.3	36.7
750.0	UG/ML	45.0	93.8	57.1	24.6
750.0	UG/ML	80.0	93.8	55.2	43.7
1000.0	UG/ML	60.0	73.3	46.5	41.9
1000.0	UG/ML	73.0	105.9	58.5	35.3
1000.0	UG/ML	54.0	93.3	60.6	29.7
P 1200.0	UG/ML	91.0	88.2	34.0	52.9
1200.0	UG/ML	136.0	81.0	27.7	86.1
1200.0	UG/ML	85.0	102.0	39.8	42.7
P 1500.0	UG/ML	186.0	61.5	19.0	155.0
1500.0	UG/ML	101.0	105.4	57.2	49.1
1500.0	UG/ML	143.0	93.3	27.4	78.6
2000.0	UG/ML	TOO TOXIC TO CLONE			
2000.0	UG/ML	TOO TOXIC TO CLONE			
2000.0	UG/ML	TOO TOXIC TO CLONE			

A ACETONE (1%)
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT p<0.05
TREND: SIGNIFICANT AT p<0.05

9C. PHENOBARBITAL (PB)

NONACTIVATION TRIAL 3		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (A)		58.0	86.5	100.0	22.3
SOLVENT CONTROL (A)		70.0	90.8	100.0	25.7
SOLVENT CONTROL (A)		96.0	96.0	100.0	33.3
SOLVENT CONTROL (A)		110.0	83.3	100.0	44.0
MMS 10 NL/ML		375.0	35.8	15.7	348.8
MMS 10 NL/ML		561.0	50.2	31.6	372.7
MMS 5 NL/ML		407.0	61.2	50.8	221.8
MMS 5 NL/ML		345.0	73.5	77.4	156.5
PB			RELATIVE TO SOLV CONTROL (%)		
600.0	UG/ML	84.0	77.5	65.1	40.5
600.0	UG/ML	102.0	89.5	70.0	42.6
600.0	UG/ML	112.0	91.0	77.4	46.0
800.0	UG/ML	94.0	88.2	58.3	39.8
800.0	UG/ML	90.0	77.7	55.1	43.3
800.0	UG/ML	101.0	90.4	49.4	41.7
P 1000.0	UG/ML	117.0	89.9	49.3	48.6
1000.0	UG/ML	124.0	87.6	44.8	52.9
1000.0	UG/ML	124.0	102.6	46.3	45.2
P 1250.0	UG/ML	130.0	90.1	49.5	53.9
1250.0	UG/ML	151.0	96.6	43.9	58.4
1250.0	UG/ML	127.0	96.0	49.3	49.4
P 1500.0	UG/ML	164.0	72.3	25.0	84.7
1500.0	UG/ML	146.0	83.3	29.2	65.5
1500.0	UG/ML	130.0	74.2	34.4	65.5
2000.0	UG/ML	LETHAL			
2000.0	UG/ML	LETHAL			
2000.0	UG/ML	LETHAL			

A ACETONE (1%)
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT p<0.05
TREND: SIGNIFICANT AT p<0.05

9D. PHENOBARBITAL (PB)

INDUCED ACTIVATION TRIAL 1		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (A)		70.0	65.8	100.0	35.4
SOLVENT CONTROL (A)		62.0	86.5	100.0	23.9
SOLVENT CONTROL (A)		78.0	70.5	100.0	36.9
SOLVENT CONTROL (A)		55.0	66.7	100.0	27.5
MCA 2.5 UG/ML		473.0	95.7	60.2	164.8
MCA 2.5 UG/ML		471.0	83.5	75.5	188.0
MCA 2.5 UG/ML		534.0	96.8	67.7	183.8
PB			RELATIVE TO SOLV CONTROL (%)		
200.0	UG/ML	51.0	87.0	79.3	27.0
200.0	UG/ML	64.0	95.3	72.1	30.9
200.0	UG/ML	75.0	92.3	67.7	37.4
400.0	UG/ML	48.0	93.0	84.0	25.8
400.0	UG/ML	62.0	90.9	71.8	31.4
400.0	UG/ML	58.0	78.5	80.1	34.0
500.0	UG/ML	66.0	107.7	87.5	28.2
500.0	UG/ML	52.0	94.6	81.9	25.3
500.0	UG/ML	68.0	94.8	72.0	33.0
600.0	UG/ML	56.0	80.1	66.8	32.2
600.0	UG/ML	48.0	77.6	61.7	28.5
600.0	UG/ML	62.0	90.9	63.7	31.4
800.0	UG/ML	70.0	99.7	55.6	32.3
800.0	UG/ML	60.0	74.4	53.8	37.1
800.0	UG/ML	106.0	88.6	40.4	55.1
1000.0	UG/ML	81.0	106.4	29.9	35.1
1000.0	UG/ML	137.0	115.8	27.0	54.5
1000.0	UG/ML	TOO TOXIC TO CLONE			

A ACETONE (1%)

10A. o-TOLUIDINE (TOL)

NONACTIVATION TRIAL 1		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)		114.0	122.2	100.0	31.1
SOLVENT CONTROL (E)		110.0	110.2	100.0	33.3
SOLVENT CONTROL (E)		100.0	90.7	100.0	36.8
SOLVENT CONTROL (E)		116.0	95.8	100.0	40.3
EMS .25 UL/ML		835.0	93.8	52.6	296.6
EMS .25 UL/ML		988.0	91.8	44.1	358.6
EMS .25 UL/ML		843.0	74.2	30.6	378.9
TOL			RELATIVE TO SOLV CONTROL (%)		
15.6	NL/ML	122.0	89.0	82.1	43.6
15.6	NL/ML	102.0	79.1	71.1	41.0
15.6	NL/ML	113.0	87.2	77.0	41.2
31.3	NL/ML	105.0	78.0	55.9	42.8
31.3	NL/ML	113.0	83.4	83.6	43.1
31.3	NL/ML	118.0	95.7	67.4	39.3
62.5	NL/ML	120.0	75.9	57.4	50.3
62.5	NL/ML	138.0	96.6	49.1	45.5
62.5	NL/ML	112.0	57.9	47.6	61.5
P 125.0	NL/ML	129.0	65.4	27.9	62.8
125.0	NL/ML	165.0	68.8	19.8	76.4
125.0	NL/ML	173.0	96.6	31.2	57.0
P 250.0	NL/ML	147.0	77.0	16.1	60.7
250.0	NL/ML	138.0	86.3	20.2	50.9
250.0	NL/ML	179.0	86.1	18.5	66.2
P 500.0	NL/ML	199.0	60.2	6.7	105.3
500.0	NL/ML	225.0	68.9	9.7	103.9

E ETHANOL (1%)
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT p<0.05
TREND: SIGNIFICANT AT p<0.05

10B. o-TOLUIDINE (TOL)

NONACTIVATION TRIAL 2		TOTAL MUTANT COLONIES	CLONING EFFI-CIENCY	RELATIVE TOTAL GROWTH (%)	MUTANT FREQUENCY (10E-6 UNITS)
SOLVENT CONTROL (E)		60.0	80.3	100.0	24.9
SOLVENT CONTROL (E)		59.0	64.0	100.0	30.7
SOLVENT CONTROL (E)		80.0	67.0	100.0	39.8
SOLVENT CONTROL (E)		62.0	67.3	100.0	30.7
MMS 10 NL/ML		551.0	24.2	9.5	760.0
MMS 10 NL/ML		380.0	19.7	6.4	644.1
TOL			RELATIVE TO SOLV CONTROL (%)		
100.0	NL/ML	61.0	76.8	34.9	38.0
100.0	NL/ML	66.0	78.7	39.4	40.1
100.0	NL/ML	71.0	83.9	40.4	40.4
200.0	NL/ML	83.0	103.1	35.5	38.5
200.0	NL/ML	61.0	80.8	23.2	36.1
200.0	NL/ML	71.0	79.6	24.0	42.6
P 300.0	NL/ML	90.0	80.3	18.2	53.6
300.0	NL/ML	64.0	55.2	12.7	55.4
300.0	NL/ML	96.0	99.2	26.1	46.3
P 400.0	NL/ML	117.0	94.7	16.1	59.1
400.0	NL/ML	107.0	83.5	11.1	61.3
400.0	NL/ML	107.0	125.1	18.6	40.9
P 500.0	NL/ML	98.0	93.7	11.4	50.0
500.0	NL/ML	171.0	103.5	8.8	79.0
500.0	NL/ML	110.0	87.0	18.6	60.4
P 600.0	NL/ML	C	78.7	10.1	C
600.0	NL/ML	109.0	103.1	10.9	50.6
600.0	NL/ML	128.0	120.5	14.7	50.8

C CONTAMINATED PLATES
E ETHANOL (1%)
P SIGNIFICANT INCREASE IN AVERAGE MUTANT FREQUENCY AT p<0.05
TREND: SIGNIFICANT AT p<0.05

dose (125 nl/ml) and high dose (5000 nl/ml) for one activation experiment (5C), but the mutant colony counts were not elevated and the responses were caused primarily by a low cloning efficiency for one member of each triplicate dose set. Additional studies indicated that DEHP toxicity correlated with the number and size of suspended droplets, which in turn was influenced by the method of dispersion. The evaluation of DEHP should be based on soluble concentrations less than 25 nl/ml, and it was assumed from the current data that such treatments would be relatively nontoxic and not mutagenic. DEHP has been tested previously in this assay system and was evaluated as negative (Kirby et al., 1982).

Diethylstilbestrol (DES)

In 2 nonactivation trials, DES at concentrations of 25 μg/ml and higher caused consistent increases in the mutant frequency (Table 6, 7A, 7B). Although the increases were small (about 2–3-fold), the mutant colony counts were clearly elevated. DES was therefore evaluated as mutagenic to L5178Y cells. It is worth noting, however, that DES was tested previously under code for the National Toxicology Program, and negative evaluations were obtained without S9. In that study, approximately 2–3-fold increases in mutant frequency were obtained for toxic treatments with 10–15 μg/ml DES in the presence of S9 mix. DES is not expected to be metabolized by the S9 system. The cause of this variability is not understood, and an irreproducible response might have been observed within both studies if a larger number of experiments had been performed. Other investigators have obtained either negative or positive and negative results (Jotz and Mitchell, 1981; Clive et al., 1979).

Hexamethylphosphoramide (HMPA)

HMPA was relatively nontoxic for concentrations up to 5000 nl/ml without S9 and did not induce any increases in the mutant frequency in two trials (Table 6, 8A–8B). With S9, two positive experiments were obtained that differed quantitatively (8C–8D). HMPA was relatively nontoxic in the first trial, and the mutant frequencies showed a positive trend with increasing dose, reaching a maximum of 1.8 times the background; the lowest concentration causing a significant response was 2000 μg/ml. This result was very similar to that reported by Jotz and Mitchell (1981). In the second activation trial, which used a different and fresh batch of S9, HMPA was toxic and induced a much larger mutagenic response. A 3-fold increase in mutant frequency was observed for the lowest tested dose of 250 nl/ml, and a 7-fold increase occurred for the 5000 nl/ml treatment, which caused an average relative total growth of 21%. These results suggested that the metabolism of HMPA, and hence its mutagenic activity, may be very sensitive to particular S9 characteristics.

Phenobarbital (PB)

PB was mutagenic without S9 at concentrations exceeding 1000 μg/ml and was excessively toxic at 2000 μg/ml (Table 6, 9A–9D). Thus, the mutagenic activity was confined to less than a 2-fold concentration range and was undetected in the first trial, which was evaluated as inconclusive due to insufficient toxicity. The mutant frequency increases were about 2–3-fold over background and were paralleled by increases in the mutant colony count. One activation experiment was performed to determine if the response would be enhanced, but no response was observed for concentrations up to 1000 μg/ml (29% relative total growth). Sodium PB was tested by Amacher et al. (1980) without S9 and evaluated as nonmutagenic. However, the results were compatible with this study because the only treatment that yielded a relative total growth less than 50% (4700 μg/ml, 14% relative total growth) also caused a 2.4-fold increase in mutant frequency.

Safrole (SAF)

SAF was not detectable as a mutagen without S9, and repeated testing with S9 was necessary to show the mutagenic potential of this chemical to mouse lymphoma cells (Table 6, 6A–6G). Two trials without S9 demonstrated the occurrence of excessive toxicity in the 80–100 nl/ml concentration range and the difficulty of obtaining treatments in the 10–30% relative total growth range. One treatment with 80 nl/ml in Trial 1 yielded 15% relative total growth and a 1.9-fold increase in mutant frequency, which suggested the possibility of detecting weak mutagenic activity if a concerted

effort is made to obtain highly toxic treatments. With S9 activation, SAF was evaluated as mutagenic in 3 of 5 trials over the same concentration range. The first induced S9 trial showed only a weak response (maximum of 1.9-fold increase in mutant frequency) that appeared to be saturated for the assayed dose range, and the repeat trial using the same S9 batch showed no response for the same treatments. A third trial with another batch of S9 showed no response. A fourth trial was performed because of the availability of a third batch of S9 that was highly active in other studies. Significant, dose-related increases in mutant colony number and frequency were obtained that resulted in a maximum 3.5-fold increase in mutant frequency at 125 nl/ml (14% average relative total growth). One noninduced S9 experiment also was performed, which showed significant responses increasing to a maximum of 2-fold at 120 nl/ml (28% average relative total growth). Thus, sufficient evidence was obtained to evaluate SAF as mutagenic with S9 activation, but the conditions for its efficient activation are not understood. Some batches of S9 (induced or noninduced) may be more active, but the particular design of the S9 activation test condition could be critical to the reliable detection of this mutagen. Jotz and Mitchell (1981) obtained very similar responses although SAF was evaluated as mutagenic without S9 activation.

o-Toluidine (TOL)

TOL was detectable as a mutagen to L5178Y cells in the absence of S9 activation. Two experiments (Table 6, 10A–10B) showed maximum responses of 2–3-fold increases over the background mutant frequency. The mutant colony counts also were elevated over the solvent control cultures. The effect of S9 activation was not tested. TOL could be easily missed in a screening program because the mutant frequency did not double until the relative total growth was approximately 10% or less. The increase in resolving power provided by the replicate treatments was important to the detection of this carcinogen as a mutagen.

The mouse lymphoma assay, as performed with coded chemicals in this laboratory, correctly identified seven of the chemicals if mammalian cell mutagenesis is equated with carcinogenic activity in rodents. Two of the carcinogens, benzene and DEHP, were not detected, whereas the noncarcinogen benzoin was one of the stronger mutagenic substances in this study. When compared to the general performance of the other mammalian cell mutation studies contributing to the Collaborative Program, the testing approach described in this report performed very well. The agreement of this study with the workgroup was the highest of all investigators (90%). Benzene was evaluated as nonmutagenic, but the workgroup considered the evidence for benzene as a mammalian cell mutagen to be inconclusive. The high agreement of this study with the workgroup evaluations was attributed to the highest resolving power made possible by the use of replicate treatments and solvent controls and the use of repeat experiments. It is also worth noting that this approach led to somewhat more conservative, rather than hypersensitive, evaluations for the presence of mutagenic activity.

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Tests for the induction of forward mutation at the thymidine kinase locus of L5178Y mouse lymphoma cells in culture

T.J. Oberly, B.J. Bewsey and G.S. Probst

Toxicology Division, Lilly Research Laboratories, Greenfield, IN (U.S.A.)

Summary

Acrylonitrile, benzene, benzoin, caprolactam, diethylhexylphthalate, diethylstilbestrol, hexamethylphosphamide, phenobarbitol, safrole and *o*-toluidine were tested in vitro for the ability to induce mammalian cell mutation in the L5178Y $TK^{+/-}$ mouse lymphoma cell forward-mutation assay with and without liver microsomal activation. Ethyl methanesulfonate (EMS) and 3-methylcholanthrene (3-MC) were also tested and served as positive controls for the nonactivated and activated assays, respectively.

Treatment with either 620 μg/ml EMS or 3, 4 or 5 μg/ml 3-MC resulted in the induction of L5178Y $TK^{-/-}$ mutants. A weak positive response was obtained with hexamethylphosphamide in the absence of metabolic activation. In addition, weak positive responses were obtained with phenobarbital, benzene, benzoin, acrylonitrile and diethylhexylphthalate only in the presence of metabolic activation. In contrast, negative responses were obtained with caprolactam, diethylstilbestrol, safrole and *o*-toluidine.

Introduction

The L5178Y mouse lymphoma cell thymidine kinase (TK) assay was first described by Clive et al. (1972, 1973) and has since been shown by several laboratories to be sensitive to a variety of mutagens and carcinogens (Amacher et al., 1979, 1980; Amacher and Turner, 1980; Clive et al., 1979; Oberly et al., 1982). In this in vitro forward mutation assay, mutagenic agents induce the heritable loss of TK activity in formerly TK-competent L5178Y cells ($TK^{+/-}$). The resulting L5178Y $TK^{-/-}$ mutants lack the salvage enzyme thymidine kinase and are therefore detected by their resistance to the lethal thymidine analogs 5-bromo-deoxyuridine (Clive et al., 1972) or trifluorothymidine (TFT) (Brown and Clive, 1977). Rat-liver microsomal enzymes (S9) have been incorporated into this test for the activation of promutagens (Amacher et al., 1980; Amacher and Turner, 1980; Clive and Spector, 1975; Clive et al., 1979; Oberly et al., 1982).

This report describes experiments in which L5178Y ($TK^{+/-}$) mouse lymphoma cells were treated in vitro with 10 compounds to evaluate mammalian cell point-mutation. The test was conducted with and without metabolic activation by an S9 liver microsomal fraction obtained from Aroclor 1254-induced rats.

Materials and methods

Chemicals

Acrylonitrile, benzene, benzoin, caprolactam, diethylhexylphthalate, diethylstilbestrol, hexamethylphosphamide, phenobarbital, safrole and *o*-toluidine were provided by the Landcaster Synthesis, Ltd. (East Gate, England) and were tested as supplied without purification or attempt to identify impurities. Ethyl methanesulfonate (EMS) and 3-methylcholanthrene (3-MC) were obtained from Eastman Kodak (Rochester, NY). Trifluorothymidine (TFT) was obtained from Sigma Chemical Co. (St. Louis, MO). All test chemicals

were dissolved in reagent grade DMSO (Fisher Scientific Co., Pittsburgh, PA, or J.T. Baker Chemical Co., Glen Ellyn, IL) and dilutions were made in R_0P medium (see below) to give final concentrations of 620 μg/ml for EMS; 3, 4 or 5 μg/ml for 3-MC and respective concentrations for each test compound. The highest concentration of DMSO (1%) did not affect survival or mutation in the control cultures.

Cell Cultures

The $TK^{+/-}$ cells (TK3.7.2C), a subline of the mouse lymphoma cell L5178Y heterozygous for thymidine kinase, were originally obtained from D. Clive (Burroughs Wellcome, Research Triangle Park, NC). Suspension cultures were initiated from stock cells stored in liquid nitrogen and maintained with $R_{10}P$ medium (see below) in continuous logarithmic growth by periodic dilution of the cultures to 3×10^5 cells/ml. All cell counts reported in this study were viable counts determined by trypan blue exclusion.

Culture media

Growth medium, designated $R_{10}P$, consisted of RPMI 1640 medium containing L-glutamine (Grand Island Biologicals, Grand Island, NY) supplemented with 200 units/ml penicillin, 200 μg/ml streptomycin, 100 μg/ml Na pyruvate, 1 mg/ml Pluronic F68 (BASF Wyandott Corp., Wyandott, MI), and 10% horse serum (Grand Island Biologicals, Grand Island, NY). Growth medium containing no serum was designated R_0P. During exposure to test substances the cells were maintained in growth medium containing 3% horse serum. Nonselective cloning medium, for the quantification of cell plating efficiency, was $R_{20}P$ containing 220 μg/ml Na pyruvate, 20% horse serum, and 0.4% (v/v) molten noble agar but without Pluronic F68. Selective cloning medium, for the quantification of $TK^{-/-}$ mutants, was cloning medium supplemented with 3 μg/ml TFT.

Preparation of liver microsomes

The microsomal activation enzymes were prepared as described by Cline and McMahon (1977) from the livers of male Fischer 344 rats, weighing approximately 200 g, treated 5 days prior with a single 500 mg/kg intraperitoneal dose of Aroclor 1254. A sterile 25% liver homogenate, prepared in 0.15 M KCl at 4°C, was centrifuged for 10 min at $9000 \times g$. The supernatant fraction (S9) was stored in 2-ml portions (equivalent to 500 mg wet weight of liver) at −80°C. Under these conditions, enzymatic activity was stable for at least one year. Each new lot of S9 was titrated with the positive control 3-MC to determine a proper S9 dilution for routine testing.

Selection of Doses

Doses of for each test compound used in the L5178Y $TK^{+/-}$ assay were selected based on results of a preliminary toxicity study in which suspension cultures of L5178Y $TK^{+/-}$ cells were exposed for 4 h to concentrations of each respective compound and suspension growth was monitored over a 2-day period. No toxicity test was performed with benzene, since preliminary toxicity information had been completed earlier at our laboratory. The highest dose selected for the mutation assay was the concentration which resulted in a 50–90% inhibition of growth in the preliminary toxicity study. When no toxicity was evident in the preliminary study, the highest dose used in the mutagenicity test was determined by its 100× stock solubility. Upon selection of the high dose, lower doses were chosen at log or geometric intervals with the expectation of providing a dose–response for toxicity in the mutation assay.

Cell preparation

Prior to initiating the L5178Y $TK^{+/-}$ assay, the $TK^{+/-}$ stock culture was cleansed of accumulated spontaneous $TK^{-/-}$ mutants (Amacher et al., 1980; Clive et al., 1979) by a 24-h incubation in $R_{10}P$-THMG medium ($R_{10}P$ medium supplemented with 6 μg/ml thymidine, 10 μg/ml hypoxanthine, 0.2 μg/ml methotrexate and 15 μg/ml glycine), followed by a 72-h incubation in similar medium lacking methotrexate ($R_{10}P$-THG). This process maintains a low spontaneous background in control cultures thus improving the sensitivity of the assay.

L5178Y $TK^{+/-}$ Assay (non-activated)

The L5178Y $TK^{+/-}$ assay was conducted essentially by the method of Clive et al. (1979) with minor modifications of Amacher et al. (1979, 1980)

and Oberly et al. (1982). Briefly, stock $TK^{+/-}$ cells harvested by centrifugation for 10 min at $500 \times g$, were resuspended in a 50% supernatant ($R_{10}P$) and 50% R_0P (serum concentration 5%) to give 1×10^6 cells/ml. 6 ml of this suspension were combined with 4 ml of R_0P (serum concentration 3%) and 0.1 ml of a $100\times$ concentrate of the appropriate test chemical or positive controls or solvent controls in a 50-ml disposable screw-gap centrifuge tube (Corning No. 25330). Incubation with the test compound was for 4 h at 37 ± 0.5 °C in a roller drum (20–30 rpm). Following incubation, the cells were washed twice by centrifugation in 10-ml portions of $R_{10}P$ and then resuspended in 20 ml of $R_{10}P$. To allow the expression of $TK^{-/-}$ mutants, the cells were incubated in a roller drum at 37 °C for 48 h. During this period of logarithmic growth, cell densities were determined every 24 h and the cells were diluted with $R_{10}P$ to 3×10^5 cells/ml daily. In the event of treatment-induced growth retardation, daily dilution to 3×10^5 cells/ml was not required.

Following this expression period each culture was adjusted to 5×10^5 cells/ml with $R_{10}P$ and 1 ml of this suspension was serially diluted (1 : 10) in R_0P with a final suspension of 100 cells/ml in $R_{10}P$. 1-ml aliquots of this suspension (100 cells) were combined with approximately 30 ml of cloning medium and allowed to gel for several minutes at room temperature, and then the plates were incubated for 12 ± 2 days at 37 °C in a 95%–5% air–CO_2 environment. Colonies of both $TK^{+/-}$ and $TK^{-/-}$ cells appeared on these plates and thus provided a measure of the number of viable L5178Y cells capable of growing in the soft-agar cloning medium.

For selection of $TK^{-/-}$ mutants, 1 ml of the 5×10^5 cell/ml suspension (500 000 cells) was combined with 30 ml of TFT-selection medium and dispensed into each of 3 petri plates and processed as described above. Only $TK^{-/-}$ colonies appeared on these plates, thus providing a measure of the number of $TK^{-/-}$ mutants capable of growing in soft agar medium.

L5178Y $TK^{+/-}$ assay (activated)

The activated assay was conducted similarly to the nonactivated assay. The $TK^{+/-}$ cells were obtained as in the nonactivated test. The enzyme mixture was prepared in R_0P and contained in a final volume of 4 ml, 45 mg Na isocitrate, 24 mg NADP and 1 ml of rat-liver S9. 4 ml of enzyme mixture (in place of R_0P used for the nonactivated test) were added to the 6 ml of cell suspension. As in the nonactivated test, the final serum concentration was 3%. All subsequent procedures were as described for the nonactivated assay.

Quantification of the L5178Y $TK^{+/-}$ assay

The number of viable colonies (growth in non-selective cloning medium) and the number of $TK^{-/-}$ mutants (growth in TFT-selection medium) were enumerated automatically using a Biotran II automated colony counter (New Brunswick Scientific Corp., New Brunswick, NJ), and the arithmetic mean of the triplicate samples was determined. Colonies derived from plates treated with the solvent control were used to establish the size discrimination standard for counting colonies derived after chemical treatment. Both large and small colonies were included in the total count. The criteria for a positive response for chemical-induced mammalian cell mutation was a dose-dependent 2-fold or greater increase in $TK^{-/-}$ frequency at 2 or more dose levels when compared to $TK^{-/-}$ frequency in solvent-treated controls. Furthermore, only cultures showing greater than 10% total survival were included in the final evaluation for a mutagenic response.

Calculations for percent suspension growth, cloning efficiency, mutation frequency, and mutation index are described in Appendix A.

Results and discussion

Acrylonitrile

As a result of a preliminary toxicity test with acrylonitrile, doses of 1, 5, 10, 20, 30, 40, 50 and 60 μg/ml were chosen for the mutagenicity assay which was conducted with and without metabolic activation (Table 1).

In the nonactivated test, values for total survival ranged from 4 to 117% of controls, and a 2.6-fold increase in mutation frequency (MF) was noted at the 50 μg/ml concentration. Although a 3.9-fold increase in MF was observed at the highest dose, the corresponding value for total survival was only 4% and therefore this culture was not included in

TABLE 1

A SUMMARY OF RESULTS FOR THE MOUSE LYMPHOMA FORWARD-MUTATION ASSAY WITH ACRYLONITRILE [a]

Treatment	Concentration (μg/ml)	Percent total survival [b]	Mutation frequency [c]		Mutation index [d]
Nonactivated Test					
DMSO [e]	(1%)	100	3.0	(2.7) [g]	(1.0)
DMSO [e]	(1%)	100	2.4		
EMS [f]	620	38	22.6		8.4
Acrylonitrile	60	4	10.5		3.9
Acrylonitrile	50	29	6.9		2.6
Acrylonitrile	40	44	4.9		1.8
Acrylonitrile	30	52	3.4		1.3
Acrylonitrile	20	81	2.7		1.0
Acrylonitrile	10	110	2.1		0.8
Acrylonitrile	5	101	2.6		1.0
Acrylonitrile	1	117	2.4		0.9
Activated test					
DMSO [e]	(1%)	100	2.4	(2.3) [g]	(1.0)
DMSO [e]	(1%)	100	2.2		
3MC [f]	3	12	10.5		4.6
Acrylonitrile	60	– [h]	–		–
Acrylonitrile	50	– [h]	–		–
Acrylonitrile	40	10	8.1		3.5
Acrylonitrile	30	12	6.4		2.8
Acrylonitrile	20	25	4.4		1.9
Acrylonitrile	10	43	3.4		1.5
Acrylonitrile	5	60	4.1		1.8
Acrylonitrile	1	62	2.2		1.0

[a] Calculations appear in Appendix A.
[b] (Suspension growth)×(cloning efficiency).
[c] $TK^{-/-}$ mutants per 1×10^5 colony-forming cells.
[d] (Mutation frequency of treated culture)/(control mutation frequency).
[e] Solvent control.
[f] Positive control.
[g] Mean of solvent control.
[h] Insufficient cells for plating.

the assessment of mutagenicity.

In the activated test, values for total survival ranged from 10 to 62% of controls and a 3.5- and 2.8-fold increase in MF was observed at the 40 and 30 μg/ml concentrations, respectively. On the basis of the results with acrylonitrile in the activated assay, this compound was judged to be mutagenic with activation in L5178Y mouse lymphoma ($TK^{+/-}$) cells.

Benzene

Former toxicity data for benzene was available in our laboratory and permitted the selection of a 500–1200 μg/ml dose range for the nonactivated test and a 100–800 μg/ml dose range for the activated test (Table 2).

In the absence of metabolic activation, doses $\geqq 1000$ μg/ml were toxic and the lower doses resulted in values for total survival that range from 5 to 60% of controls. No culture in the non-activated test showed a two-fold or greater increase in MF over background.

In the activated test, values for total survival ranged from 8 to 45% of controls and four cultures

TABLE 2

A SUMMARY OF RESULTS FOR THE MOUSE LYMPHOMA FORWARD-MUTATION ASSAY WITH BENZENE [a]

Treatment	Concentration (μg/ml)	Percent total survival [b]	Mutation frequency [c]	Mutation index [d]
Nonactivated Test				
DMSO [e]	(1%)	100	1.8 (2.2) [g]	(1.0)
DMSO [e]	(1%)	100	2.6	
EMS [f]	620	18	57.2	26.0
Benzene	1200	– [h]	–	–
Benzene	1100	– [h]	–	–
Benzene	1000	– [h]	–	–
Benzene	900	5	3.7	1.7
Benzene	800	27	1.8	0.8
Benzene	700	55	1.9	0.9
Benzene	600	49	2.6	1.2
Benzene	500	60	2.2	1.0
Activated Test				
DMSO [e]	(1%)	100	2.0 (2.0) [g]	(1.0)
DMSO [e]	(1%)	100	2.0	
3MC [f]	5	2	30.7	15.4
Benzene	800	8	3.8	1.9
Benzene	700	16	3.0	1.5
Benzene	600	9	4.1	2.1
Benzene	500	11	4.9	2.5
Benzene	400	16	5.0	2.5
Benzene	300	33	5.1	2.6
Benzene	200	40	3.3	1.7
Benzene	100	45	4.6	2.3

[a] Calculations appear in Appendix A.
[b] (Suspension growth)×(cloning efficiency).
[c] $TK^{-/-}$ mutants per 1×10^5 colony-forming cells.
[d] (Mutation frequency of treated culture)/(control mutation frequency).
[e] Solvent control.
[f] Positive control.
[g] Mean of solvent control.
[h] No surviving cultures.

showed a 2–3-fold increase in MF over background. In each of the cultures that showed increased MF the corresponding values for total survival exceeded 10%. Consequently, benzene was judged to be mutagenic with activation in the L5178Y cells.

Benzoin

As a result of a preliminary toxicity test with benzoin, doses of 1, 10, 50, 100, 250, 500, 750 and 1000 μg/ml were selected for the non-activated mutagenicity assay; and doses of 0.1, 0.5, 1.0, 2.5, 5, 7.5, 10 and 20 μg/ml were chosen for the mutagenicity test with activation (Table 3).

For the nonactivated test, excessive toxicity precluded the cloning of cells from the two highest dose groups. The remaining dose groups showed a dose-related response for total survival which ranged from 4 to 92%. No increase in MF was observed in the nonactivated test.

With activation, cells from the highest dose group were not cloned due to excessive toxicity, while cells from the 0.1–10 μg/ml dose groups showed values of 10 to 83% for total survival. A

TABLE 3

A SUMMARY OF RESULTS FOR THE MOUSE LYMPHOMA FORWARD-MUTATION ASSAY WITH BENZOIN [a]

Treatment	Concentration (μg/ml)	Percent total survival [b]	Mutation frequency [c]	Mutation index [d]
Nonactivated test				
DMSO [e]	(1%)	100	3.1 (3.25) [g]	(1.0)
DMSO [e]	(1%)	100	2.4	
EMS [f]	620	12	93.3	28.7
Benzoin	1000	– [h]	–	–
Benzoin	750	– [h]	–	–
Benzoin	500	4	2.8	0.9
Benzoin	250	20	2.6	0.8
Benzoin	100	34	2.6	0.8
Benzoin	50	51	3.2	1.0
Benzoin	10	92	4.3	1.3
Benzoin	1	80	4.1	1.3
Activated test				
DMSO [e]	(1%)	100	2.7 (2.4) [g]	(1.0)
DMSO [e]	(1%)	100	2.1	
3MC [f]	5	6	26.9	11.2
Benzoin	20	– [h]	–	–
Benzoin	10	10	8.6	3.6
Benzoin	7.5	21	5.3	2.2
Benzoin	5	24	5.0	2.1
Benzoin	2.5	46	4.8	2.0
Benzoin	1	67	4.1	1.7
Benzoin	0.5	77	3.2	1.3
Benzoin	0.1	83	2.4	1.0

[a] Calculations appear in Appendix A.
[b] (Suspension growth)×(cloning efficiency).
[c] $TK^{-/-}$ mutants per 1×10^5 colony-forming cells.
[d] (Mutation frequency of treated culture)/(control mutation frequency).
[e] Solvent control.
[f] Positive control.
[g] Mean of solvent control.
[h] Insufficient cells for plating.

2-fold or greater increase in MF was noted in the cultures from the 4 highest treatment levels and the corresponding values for total survival were $\geq$ 10% in each case. It was concluded that benzoin was mutagenic to L5178Y cells only with activation.

Caprolactam

Caprolactam was not toxic at concentrations up to 1000 μg/ml and therefore the upper dose for mutagenicity testing was based on the highest soluble concentration attainable. Caprolactam doses of 500, 1000, 2500, 4000, 5500, 7000, 8500 and 10000 μg/ml were chosen for the mutagenicity assay both with and without metabolic activation (Table 4).

In the nonactivated test, a dose-related response for toxicity was not evident, and no toxicity was observed in the test with metabolic activation. In addition, a treatment-related increase in MF was not evident in either the nonactivated or activated test even though unusually high doses were

TABLE 4

A SUMMARY OF RESULTS FOR THE MOUSE LYMPHOMA FORWARD-MUTATION ASSAY WITH CAPROLACTAM [a]

Treatment	Concentration (μg/ml)	Percent total survival [b]	Mutation frequency [c]		Mutation index [d]
Nonactivated test					
DMSO [e]	(1%)	100	2.6	(2.55) [g]	(1.0)
DMSO [e]	(1%)	100	2.5		
EMS [f]	620	14	65.2		25.6
Caprolactam	10000	60	3.0		1.2
Caprolactam	8500	77	3.7		1.5
Caprolactam	7000	56	3.6		1.4
Caprolactam	5500	76	2.9		1.1
Caprolactam	4000	83	2.1		0.8
Caprolactam	2500	76	3.5		1.4
Caprolactam	1000	88	2.9		1.1
Caprolactam	500	89	3.1		1.2
Activated test					
DMSO [e]	(1%)	100	2.8 (2.65) [g]		(1.0)
DMSO [e]	(1%)	100	2.5		
3MC [f]	5	2	36.5		13.8
Caprolactam	10000	121	2.5		0.9
Caprolactam	8500	107	3.1		1.2
Caprolactam	7000	108	2.7		1.0
Caprolactam	5500	105	2.4		0.9
Caprolactam	4000	110	2.9		1.1
Caprolactam	2500	105	2.6		1.0
Caprolactam	1000	133	2.6		1.0
Caprolactam	500	122	2.2		0.8

[a] Calculations appear in Appendix A.
[b] (Suspension growth)×(cloning efficiency).
[c] $TK^{-/-}$ mutants per 1×10^5 colony-forming cells.
[d] (Mutation frequency of treated culture)/(control mutation frequency).
[e] Solvent control.
[f] Positive control.
[g] Mean of solvent control.

examined. Consequently, caprolactam was judged to be nonmutagenic in the L5178Y mouse lymphoma $TK^{+/-}$ forward-mutation assay.

Diethylhexylphthalate

As a result of a preliminary toxicity test with diethylhexylphthalate, doses of 10, 20, 30, 40, 50, 100, 200 and 400 μg/ml and 1.0, 2.5, 5.0, 7.5, 10, 20, 40 and 80 μg/ml were chosen for the nonactivated and activated mutagenicity tests, respectively (Table 5).

A dose-dependent decrease in values for total survival was obtained in the nonactivated mutagenicity test and these values ranging from 5 to 73% of controls. A 2-fold or greater increase in MF was seen at 2 dose levels in the nonactivated test; however, the value for total survival exceeded 10% at only one of these dose levels.

In the activated test, cells from the highest dose (80 μg/ml) were not plated due to excessive toxicity. Cultures from subsequent doses showed values for total survival of 5 to 90%.

A dose-related decrease in total survival was also observed in the activated test. In addition,

TABLE 5

A SUMMARY OF RESULTS FOR THE MOUSE LYMPHOMA FORWARD-MUTATION ASSAY WITH DIETHYLHEXYLPHTHALATE [a]

Treatment	Percent			
	Concentration (μg/ml)	total survival [b]	Mutation frequency [c]	Mutation index [d]
Nonactivated test				
DMSO [e]	(1%)	100	3.8 (3.8) [g]	(1.0)
DMSO [e]	(1%)	100	3.8	
EMS [f]	620	12	112.3	29.6
DEHP *	400	12	6.8	1.8
DEHP	200	5	10.1	2.7
DEHP	100	9	6.8	1.8
DEHP	50	14	5.9	1.6
DEHP	40	12	9.8	2.6
DEHP	30	25	4.7	1.2
DEHP	20	37	4.5	1.2
DEHP	10	73	4.8	1.3
Activated test				
DMSO [e]	(1%)	100	3.5 (3.35) [g]	(1.0)
DMSO [e]	(1%)	100	3.2	
3MC [f]	3	7	30.2	9.0
DEHP *	80	– [h]	–	–
DEHP	40	5	11.2	3.3
DEHP	20	10	9.0	2.7
DEHP	10	44	6.2	1.9
DEHP	7.5	65	6.6	2.0
DEHP	5.0	84	4.4	1.3
DEHP	2.5	90	4.2	1.3
DEHP	1.0	63	4.9	1.5

* Di(2-ethylhexyl)phthalate.

[a] Calculations appear in Appendix A.

[b] (Suspension growth)×(cloning efficiency).

[c] $TK^{-/-}$ mutants per 1×10^5 colony-forming cells.

[d] (Mutation frequency of treated culture)/(control mutation frequency).

[e] Solvent control.

[f] Positive control.

[g] Mean of solvent control.

[h] Insufficient cells for plating.

cultures from three dose levels gave a 2-fold or greater increase in MF and two of these showed $\geqq 10\%$ survival. Under these conditions, it was concluded that diethylhexylphthalate was mutagenic in the L5178Y mouse lymphoma $TK^{+/-}$ forward-mutation assay with metabolic activation.

Diethylstilbestrol

A preliminary toxicity test was performed with diethylstilbestrol; however, due to extremely irregular toxicity a total of three mutagenicity tests were performed. The final test is reported here since it provided results that were most clearly dose-related (Table 6). In the nonactivated test the dose range was 10, 50, 100, 110, 120, 130, 140 and 150 μg/ml. Doses of 0.1, 1, 5, 10, 20, 25, 30 and 35 μg/ml were selected for the test with metabolic activation.

TABLE 6

A SUMMARY OF RESULTS FOR THE MOUSE LYMPHOMA FORWARD-MUTATION ASSAY WITH DIETHYLSTILBESTROL [a]

Treatment	Percent			
	Concentration (μg/ml)	total survival [b]	Mutation frequency [c]	Mutation index [d]
Nonactivated test				
DMSO [e]	(1%)	100	2.7 (2.8) [g]	(1.0)
DMSO [e]	(1%)	100	2.9	
EMS [f]	620	20	81.5	29.1
Diethylstilbestrol	150	22	4.6	1.6
Diethylstilbestrol	140	50	2.6	0.9
Diethylstilbestrol	130	62	3.0	1.1
Diethylstilbestrol	120	53	4.0	1.4
Diethylstilbestrol	110	52	2.9	1.0
Diethylstilbestrol	100	66	2.8	1.0
Diethylstilbestrol	50	71	3.0	1.1
Diethylstilbestrol	10	59	3.5	1.3
Activated test				
DMSO [e]	(1%)	100	3.2 (3.55) [g]	(1.0)
DMSO [e]	(1%)	100	3.9	
3MC [f]	3	6	37.6	10.6
Diethylstilbestrol	35	– [h]	–	–
Diethylstilbestrol	30	– [h]	–	–
Diethylstilbestrol	25	5	4.2	1.2
Diethylstilbestrol	20	20	5.0	1.4
Diethylstilbestrol	10	– [h]	–	–
Diethylstilbestrol	5	4	9.4	2.6
Diethylstilbestrol	1	28	4.5	1.3
Diethylstilbestrol	0.1	100	4.8	1.4

[a] Calculations appear in Appendix A.
[b] (Suspension growth)×(cloning efficiency).
[c] TK $^{-/-}$ mutants per 1×10^5 colony-forming cells.
[d] (Mutation frequency of treated culture)/(control mutation frequency).
[e] Solvent control.
[f] Positive control.
[g] Mean of solvent control.
[h] Insufficient cells for plating.

For each dose group of the nonactivated test, sufficient cells were available for cloning, and values for total survival ranged from 22 to 71% of controls. In each of these cultures there was no treatment related increase in MF.

Irregular toxicity was also noted in the activated test and this was consistent with the results of the 2 prior mutagenicity tests (data not shown). Cells of 3 dose groups were not plated due to severe toxicity and values for total survival ranged from 4 to 100%. No appreciable increase in MF was observed in the activated test with the exception of one culture (5 μg/ml) which showed a 2.6-fold increase in MF over background; however, survival was less than 10%. On the basis of these results, diethylstilbestrol was judged not to be mutagenic in the L5178Y cells.

Hexamethylphosphamide

As a result of the findings in a preliminary toxicity test, hexamethylphosphamide doses of 10, 50, 100, 250, 500, 750, 1000 and 1250 μg/ml were

TABLE 7

A SUMMARY OF RESULTS FOR THE MOUSE LYMPHOMA FORWARD-MUTATION ASSAY WITH HEXAMETHYLPHOSPHAMIDE [a]

Treatment	Percent			
	Concentration (μg/ml)	total survival [b]	Mutation frequency [c]	Mutation index [d]
Nonactivated test				
DMSO [e]	(1%)	100	5.2 (4.75) [g]	(1.0)
DMSO [e]	(1%)	100	4.3	
EMS [f]	620	17	70.3	14.8
Hexamethylphosphamide	1250	83	6.1	1.3
Hexamethylphosphamide	1000	120	5.7	1.2
Hexamethylphosphamide	750	105	4.8	1.0
Hexamethylphosphamide	500	82	7.3	1.5
Hexamethylphosphamide	250	119	10.5	2.2
Hexamethylphosphamide	100	86	13.3	2.8
Hexamethylphosphamide	50	114	4.1	0.9
Hexamethylphosphamide	10	110	11.0	2.3
Activated test				
DMSO [e]	(1%)	100	2.9 (3.05) [g]	(1.0)
DMSO [e]	(1%)	100	3.2	
3MC [f]	3	9	31.5	10.3
Hexamethylphosphamide	1250	87	2.9	1.0
Hexamethylphosphamide	1000	50	6.1	2.0
Hexamethylphosphamide	750	64	3.1	1.0
Hexamethylphosphamide	500	91	2.7	0.9
Hexamethylphosphamide	250	76	1.8	0.6
Hexamethylphosphamide	100	80	2.4	0.8
Hexamethylphosphamide	50	79	4.4	1.4
Hexamethylphosphamide	10	91	3.3	1.1

[a] Calculations appear in A.
[b] (Suspension growth)×(cloning efficiency).
[c] $TK^{-/-}$ mutants per 1×10^5 colony-forming cells.
[d] (Mutation frequency of treated culture)/(control mutation frequency).
[e] Solvent control.
[f] Positive control.
[g] Mean of solvent control.

chosen for the mutagenicity assay conducted with and without metabolic activation (Table 7).

Minimal toxicity was observed in the non-activated test and values for total survival ranged from 82 to 120%. These values were not clearly related to dose. Three cultures in the nonactivated test exhibited a greater than 2-fold increase in MF over background, however higher dose levels did not show a similar effect.

Toxicity was also observed in the activated test where values for total survival ranged from 50 to 91%, however this effect did not show a clear relationship to dose. One culture in the activated test showed a 2-fold increase in MF, however, similar to the nonactivated test, this effect was not evident at the next higher dose level.

Hexamethylphosphamide was, therefore, considered to be mutagenic in the L5178Y mouse lymphoma $TK^{+/-}$ forward-mutation assay without metabolic activation.

TABLE 8

A SUMMARY OF RESULTS FOR THE MOUSE LYMPHOMA FORWARD-MUTATION ASSAY WITH PHENOBARBITAL [a]

Treatment	Percent			
	Concentration (μg/ml)	total survival [b]	Mutation frequency [c]	Mutation index [d]
Nonactivated test				
DMSO [e]	(1%)	100	3.0 (2.4) [g]	(1.0)
DMSO [e]	(1%)	100	1.8	
EMS [f]	620	31	59.3	24.7
Phenobarbital	2500	5	7.4	3.1
Phenobarbital	2250	48	1.9	0.8
Phenobarbital	2000	54	2.1	0.9
Phenobarbital	1750	55	3.8	1.6
Phenobarbital	1500	59	4.0	1.7
Phenobarbital	1250	79	3.8	1.6
Phenobarbital	1000	91	3.5	1.5
Phenobarbital	750	97	2.7	1.1
Activated test				
DMSO [e]	(1%)	100	2.7 (2.8) [g]	(1.0)
DMSO [e]	(1%)	100	2.9	
3MC [f]	3	12	42.0	14.0
Phenobarbital	2000	12	7.7	2.6
Phenobarbital	1750	28	4.1	1.4
Phenobarbital	1500	14	6.7	2.2
Phenobarbital	1250	76	3.7	1.2
Phenobarbital	1000	87	3.4	1.1
Phenobarbital	750	93	3.2	1.1
Phenobarbital	500	94	2.5	0.8
Phenobarbital	250	99	2.8	0.9

[a] Calculations appear in Appendix A.
[b] (Suspension growth)×(cloning efficiency).
[c] TK $^{-/-}$ mutants per 1×10^5 colony-forming cells.
[d] (Mutation frequency of treated culture)/(control mutation frequency).
[e] Solvent control.
[f] Positive control.
[g] Mean of solvent control.

Phenobarbital

Based on the results obtained from a preliminary toxicity test with phenobarbital, doses of 750, 1000, 1250, 1500, 1750, 2000, 2250 and 2500 μg/ml were chosen for the nonactivated mutagenicity test while 250, 500, 750, 1000, 1250, 1500, 1750 and 2000 μg/ml were chosen for the activated mutagenicity test (Table 8).

A dose-related response for total survival was noted in the nonactivated test and these values ranged from 5 to 97%. No appreciable mutation was noted in the nonactivated assay with exception that the highest dose (2500 μg/ml) resulted in a 3.1-fold increase in MF. Total survival for this culture (2500 μg/ml) was less than 10% and it was therefore not considered in the assessment of the mutagenesis of phenobarbital.

A dose-related decrease in values for total survival was observed in the activated test. Two cultures in this test showed a > 2-fold increase in MF over background and this effect was generally dose-dependent. Although survival was low at both these dose levels, it nonetheless did exceed 10%. Under the conditions described, phenobarbital was

TABLE 9

A SUMMARY OF RESULTS FOR THE MOUSE LYMPHOMA FORWARD-MUTATION ASSAY WITH SAFROLE [a]

Treatment	Concentration (μg/ml)	Percent total survival [b]	Mutation frequency [c]	Mutation index [d]
Nonactivated test				
DMSO [e]	(1%)	100	4.1 (3.8) [g]	(1.0)
DMSO [e]	(1%)	100	3.5	
EMS [f]	620	4	198.8	52.3
Safrole	70	29	2.6	0.7
Safrole	60	106	2.7	0.7
Safrole	50	95	4.1	1.1
Safrole	40	136	2.8	0.7
Safrole	30	95	4.1	1.1
Safrole	20	86	2.8	0.7
Safrole	10	123	3.1	0.8
Safrole	1	162	2.0	0.5
Activated test				
DMSO [e]	(1%)	100	3.6 (3.5) [g]	(1.0)
DMSO [e]	(1%)	100	3.4	
3MC [f]	4	3	36.7	10.5
Safrole	5.0	27	5.6	1.6
Safrole	2.5	34	5.5	1.6
Safrole	1.0	12	3.9	1.1
Safrole	0.75	77	3.8	1.1
Safrole	0.50	83	5.8	1.7
Safrole	0.25	92	4.2	1.2
Safrole	0.10	87	3.7	1.1
Safrole	0.05	95	3.2	0.9

[a] Calculations appear in Appendix A.
[b] (Suspension growth)×(cloning efficiency).
[c] $TK^{-/-}$ mutants per 1×10^5 colony-forming cells.
[d] (Mutation frequency of treated culture)/(control mutation frequency).
[e] Solvent control.
[f] Positive control.
[g] Mean of solvent control.

judged to be mutagenic in the L5178Y mouse lymphoma $TK^{+/-}$ assay only with metabolic activation.

Safrole

A preliminary toxicity test with safrole resulted in the selection of 1, 10, 20, 30, 40, 50, 60 and 70 μg/ml as doses for mutagenicity testing without metabolic activation and 0.05, 0.1, 0.25, 0.50, 0.75, 1.0, 2.5 and 5.0 μg/ml with metabolic activation (Table 9).

In the non-activated test, the highest dose, 70 μg/ml, resulted in a value of 29% for total survival, however, all lower doses gave values for total survival of near 100%. No dose-related increase in MF was seen in the nonactivated test.

A dose-related cytotoxic effect was observed in the activated phase of the test. In addition, a slight increase in MF was noted with several cultures, but none of these showed values which were greater than two-fold over controls. As a result of these findings, safrole was judged not to be mutagenic in the L5178Y cells.

o-Toluidine

Based on the results from a preliminary toxicity

TABLE 10

A SUMMARY OF RESULTS FOR THE MOUSE LYMPHOMA FORWARD-MUTATION ASSAY WITH *o*-TOLUIDINE [a]

Treatment	Concentration (μg/ml)	Percent total survival [b]	Mutation frequency [c]		Mutation index [d]
Nonactivated test					
DMSO [e]	(1%)	100	0.9	(1.55) [g]	(1.0)
DMSO [e]	(1%)	100	2.2		
EMS [f]	620	17	87.6		56.5
o-Toluidine	1000	6	3.1		2.0
o-Toluidine	875	9	3.9		2.5
o-Toluidine	750	9	2.5		1.6
o-Toluidine	625	19	2.9		1.9
o-Toluidine	500	46	0.9		0.6
o-Toluidine	375	63	1.8		1.2
o-Toluidine	250	56	2.1		1.4
o-Toluidine	125	59	2.3		1.5
Activated test					
DMSO [e]	(1%)	100	2.4 (2.65) [g]		(1.0)
DMSO [e]	(1%)	100	2.9		
3MC [f]	3	12	33.5		12.6
o-Toluidine	400	7	4.8		1.8
o-Toluidine	300	20	4.9		1.8
o-Toluidine	200	32	3.8		1.4
o-Toluidine	100	56	3.2		1.2
o-Toluidine	75	33	3.5		1.3
o-Toluidine	50	61	1.6		0.6
o-Toluidine	25	76	3.8		1.4
o-Toluidine	10	107	2.5		0.9

[a] Calculations appear in Appendix A.
[b] (Suspension growth)×(cloning efficiency).
[c] $TK^{-/-}$ mutants per 1×10^5 colony-forming cells.
[d] (Mutation frequency of treated culture)/(control mutation frequency).
[e] Solvent control.
[f] Positive control.
[g] Mean of solvent control.

test with *o*-toluidine, doses of 125, 250, 375, 500, 625, 750, 875 and 1000 μg/ml were chosen for the nonactivated mutagenicity test and 10, 25, 50, 75, 100, 200, 300 and 400 μg/ml for the activated test (Table 10).

A dose-related response for total survival was observed with *o*-toluidine in both the nonactivated and activated tests with values ranging from 6 to 63% and 7 to 107%, respectively. Two cultures from the nonactivated test showed a 2-fold or greater increase in MF, however, both cultures also showed total survival of less than 10%. In the activated test several cultures showed an increase in MF yet these were not 2-fold greater than the control. Consequently, *o*-toluidine was judged not to be mutagenic in the L5178Y mouse lymphoma $TK^{+/-}$ forward-mutation assay.

The spontaneous mutation frequencies in control cultures and the levels of activity of the positive controls (EMS and 3MC) were within acceptable ranges in each of these experiments and confirmed the sensitivity and reproducibility of the test system.

Appendix A

Calculations

Percent suspension growth

A.

$$\frac{\text{Day 2 cell count (viable cells/ml)}}{3 \times 10^5 \text{ (or day 1 cell count if } < 300\,000)} = \text{day 2 culture replication}$$

B. Day 1 cell count (viable cells $\times 10^6$/ml) $\times$ A = total replication

C. Percent suspension growth:

1. Solvent control = 100%
2.

$$\frac{\text{Positive control or test doses (see B above)}}{\text{solvent control * (see B above)}} \times 100\%$$

* Mean of two solvent controls.

Cloning efficiency

$$\frac{\text{Avg. No. colonies on nonselective plates in treated culture}}{\text{Avg. No. colonies on nonselective plates of solvent control}} \times 100\%$$

Percent total survival = % suspension growth $\times$ cloning efficiency

Mutation Frequency (MF)

The number of $TK^{-/-}$ mutants per 1×10^5 colony forming cells (CFC) plated into the selective cloning medium.

1. CFC = mean number of colonies on nonselective plate $\times$ 5000
2. Product of Step (1) $\div$ 1×10^5
3. MF = mean number of colonies on selective plates $\div$ quotient of Step 2

Mutation index =

$$\frac{\text{Mutation frequency of treated culture}}{\text{Mutation frequency of solvent control}}$$

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Assays for the induction of mutations to 6-thioguanine and ouabain resistance in Chinese hamster ovary (CHO) cells in culture

M.Z. Zdzienicka and J.W.I.M. Simons

Department of Radiation Genetics and Chemical Mutagenesis, State University of Leiden (The Netherlands)

Summary

Within the framework of the IPCS Collaborative Study on in vitro Short-Term Tests the mutagenicity of 5 compounds was studied (HMPA, TOL, BEN, SAF and CAP). Mutations were determined in Chinese hamster ovary (CHO) cells at the HPRT-locus (hypoxanthine-guanine phosphoribosyl transferase) and at the Na^+/K^+ ATPase locus. The cells were treated over a period of 4 days in the presence and the absence of a metabolic activation system. Metabolic activation was achieved by cocultivation of the cells with primary Syrian hamster embryo cells.

5 compounds were tested for mutagenicity at the HPRT and the Na/K-ATPase loci in CHO cells by means of a 4-day treatment in the absence and presence of a metabolic activation system. As a metabolic activation system, cocultivation with lethally-irradiated Syrian hamster embryo cells was used. No indication for mutagenicity was observed. *o*-toluidine in the presence of the metabolic activation system should be tested further before a definite conclusion on its mutagenic activity at the Na/K-ATPase locus can be drawn.

Materials and methods

Culture

The experiments were carried out with Chinese hamster ovary (CHO) cells. For each individual experiment one ampule, containing approximately 10^6 cells was taken from a stock, stored in liquid nitrogen. The cells were seeded in a petri dish, 145 mm in diameter (P_{145}, Greiner) containing 25 ml of Ham's F10 medium, modified by the omission of hypoxanthine and supplemented with 10% newborn calf serum, penicillin (100 μg/ml) and streptomycin (0.1 mg/ml). The cells were incubated in a humidified CO_2-incubator (5% CO_2) at 37°C. For subcultures a trypsin solution (0.5%) containing EDTA (0.02%) was used. After 3 days, cells were subcultured and seeded in P_{145} dishes at a density of 3.5×10^5 cells per dish. After 3 more days, the cells were trypsinized and seeded at a density of 10^6 cells per roller bottle, each containing 50 ml of medium.

Treatment

Chemicals were stored at 4°C in a refrigerator until use. The compounds were dissolved or diluted in DMSO (Merck). This stock solution was added to the cells in the roller bottles in the appropriate amount to obtain the desired concentration. The compounds were added 4 h after seeding the cells and the treatment occurred over 4 consecutive days. Metabolic activation of the compounds was achieved by cocultivation with irradiated primary Syrian hamster embryo cells (4000 rad), which were added to the roller bottle at the same time as the chemical, 5×10^6 cells per bottle.

Determination of mutant frequencies

After treatment the cells were washed 3 times with PBS, trypsinized and seeded for expression of induced mutations at a density of 3.5×10^5

TABLE 1

CYTOTOXICITY OF THE COMPOUNDS

Compound		Experimental groups			
		1	2	3	4
HMPA	Concentration (μl/ml)	0.0	2.0	5.0	10.0
	Cell number (%)	1.00	0.76	0.28	0.01
TOL	Concentration (μl/ml)	0.00	0.10	0.25	0.50
	Cell number (%)	1.00	1.01	0.74	0.26
		1.00	0.77	0.33	0.05
BEN	Concentration (μl/ml)	0.0	0.5	1.0	
	Cell number (%) expt. 1	1.00	1.03	0.87	
	Cell number (%) expt. 2	1.00	0.92	1.07	
SAF	Concentration (μl/ml)	0.0	0.5	1.0	2.5
	Cell number (%)	1.00	0.76	0.10	0.00
CAP	Concentration (mg/ml)	0.0	1.0	2.0	
	Cell number (%)	1.00	1.01	0.89	
B(α)P	Concentration (μg/ml)	0.0	1.0	10.0	50.0
	Cell number (%)	1.00	1.20	1.10	1.07
EMS	Concentration (μl/ml)	0.0	0.2		
	Cell number (%)	1.00	0.83		

The cells were grown for 4 days in the presence of the compound, after which the cell number was determined. Untreated cells increased about 40-fold during this period.

cells/P_{145}, 3 dishes per experimental group. After 4 days, the cells were trypsinized and seeded for selection in medium containing ouabain (1 mM) 2.5×10^5 cells/P_{90}, 10 P_{90} per group. The remainder of the cells were seeded again at a density of 3.5×10^5 cells/P_{145}, 3 dishes per group. After 4 more days (8 days after treatment) the cells were seeded in medium containing 6-thioguanine (5 μg/ml), 10^5 cells/P_{90}, 20 P_{90} per group. Next to seeding for selection, cells were seeded in standard medium for the determination of the cloning efficiency, 150 cells/P_{90}, 5 P_{90} per group. The mutant frequencies were calculated by correcting the observed mutant frequencies with the corresponding cloning efficiencies.

Results and discussion

The cytotoxicity of the compounds was measured by the determination of the cell number at the end of the 4-day treatment (Table 1). For the chosen concentrations cytotoxicity in the absence of a metabolic activation system was observed for hexamethylphosphoramide, *o*-toluidine, and safrole, but not for benzene and caprolactam. The cytotoxicity in the presence of a metabolic activation system could not be ascertained in this way as the metabolic activation system consisted out of cocultivation with Syrian hamster cells, which are also present in the cell population after the trypsinization. Because the trypsinized cells were seeded immediately in agar to determine transformation frequencies, another indication for cytotoxicity both in the absence and presence of the metabolic activation system was obtained from the cloning efficiencies in agar (Zdzienicka et al., this volume). The mutation frequencies for 6-thioguanine resistance and ouabain resistance, observed after treatment, are given in Table 2 and the conclusions drawn from these experiments are shown in Table 3. Induction of mutation was not observed for any of the compounds. For *o*-toluidine a conclusion could not yet be reached for mutation at the Na/K-ATPase locus.

TABLE 2

MUTAGENICITY OF THE COMPOUNDS

Compound		Metabolic activation	Experimental groups			
			1	2	3	4
HMPA	concentration (μl/ml)		0.0	2.0	5.0	
	HPRT ($\times 10^{-5}$)	−	0.1	1.3	2.7	
		+	2.7	3.2	3.0	
	Oua^R ($\times 10^{-6}$)	−	0.8	0.0	0.0	
		+	0.0	0.0	0.9	
TOL	concentration (μl/ml)		0.00	0.10	0.25	0.50
	HPRT ($\times 10^{-5}$)	−	2.7	0.6	3.0	2.2
			0.3	5.6	0.6	4.7
	HPRT ($\times 10^{-5}$)	−	0.4	0.7	1.9	0.2
		+	1.2	1.1	0.3	0.5
	Oua^R ($\times 10^{-6}$)	−	0.0	1.4	0.0	0.0
		+	0.0	2.9	4.1	1.4
BEN	concentration (μl/ml)		0.0	0.5	1.0	
	$HPRT^-$ ($\times 10^{-5}$)	−	0.9	0.3	0.6	
		+	0.3	0.2	0.8	
	HPRT ($\times 10^{-5}$)	−	2.6	2.8	3.4	
		+	1.7	1.1	2.9	
	Oua^R ($\times 10^{-6}$)	−	0.0	0.0	0.0	
		+	0.0	0.0	0.0	
SAF	concentration (μl/ml)		0.0	0.5	1.0	
	HPRT ($\times 10^{-5}$)	−	1.7	1.1	0.2	
		+	2.5	1.1	0.1	
	Oua^R ($\times 10^{-6}$)	−	0.6	2.5	0.5	
		+	0.9	0.0	0.5	
CAP	concentration (mg/ml)		0.0	1.0	2.0	
	$HPRT^-$ ($\times 10^{-5}$)	−	2.0	0.9	0.2	
		+	0.5	0.1	0.0	
	Oua^R ($\times 10^{-6}$)	−	1.3	0.8	0.4	
		+	0.5	0.0	0.4	
B[*a*]P	concentration (μg/ml)		0.0	10.0	50.0	
	$HPRT^-$ ($\times 10^{-5}$)	−	1.2	1.1	0.9	
		+	0.5	32.0	29.9	
	Oua^R ($\times 10^{-6}$)	−	0.6	0.0	0.8	
		+	0.6	9.1	35.0	
EMS	concentration (μl/ml)		0.0	0.2		
	HPRT ($\times 10^{-5}$)	−	2.0	24.7		
	Oua^R ($\times 10^{-6}$)	−	1.3	155.8		

TABLE 3

CONCLUSIONS ON THE MUTAGENICITY OF THE COMPOUNDS

Compound	HPRT		Oua^R	
	− metabolic activation	+ metabolic activation	− metabolic activation	+ metabolic activation
Hexamethylphosphoramide	−	−	−	−
o-Toluidine	−	−	−	?
benzene	−	−	−	−
Safrole	−	−	−	−
Caprolactam	−	−	−	−

Reference

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Assays for the induction of gene mutations at the thymidine kinase and the Na^+/K^+ ATPase loci in two different mouse lymphoma cell lines in culture

J.A. Styles, P. Clay and M.F. Cross

Imperial Chemical Industries PLC, Central Toxicology Laboratory, Alderley Park, Cheshire (Great Britain)

Summary

Studies conducted on the test chemicals gave positive results for hexamethylphosphoramide (HMPA), safrole (SAF), *o*-toluidine (TOL), benzene (BEN), diethylstilboestrol (DES), phenobarbitone (PB) and benzoin (ZOIN). Diethylhexylphthalate (DEHP) and acrylonitrile (ACN) gave negative results. No conclusive result was obtained with caprolactam (CAP).

The L5178Y mouse lymphoma gene mutation assay, adapted to a multiwell plating method, was used to test the selected chemicals for their ability to induce mutations at the thymidine kinase and Na^+/K^+ ATPase loci.

Materials and methods

Cell lines

Two cell lines were used in this study: a L5178Y $TK^{+/+}$ cell line obtained from J. Cole (MRC, Cell Mutation Unit, Sussex University); and a L5178Y clone $372^{+/-}$ cell line obtained from L. Weissbecker, Philip Morris Research Center Richmond, VA (U.S.A.) (originally from D. Clive, Burroughs Wellcome Co., Research Triangle Park, NC, U.S.A.). The $TK^{+/+}$ cell line was used in experiments when only ouabain was used for selection and the $TK^{+/-}$ when selecting for mutants with trifluorothymidine (TFT) and when comparing ouabain mutants to TFT mutants. Cell stocks were stored in a liquid nitrogen freezer. The stock cell cultures were maintained in an exponentially growing suspension culture is roller bottles in a 37°C hotroom on a Bellco roller. The normal maintenance medium was RPMI 1640 supplemented with 4 mM L-glutamine; 200 units/ml of penicillin/streptomycin and 10% donor horse serum.

Preparation of dosing solutions

All test chemicals were stored at 4°C until used. Dosing solutions were prepared immediately prior to each assay and were made up in DMSO at 100 times the final exposure concentration, giving a final concentration of DMSO in the cultures of 1%. Positive controls were prepared in the same way. These were: benzo[α]pyrene (Bαp) at a final concentration of 10 μg/ml when S9 mix was used, and ethyl methanesulphonate (EMS) at a final concentration of 1000 μg/ml when S9 mix was omitted. DMSO at a concentration of 1% v/v was the negative control in the presence or absence of S9 mix.

Preliminary cytotoxicity testing

To determine the toxicity of a test chemical, a range of dilutions of it were assayed for their effect on cell survival. From these data and/or historial data, dose levels were chosen for the mutation assay to give a wide range of toxicity. If no toxicity occurred, a top dose of 2000 μg/ml for solids was used and for liquids, the test chemical added at 1% v/v to the cultures.

Metabolic activation

Chemicals were tested both with and without auxiliary metabolism (S9 mix). S9 was prepared aseptically from Sprague–Dawley rats dosed with

500 mg/kg body weight of Aroclor 1254 five days before sacrifice. The S9 was stored at −70 °C until used. The S9 mix was prepared by mixing one part S9 to three parts of cofactor solution (33 mM KCl, 8 mM $MgCl_2$, 5 mM glucose 6-phosphate, 4 mM NADP and 100 mM K_2HPO_4 in RPMI 1640 medium) and was added at 20% v/v to cultures just prior to the addition of the test chemical. The equivalent amount of maintenance medium was added at 20% v/v to cultures without S9 mix to maintain the same volume and cell concentration.

Mutation assay

Mutant cells were detected by their growth in medium supplemented with either TFT (2 μg/ml) (Clive et al., 1982, 1979) or ouabain (730 μg/ml) (Brusick, 1980) when dispensed into multiwell plates (Gatehouse, 1978; Kraemer et al., 1980; Thilly et al., 1980). The experimental procedure was adapted from that described by Cole (1983).

For each dose level of test chemical or control, 1×10^7 cells from an exponentially growing culture were exposed for 2 h in a 50-ml centrifuge tube placed on a roller in a room maintained at 37 °C. The test chemical was removed by centrifugation and the cell pellet resuspended in fresh medium. Immediately following treatment, an aliquot of cells from each dose level was taken, diluted to 8 cells/ml in RPMI supplemented with 20% horse serum and dispensed into one 96-well plate per dose level at 1.6 cells/well to estimate percentage survival relative to the negative control. The main posttreated cultures were then grown on the roller for the predetermined expression time (Clive et al., 1972, 1979; Cole and Arlett, 1976; Moore et al., 1981), removing only to dilute as appropriate to maintain the cells in exponential growth.

On reaching the expression time (usually 48 h, but sometimes extended to 72 h), the concentration of cells in each culture was counted and diluted with RPMI supplemented with 20% horse serum to give 3 series: (A) 1×10^4/ml in medium supplemented with 2.0 μg/ml TFT; (B) 2×10^5/ml in medium supplemented with 730 μg/ml ouabain; (C) 8 cells/ml to determine plating efficiency (viability) for (A) and (B) above.

These were then dispensed at 200 μl/well into 96-well plates [2 plates/dose for the mutation series (A) and (B), 1 plate per dose for plating efficiency (C)]. The microwell cultures were placed in humidified incubators with atmospheres enriched with 5% CO_2. After 10 days, the plates were scored for wells showing no growth. The mutation frequency for each dose was calculated using the following formula (Thilly et al., 1980):

$$\text{Mutation frequency} = \left[-\ln(x_s/n_s)/-\ln(x_0/n_0)\right] \times \text{dilution factor}$$

where, dilution factor = difference in cell concentrations between plating efficiency plates and mutation plates; x_s = number of empty wells with selective agent (mutation plates); n_s = total number of wells plated with selective agent; x_0 = number of empty wells without selective agent (plating efficiency plates); n_0 = total number of wells plated without selective agent.

An assay was considered valid if the following criteria were met: (i) the positive controls showed the expected responses; (ii) there was a dose-related toxicity range (or the compound was tested to its solubility limits or to maximum toxicity); (iii) the plating efficiency was considered adequate.

Statistics

A statistical analysis of the results was applied in certain cases of equivocal results (Thomas et al., unpublished). The statistical analysis was based on the same distributional assumptions as that of Furth et al. (1981). The distribution of empty wells was assumed binomial with a parameter given by zero term of a poisson distribution of viable cells per well. The log mean of the poisson distribution was expressed as a linear function of log mutant frequencies, log survival and log dilution factors. This relation was used to estimate log mutant frequencies. The estimation of log mutant frequencies was asymptotically normal which led to large sample tests of heterogeneity of mutant frequencies from standard normal theory.

Results and discussion

The results of assays carried out on individual chemicals are given in Table 2. A summary of test results is given in Table 1.

HMPA gave a clear positive result in the absence of S9 mix. The negative result with S9 and ouabain was invalid since BαP failed to elicit a positive response.

Safrole gave a clear positive result without S9 at the TK locus; the positive results with or without S9 mix at the oua locus were weak but reproducible. The repeat test without S9 required statistical analysis to arrive at a positive result.

o-Toluidine was considered positive on the basis of a single strong result without S9 at the TK locus. The first test was negative at both loci.

Benzene produced an unequivocal positive result with S9 at the oua locus but a weak one with S9 at the TK locus. The test failures without S9 at both loci were due to excessive toxicity of benzene.

DEHP gave a clear negative result.

Acrylonitrile showed no evidence of toxicity at the TK locus in two attempts to test the chemical. Similarly, two attempts to test the activity at the oua locus also gave no toxicity without S9. A single negative result with S9 at the oua locus led to acrylonitrile being classified as negative.

Diethylstilboestrol produced clear positive results with S9 at both loci, while in the absence of S9 the results were negative.

Phenobarbitone at the TK locus gave an unequivocal positive result with S9 but a repeat experiment gave a negative response. The test with S9 at the oua locus failed because BαP gave a negative result; in the absence of S9 there was a negative result but a repeat test gave a weak positive response.

Caprolactam did not allow a definitive conclusion since it did not produce any evidence of toxicity to accompany the negative mutagenicity result.

Benzoin produced an overall positive result because the single positive response without S9 at the oua locus was a very clear effect.

The spontaneous mutation frequency observed for the TK locus during the study was $11.7 \times 10^{-4} \pm 12.2$ (SD) $n = 21$, while that for the oua locus was $19.1 \times 10^{-6} \pm 16.2$ (SD) $n = 26$.

The results, limited as they are with respect to numbers of chemicals and reproducibility, indicate that the two loci are comparable in their sensitivity, irrespective of their accuracy. There are only two major contrasting results: *o*-toluidine and benzoin, although the positive responses given by safrole (without S9 in TK and ±S9 in oua) and phenobarbitone (with S9 in TK and without S9 in oua) are also noteworthy in that they suggest that

TABLE 1

SUMMARY OF TEST RESULTS

Compound	Selective agent				Combined result
	TFT		Oua		
	+S9	−S9	+S9	−S9	
HMPA	−	+	I	+	+
Safrole	−	+	+/+^{R}	+/+^{R}	+
o-Toluidine	−/−^{R}	−/+^{R}	−/−^{R}	−/−^{R}	+
Benzene	+	I	+	I	+
DEHP	−	−	−	−	−
Acrylonitrile	I	I	−	I	−
DES	+	−	+	−	+
Phenobarbitone	−/+^{R}	−	I	−/+^{R}	+
Caprolactam	NT	I	NT	I	I
Benzoin	−	−	NT	+/−^{R}	+

I, test system invalid; no conclusion.
R, repeat test.
NT, not tested.

TABLE 2

RESULTS OF TESTING 10 WHO/IPCS CHEMICALS IN THE L5178Y TK$^{+/-}$ ASSAY

Dose (μg/ml)	+ or − S9	P.E. positive wells/plate	Mutants positive wells/plate	% Survival	Mutation frequency
Compound: HMPA					($\times 10^{-4}$)
Selective agent: TFT					
10300	+	15	24	65	7.5
5150	+	15	19	77	5.7
2575	+	9	16	46	8.1
1287.5	+	21	23	73	4.9
0	+	16	24	100	6.9
10300	−	3	13	104	20.2
5150	−	11	24	72	10.4
2575	−	12	29	72	11.9
1287.5	−	12	19	88	7.3
0	−	16	32	100	9.8
Compound: HMPA					($\times 10^{-6}$)
Selective agent: ouabain					
10300	+	15	20	65	30.3
5150	+	15	26	77	40.9
2575	+	9	15	46	38.0
1287.5	+	21	17	73	17.4
0	+	16	34	100	52.8
10300	−	3	9	104	68.2
5150	−	11	28	72	62.3
2575	−	12	21	72	40.7
1287.5	−	12	12	88	22.0
0	−	16	24	100	34.7
Compound: safrole (1)					($\times 10^{-4}$)
Selective agent: TFT					
54.8	+	30	29	83	7.7
27.4	+	70	41	104	3.4
13.7	+	54	31	113	3.8
6.9	+	62	39	179	4.0
0	+	45	39	100	6.5
54.8	−	26	37	73	12.3
27.4	−	27	35	76	11.0
13.7	−	39	31	78	6.0
6.9	−	37	25	81	5.0
0	−	42	26	100	4.4
Compound: safrole (1)					($\times 10^{-6}$)
Selective agent: ouabain					
54.8	+	30	15	83	18.1
27.4	+	70	27	104	10.1
13.7	+	54	12	113	6.5
6.9	+	62	22	179	10.0
0	+	45	8	100	5.5
54.8	−	26	17	73	24.7
27.4	−	27	20	76	28.3
13.7	−	39	21	78	18.9
6.9	−	37	17	81	16.0
0	−	42	10	100	7.6

TABLE 2 (continued)

Dose (μg/ml)	+ or − S9	P.E. positive wells/plate	Mutants positive wells/plate	% Survival	Mutation frequency
Compound: safrole (2)					($\times 10^{-6}$)
Selective agent: ouabain					
109.5	+	28	84	5	241.2
54.8	+	45	67	66	75.7
27.4	+	52	60	83	50.3
13.7	+	42	58	115	64.4
0	+	55	58	100	43.6
109.5	−	–	–	2.5	–
54.8	−	69	87	50	74.6
27.4	−	54	66	70	56.3
13.7	−	66	71	93	46.3
0	−	52	55	100	43.6
Compound: *o*-toluidine (1)					(10^{-6})
Selective agent: TFT					
1004	+	36	24	45	4.9
100	+	35	15	88	3.0
10	+	51	27	116	3.5
1	+	40	15	100	2.5
0	+	53	29	100	3.5
1004	−	46	26	31	3.9
100	−	53	24	90	2.9
10	−	47	23	166	3.3
1	−	54	33	83	4.1
0	−	50	30	100	4.1
Compound: *o*-toluidine (1)					($\times 10^{-6}$)
Selective agent: ouabain					
1004	+	36	12	45	11.4
100	+	35	4	88	3.8
10	+	51	19	116	11.6
1	+	40	24	100	21.3
0	+	53	12	100	6.7
1004	−	46	27	31	20.3
100	−	53	20	90	11.6
10	−	47	23	166	16.3
1	−	54	12	83	6.5
0	−	50	32	100	22.0
Compound: *o*-toluidine (2)					($\times 10^{-4}$)
Selective agent: TFT					
1004	+	42	45	59	8.8
100	+	54	55	100	8.2
10	+	51	55	105	8.9
1	+	70	52	111	4.8
0	+	45	42	100	7.3
1004	−	48	58	58	10.7
100	−	45	38	83	6.4
10	−	42	49	96	9.9
1	−	56	52	91	7.1
0	−	64	39	100	3.8

TABLE 2 (continued)

Dose (μg/ml)	+ or − S9	P.E. positive wells/plate	Mutants positive wells/plate	% Survival	Mutation frequency
Compound: *o*-toluidine (2)					($\times 10^{-6}$)
Selective agent: ouabain					
1004	+	42	13	59	10.1
100	+	54	4	100	2.1
10	+	51	15	105	9.0
1	+	70	5	111	1.6
0	+	45	10	100	7.0
1004	−	48	11	58	7.0
100	−	45	16	83	11.5
10	−	42	16	96	12.7
1	−	56	7	91	3.5
0	−	64	21	100	9.0
Compound: benzene					($\times 10^{-4}$)
Selective agent: TFT					
500	+	45	29	5	4.5
250	+	50	36	22	5.1
125	+	63	34	27	3.3
62.5	+	68	27	40	2.1
0	+	72	38	100	2.9
500	−	38	23	3	4.3
250	−	52	26	6	3.2
125	−	59	32	8	3.4
62.5	−	69	27	18	2.1
0	−	61	24	100	2.3
Compound: benzene					($\times 10^{-6}$)
Selective agent: ouabain					
500	+	45	8	5	5.5
250	+	50	11	22	6.6
125	+	63	18	27	7.8
62.5	+	68	13	40	4.7
0	+	72	5	100	1.2
500	−	38	10	3	8.7
250	−	52	5	6	2.7
125	−	59	3	8	1.3
62.5	−	69	5	18	1.7
0	−	61	19	100	8.7
Compound: DEHP					($\times 10^{-4}$)
Selective agent: TFT					
9810	+	49	47	83	7.5
1962	+	51	49	62	7.5
392	+	62	36	73	2.0
78	+	58	40	108	4.5
0	+	36	42	100	9.8
9810	−	59	42	102	4.8
1962	−	60	48	102	5.7
392	−	82	57	115	3.7
78	−	59	32	98	3.4
0	−	62	34	100	3.3

TABLE 2 (continued)

Dose (μg/ml)	+ or − S9	P.E. positive wells/plate	Mutants positive wells/plate	% Survival	Mutation frequency
Compound: DEHP					($\times 10^{-6}$)
Selective agent: ouabain					
9810	+	49	7	83	4.2
1962	+	51	5	62	2.2
392	+	62	5	73	1.6
78	+	58	2	108	0.5
0	+	36	8	100	7.4
9810	−	59	2	102	0.4
1962	−	60	3	102	1.3
392	−	82	13	115	3.0
78	−	59	4	98	1.8
0	−	62	9	100	3.8
Compound: acrylonitrile (1)					($\times 10^{-4}$)
Selective agent: TFT					
100	+	64	39	105	3.8
50	+	69	44	105	3.9
25	+	67	25	93	2.0
12.5	+	66	39	95	3.6
0	+	36	42	100	9.8
100	−	72	41	112	3.2
50	−	68	47	117	4.4
25	−	60	36	102	3.8
12.5	−	67	37	124	3.3
0	−	62	34	100	3.3
Compound: acrylonitrile (1)					($\times 10^{-6}$)
Selective agent: ouabain					
100	+	64	9	105	3.6
50	+	69	5	105	1.7
25	+	67	10	93	3.7
12.5	+	66	6	95	2.2
0	+	36	8	100	7.4
100	−	72	13	112	4.2
50	−	68	4	117	1.4
25	−	60	8	102	3.5
12.5	−	67	12	124	4.5
0	−	62	9	100	3.8
Compound: acrylonitrile (2)					($\times 10^{-6}$)
Selective agent: ouabain					
50	+	36	6	22	5.5
25	+	70	10	53	3.4
12.5	+	72	4	71	1.2
6.25	+	65	7	87	2.7
0	+	66	8	100	3.0

TABLE 2 (continued)

Dose (μg/ml)	+ or − S9	P.E. positive wells/plate	Mutants positive wells/plate	% Survival	Mutation frequency
Compound: DES					($\times 10^{-4}$)
Selective agent: TFT					
50	+	21	43	70	19.2
25	+	33	29	91	6.8
12.5	+	21	29	116	11.7
6.25	+	30	36	63	10.0
0	+	31	33	100	8.6
25	−	63	32	132	3.0
12.5	−	53	38	129	5.0
6.25	−	46	37	68	6.0
0	−	24	37	100	13.5
Compound: DES					($\times 10^{-6}$)
Selective agent: ouabain					
50	+	21	19	70	35.7
25	+	33	6	91	6.1
12.5	+	21	22	116	42.2
6.25	+	30	12	63	14.3
0	+	31	10	100	11.3
25	−	63	7	132	2.8
12.5	−	53	13	129	7.2
6.25	−	46	25	68	18.5
0	−	24	23	100	38.1
Compound: phenobarbital (1)					($\times 10^{-4}$)
Selective agent: TFT					
2000	+	9	33	14	34.2
1000	+	13	33	20	23.2
500	+	17	28	28	14.2
250	+	14	22	35	13.2
0	+	13	20	100	12.8
Compound: phenobarbital (1)					($\times 10^{-6}$)
Selective agent: ouabain					
2000	+	9	27	14	134.2
1000	+	13	11	20	33.5
500	+	17	3	28	6.5
250	+	14	13	35	36.9
0	+	13	3	100	8.7
Compound: phenobarbital (2)					($\times 10^{-4}$)
Selective agent: TFT					
2000	+	19	59	90	34.6
1000	+	52	61	130	10.3
500	+	67	68	175	8.2
250	+	50	59	130	10.4
0	+	12	39	100	31.2
2000	−	31	47	3	13.8
1000	−	25	49	31	18.9
500	−	46	53	38	9.9
250	−	48	59	41	11.0
0	−	26	44	100	15.5

TABLE 2 (continued)

Dose (μg/ml)	+ or − S9	P.E. positive wells/plate	Mutants positive wells/plate	% Survival	Mutation frequency
Compound: phenobarbital (2)					($\times 10^{-6}$)
Selective agent: ouabain					
2000	+	19	7	90	13.7
1000	+	52	6	130	3.3
500	+	67	18	175	6.9
250	+	50	12	130	7.3
0	+	12	4	100	12.7
2000	−	31	6	3	6.6
1000	−	25	8	31	11.5
500	−	46	13	38	8.9
250	−	48	15	41	9.8
0	−	26	9	100	12.5
Compound: phenobarbital (3)					($\times 10^{-6}$)
Selective agent: ouabain					
2000	−	57	72	64	61.6
1000	−	58	68	81	53.2
500	−	45	62	93	65.6
250	−	68	67	70	38.9
0	−	53	51	100	37.7
Compound: caprolactam					($\times 10^{-4}$)
Selective agent: TFT					
1000	−	39	88	98	38.1
500	−	38	94	100	61.5
250	−	21	91	105	95.8
125	−	30	95	107	97.5
0	−	29	82	100	42.8
Compound: caprolactam					($\times 10^{-6}$)
Selective agent: ouabain					
1000	−	39	15	98	13.0
500	−	38	20	100	18.5
250	−	21	19	105	35.7
125	−	30	29	107	38.4
0	−	29	26	100	35.1
Compound: benzoin (1)					($\times 10^{-4}$)
Selective agent: TFT					
1000	+	19	69	4	46.0
500	+	33	71	3	25.6
250	+	28	58	9	21.5
125	+	31	74	10	30.2
0	+	33	83	100	38.0
1000	−	7	30	15	40.0
500	−	27	42	19	13.9
250	−	40	45	66	9.4
125	−	45	52	57	9.9
0	−	29	82	100	42.8

TABLE 2 (continued)

Dose (μg/ml)	+ or − S9	P.E. positive wells/plate	Mutants positive wells/plate	% Survival	Mutation frequency
Compound: benzoin (1)					($\times 10^{-6}$)
Selective agent: ouabain					
500	−	27	32	19	49.1
250	−	40	29	66	26.7
125	−	45	19	57	13.9
0	−	29	26	100	35.1
Compound: benzoin (2)					($\times 10^{-6}$)
Selective agent: ouabain					
250	−	34	92	16	290.8
125	−	38	83	24	158.7
62.5	−	26	67	57	151.6
0	−	53	51	100	37.7

a substantial contribution is made by the target cells' metabolic system to the activation of the test chemical.

Although the plating efficiency fell below 20% on occasions, most of the experiments attained a level between 30% and 50% and some were higher. Plating efficiencies of this order were typical of this assay at the time of the study so the experiments were considered to be comparable. Since completing this study, plating efficiences have been improved and presently average around 75%.

At the outset of the study it was decided that the outcome of each experiment should either be classified as positive, negative or invalid. Time did not permit the repetition of experiments until a consistent result was obtained, hence the final decision was often based on the occurrence of a single reasonably credible result.

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Tests with the Chinese hamster V79 inhibition of metabolic cooperation assay

Eugene Elmore, Elisabeth A. Korytynski and Mary P. Smith

In Vitro Toxicology, Northrop Services, Inc.– Environmental Sciences, Research Triangle Park, NC 27709 (U.S.A.)

Summary

The Chinese hamster V79 inhibition of metabolic cooperation assay was evaluated as a potential in vitro bioassay system to complement the bacterial tests for detecting compounds with carcinogenic potential. Three chemicals, diethylhexylphthalate, *o*-toluidine and acrylonitrile, produced moderate to strong positive responses; three chemicals, safrole, phenobarbital and benzoin, produced weakly positive responses; and four chemicals, hexamethylphosphoramide, diethylstilbestrol, benzene and caprolactam, produced negative responses in the assay. The results from these studies and others suggest that the Chinese hamster V79 inhibition of metabolic cooperation assay for epigenetic chemicals could complement the bacterial tests for environmental toxicants; however, additional studies are needed to fully assess the potential of this relatively new assay.

The development and validation of in vitro bioassays to detect epigenetic chemicals, which act primarily through nongenetic mechanisms, is of great importance since many environmentally important chemicals fall into this category. In addition, assays that respond to epigenetic chemicals may also detect agents whose primary effect is genetic but which also exhibit epigenetic activity. Chemicals that could potentially be detected by such assays include cocarcinogens, tumor promoters, teratogens, toxins that alter reproductive development, neurotoxins, and some carcinogens. The carcinogens selected by the International Program for Chemical Safety (IPCS) for these studies were those with hard-to-detect genetic activity in bacterial mutagenicity assays. The Chinese hamster V79 inhibition of metabolic cooperation assays meets the requirements of an epigenetic assay because it has been shown to be responsive to many of the classes of compounds known to act through epigenetic mechanisms.

The inhibition of metabolic cooperation assay is based on the enhanced recovery of hypoxanthine–guanine phosphoribosyl transferase (HG–PRT)-deficient mutants from high-density populations of HG–PRT proficient wild-type cells. HG–PRT is an enzyme from an X-linked recessive locus in mammalian cells. This salvage enzyme catalyzes the conversion of hypoxanthine and guanine to the corresponding nucleoside 5′-monophosphate (Chu, 1971; Caskey and Kruh, 1979). The enzyme can also catalyze the phosphorylation of several purine analogues, 6-thioguanine(6-TG), 8-azaguanine(8-AG) and 6-mercaptopurine (Chu, 1971; Caskey and Kruh, 1979). The product is then further phosphorylated and incorporated into both DNA and RNA (Nelson et al., 1975) resulting in cell death. Cells that are HG–PRT-deficient ($HG–PRT^-$) cannot phosphorylate the purines and are therefore resistant to cell killing by the purine analogues.

When mammalian cells are cultured at a density that permits frequent contact between cells, gap junctions can form at the points of contact. Cells connected with each other via gap junctions can transfer nutrients, ions, and other molecules di-

rectly to one another resulting in metabolic cooperation (Cox et al., 1976). Metabolic cooperation is known to play an important role in the selection and recovery of HG–PRT$^-$ cells using many of the purine analogues (Van Zeeland et al., 1972). When HG–PRT$^-$ cells make contact with cocultured HG–PRT$^+$ cells and gap junctions form, the phosphorylated base analogues can be transferred from the HG–PRT$^+$ cells to the HG–PRT$^-$ cells (Van Zeeland et al., 1972). Increasing the number of HG–PRT$^+$ cells in cultures containing both HG–PRT$^+$ and HG–PRT$^-$ cells will result in an increased probability of cell-to-cell contact and gap junction formation, and a decreased probability of recovery of HG–PRT$^-$ cells in purine-analogue selective medium. Under these conditions, the HG–PRT$^-$ cells will not form colonies. The degree of inhibition of colony formation depends upon (a) the size of the cells and the surface area each cell occupies in the dish used in the selection; (b) the presence in the media of various purine bases and/or other agents that might influence the sensitivity of the cells to purine analogues; (c) the concentration of the purine analogue used for selection, which will determine the number of doublings permitted before cell death; and (d) the presence of agents which modulate gap junctional communication.

The experiments of Yotti et al. (1979), Newbold and Amos (1981), and Umeda et al. (1980) have clearly demonstrated that the decrease in HG–PRT$^-$ cell recoverability at high cell densities can be inhibited by adding the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) to the selective media. Using direct communication assays, Fitzgerald and Murray have also shown that TPA inhibits junctional communication between various cell types (Murray and Fitzgerald, 1979; Fitzgerald and Murray, 1980; Fitzgerald et al., 1983) thereby offering a potential explanation for the observed phenomenon. The results from several laboratories using the Chinese hamster V79 inhibition of metabolic cooperation assay are presented in Table 1. The data suggest that the assay has broad applicability for evaluating chemicals that produce a variety of chronic human health effects and has potential as a preliminary assay to complement the bacterial assays for screening environmental toxicants.

TABLE 1

CHEMICALS TESTED IN THE CHINESE HAMSTER V79 INHIBITION OF METABOLIC COOPERATION ASSAY

Compound	Reported in vitro response
Trosko et al. (1981, 1982)	
12-*O*-Tetradecanoyl-phorbol-13-acetate (TPA)	+
Phorbol-12,13-didecanoate (PDD)	+
4αPDD	–
4-*O*-Methyl TPA	+
Phorbol-12,13-diacetate (PDA)	+
Phorbol-12,13-dibutyrate (PDBu)	+
Phenobarbital	+
Butylated hydroxytoluene	+
Mezerein	+
Melittin	+
DDT	+
Lindane	+
Chlordane	–
Anthralin	+
Saccharin (pure)	+
Saccharin (impure)	+
Tween 60	+
Tween 80	+
Cholesterol	–
Deoxycholic acid	+
Lithocholic acid	+
Taurodeoxycholic acid	–
Cytocalasin B	+
Cytocalasin D	–
Dinitrofluorobenzene	+
Polybromobiphenyl	+
3′,4′,5′,3,4,5-Hexabromobiphenyl	–
2′,4′,5′,2,4,5-Hexabromobiphenyl	+
3′,4′,5′,2,4,5-Hexabromobiphenyl	+
3′,4′,2,5,5-Pentabromobiphenyl	+
2′,4′,5′,2,3,4,5-Heptabromobiphenyl	+
2′,3′,4′,5′,2,3,4,5-Octobromobiphenyl	+
Heptachlor epoxide	+
Hydroquinone	+
Benzoyl peroxide	+
Valium	+
Methyl clofenopate	–
Nafenopin	+
[4-Chloro-6-(2,3-xylidino) 2-pyrimidinylthio]acetic acid (Wy-14643)	+
4-Chloro-6-(2,3-zylidino)2-pyrimidinylthio (*N*-β-hydroxyethyl)-acetamide (BR 931)	–
Diphenylhydantoin	+
9-Methyl-pteroylglutamic acid	+
Mestranol	+

TABLE 1 (continued)

Compound	Reported in vitro response
Ethinyl estradiol	+
Norethynodrel	+
Ethyl alcohol	−
Retinoic acid	−
Dibutyryl cAMP	−
DMSO	−
Epidermal growth factor	−
Fibroblast derived growth factor	−
Formaldehyde	−
Thalidomide	−
Umeda et al. (1980)	
Concanavalin A	+
Lithocholic acid	+
Chaetoglobosin A	+
3-Hydroxyanthranilic acid	+
Urea	+
Anthralin	+
Saccharin	+
Phenobarbital	−
Sodium deoxycholate	−
Adrenaline	−
Dispase	−
Elastinal	−
Pepstatin	−
Polymyxin B	−
EDTA	−
Malcolm and Mills (1984)	
Di-(ethylhexyl) phthalate (DEHP)	+
Catechol	+
Quinol	+
Hydroxyquinol	+
Butylated hydroxytoluene (BHT)	+
Trisodium nitrilotriacetate	+
Dimethyl sulfoxide (DMSO)	+
4-Chlorobiphenyl	+
Sodium cyclamate	+
1,4-Benzoquinone	+
Phenyl sulfate	+
Ethanol	+
Phenol	−
3,4-Benzopyrene	−
Phenylglucuronide	−
2-Methoxyphenol	*
Welsch and Stedman (1984)	
TPA	+
Warfarin	+
Diphenylhydantoin	+
2,4,5,2′,4′,5′-hexabromobiphenyl	+
Ethylene glycol monomethyl ether	+
Ethylene glycol monoethyl ether	+
Ethylene glycol monobutyl ether	+
Ethylene glycol mono-*n*-propyl ether	+
Ethylene glycol monoisopropyl ether	+
Saccharin	−
Ascorbic acid	−
Newbold and Amos (1981)	
Benzo[*e*]pyrene	−
Benzo[*a*]pyrene	−
Benz[*a*]anthracene	−
Dimethylbenz[*a*]anthracene	−
Barrett et al. (1982)	
DES	−
Warren et al. (1982)	
Nitro-fluorobenzene	+
Trosko and Horrobin (1980)	
Diazepam	+
Tsushimoto et al. (1982)	
Various polybrominated biphenyls	+

+, compounds reported to inhibit metabolic cooperation.
−, compounds reported to be negative in the inhibition of metabolic cooperation assay.
*, compound reported to enhance metabolic cooperation.

The objectives for this IPCS collaborative study were to (1) identify complementary assay systems which, when coupled with the bacterial tests, could provide improved resolution relative to the bacterial tests alone, and (2) evaluate those short-term tests that might detect carcinogens with non-genetic mechanisms of action. The Chinese hamster V79 inhibition of metabolic cooperation assay meets both of these objectives. Evaluation of the carcinogenic and noncarcinogenic chemicals selected by the IPCS in this relatively new assay was of considerable interest.

Materials and methods

Cells and growth conditions

The 6-thioguanine-sensitive wild-type (6-TG^s), and X-ray-induced 6-thioguanine-resistant mutant

(6-TG^r) Chinese hamster V79 cells were kindly provided by J.E. Trosko, Michigan State University, East Lansing, MI. The cells were routinely maintained in modified Eagle's minimal essential medium with Earle's salts with a 50% increase in all vitamins and essential amino acids except glutamine, 100% increase in the nonessential amino acids, 1 mM pyruvate (Grand Island Biological Company, Grand Island, NY) and 3% fetal bovine serum (FBS) (Sterile Systems, Logan, UT). The cultures were trypsinized using 0.01% trypsin (TRL3, Worthington Biochemical, Freehold, NJ) in calcium- and magnesium-free phosphate-buffered saline (PBS). All cultures were incubated at 37°C in a humidified 5% CO_2 in air atmosphere. The cultures were free of microbial contamination.

The cultures were passaged 2–3 times per week. All cultures were passaged on the day before use in the metabolic cooperation or cytotoxicity assays. All cultures were used within 2 months of thawing. Stock cultures were cryopreserved in culture medium containing 5% FBS and 5% dimethyl sulfoxide (DMSO).

The test chemicals, obtained from Ashby (Imperial Chemical Industries, Ltd., Cheshire, Great Britain) were stored in the dark at −20°C until ready for use. The test chemicals were: diethylhexylphthalate (DEHP), *o*-toluidine (TOL), acrylonitrile (ACN), safrole (SAF), phenobarbital (PB), benzoin (ZOIN), diethylstilbestrol (DES), hexamethylphosphoramide (HMPA), benzene (BEN) and caprolactam (CAP). All test chemicals were dissolved in DMSO. The volume of the solvent added per dish was the same for all test chemical dilutions. The highest concentration of DMSO added was 0.5%.

Controls

12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was used at 4 ng/ml as the positive control. Solvent controls were used for both the cytotoxicity assay and the metabolic cooperation assay. A cytotoxicity assay was done in parallel to the metabolic cooperation assay.

Preliminary cytotoxicity assays

All test chemicals were initially evaluated in assays designed to determine the concentration ranges that produced little or no cytotoxicity. The first assay was done by seeding 4×10^5 6-TG^r cells in each of 10 or 11 dishes. After a 4-h attachment period, the test chemical was added to each dish in half-log dilutions ranging from 300 μg/ml to 0.001 μg/ml. Following a 3-day growth period, the dishes were evaluated microscopically to determine the presence of cytotoxicity. Concentrations were selected, which ranged from nontoxic to marginally cytostatic, for the next cytotoxicity assay. In the second cytotoxicity assay, 100 6-TG^r cells were seeded in each dish for a total of 4 dishes per assay point. After a 4-h attachment period, the appropriate concentration of test chemical was added to each dish. Following a 3-day growth period, the dishes were refed with culture media without test compound and the incubation continued. After a 6-day growth period, the cultures were rinsed, and fixed and stained with 0.4% methylene blue in 30% methanol. The relative cytotoxicity was estimated by comparing to the solvent control cloning efficiency from the same experiment. The relative cytotoxicity was used to select the concentrations for use in the metabolic cooperation assay. If a concentration that resulted in approximately a 20%–30% inhibition of colony formation relative to the controls could not be determined, the assay was repeated using a narrower dose range.

Inhibition of metabolic cooperation assay

The assay was adapted from that described by Yotti et al. (1979) and Tsushimoto et al. (1982). Briefly, 100 6-TG^r cells were seeded with 4×10^5 6-TG^s cells in each 60-mm dish. Following an attachment period of 4 h, the appropriate concentrations of test and control chemicals were added to each dish for a total of 4–8 dishes for each test or control point. Care was taken to ensure that the chemical did not layer on top of the culture media and that adequate mixing of the chemical occurred. 6-Thioguanine (6-TG) was added to each dish to obtain a final concentration of 10 μg/ml. The dishes were returned to incubation. The culture medium containing test or control chemical and 6-TG was removed after 3 days and replaced with culture medium containing 6-TG but without added chemicals. On the sixth or seventh day, the culture dishes were rinsed with

PBS, fixed and stained using 0.4% methylene blue in 30% methanol. The 6-TG^r colonies present on each dish were counted and compared to the number of colonies expected, which was estimated from the solvent control cloning efficiency assay. For the cloning efficiency assay, done in parallel to the metabolic cooperation assay, all procedures were similar to that in the metabolic cooperation assay except that no 6-TG^s cells were seeded with the 6-TG^r cells.

Criteria for a valid assay

For an experiment to be considered valid, the following criteria were met: (a) the relative recovery of the solvent controls should not exceed 33% of the total number of recoverable cells seeded determined from the absolute cloning efficiency of the solvent controls, (b) the mutant colony recovery efficiency with the TPA control should represent a 2-fold enhancement over that observed in the solvent control, and (c) the cytotoxicity in the parallel experiment should not exceed 30%.

Criteria for a positive response

A test chemical concentration was considered as producing a positive response if the enhanced recovery equaled or exceeded 10% of the enhanced recovery observed with the positive control in the same experiment. A test chemical was considered positive in an experiment if two consecutive chemical concentrations produced positive responses in the same experiment. Test chemicals that were positive in two or more experiments were considered positive in this study. Test chemicals that were positive in one of two or more experiments were considered weak (or suspect) in this study. A test chemical that did not produce a positive experimental response was considered negative in this study.

The enhancement of recovery was determined by the formula:

$$\frac{tc - sc}{pc - sc} \times 100$$

where *tc* is the absolute 6-TG mutant recovery with the test or control chemical concentration, *sc* is the absolute 6-TG mutant recovery with the solvent control, and *pc* is the absolute 6-TG mutant recovery with the positive control (TPA). No correction was made for the cytotoxicity observed with each test or control condition.

Results

The data from the preliminary cytotoxicity assays were used to select the concentrations for each test chemical for the metabolic cooperation assays and are not included in this report. The results of the inhibition of metabolic cooperation assays are presented in Tables 2–11. Two metabolic cooperation assays were done with HMPA and DES and three assays were done with SAF, TOL, BEN, DEHP, ACN, PB, CAP and ZOIN. The tables contain data for each metabolic cooperation assay and its parallel cytotoxicity assay.

DEHP produced positive responses in all 3 experiments (Table 2). The cytotoxicity observed with DEHP did not exceed 30% with the exception of the highest concentration in the third experiment. All concentrations evaluated in the first two experiments produced positive responses. In the third experiment, 3 concentrations produced positive responses. The highest concentration was considered as invalid due to the high cytotoxicity. DEHP was therefore considered as positive in this study.

TOL produced positive responses in 2 of 3 experiments (Table 3). All chemical concentrations with valid data points produced positive responses. The cytotoxicity observed at 25 μg/ml in the first experiment and at 20- and 25-μg/ml in the second experiment exceeded the limits set for valid data points and the data were not considered. TOL was therefore considered positive in this study.

ACN produced positive responses in all 3 experiments (Table 4), however, the criteria for responses with consecutive concentrations were met in only 2 experiments. ACN was not cytotoxic at any concentration evaluated in the metabolic cooperation assays. ACN was considered positive in this study.

SAF produced positive responses at consecutive concentrations in only one experiment (Table 5). In a second experiment SAF produced a positive response at one test concentration. The responses observed with SAF were weak in both experiments

TABLE 2

RESULTS OF THE INHIBITION OF METABOLIC COOPERATION ASSAYS WITH DIETHYLHEXYLPHTHALATE (DEHP)

	Absolute cloning efficiency [a]	Relative survival % [b]	Absolute 6-TG mutant recovery [a]	Relative enhancement of recovery % [c]
Expt. 1				
Solvent control	73.3	100.0	12.3	0
TPA	69.8	95.2	49.5	100.0
Chemical concentration (μg/ml)				
25	51.3	70.0	56.8	119.4
50	43.0	58.7	31.3	51.1
100	67.0 (3)	91.5	20.8	22.8
150	66.3	90.4	25.8	36.3
200	68.3	93.2	30.8	49.7
Expt. 2				
Solvent control	43.0 (1)	100.0	12.0	0
TPA	56.3	128.6	42.0	100.0
Chemical concentration (μg/ml)				
25	48.8	111.4	38.0	86.7
50	39.5	90.3	32.0	66.6
100	51.3	117.3	21.0	30.0
150	41.0	93.7	23.3	37.5
200	42.7 (3)	97.5	24.3	40.8
Expt. 3				
Solvent control	87.6 (5)	100.0	5.0 (10)	0
TPA	85.3 (3)	97.4	62.2 (10)	100.0
Chemical concentration (μg/ml)				
0.5	85.3	97.3	7.6 (7)	4.5
1.0	83.8	95.6	4.9 (8)	(−)
5.0	87.0 (2)	91.3	13.7 (7)	15.2
10.0	77.8	88.8	36.5 (8)	55.0
25.0	80.0 (3)	91.3	38.4 (7)	58.4
50.0	47.3	53.9	24.8 (8)	34.5 *

[a] Each value represents the mean of 4 or more dishes. If the number of dishes used to obtain the mean was different than 4, the number of dishes used for that concentration is indicated in parentheses.

[b] The relative survival was calculated relative to the solvent control.

[c] The relative enhancement was determined as described in the methods.

* This point was considered as invalid due to cytotoxicity.

The values underlined were considered positive.

with relative recovery efficiency enhancement ranging from 13 to 18%. SAF was not cytotoxic at concentrations that produced positive responses. SAF was considered weakly positive (or suspect) in this study.

PB produced positive responses at consecutive concentrations in only one experiment (Table 6). In a second experiment PB produced a positive response at one test concentration. PB was not cytotoxic at any concentration evaluated in this study. The responses observed with PB were weak with relative recovery efficiency enhancement ranging from 12 to 24%. PB was considered weakly positive (or suspect) in this study.

ZOIN produced positive responses at 3 consecutive concentrations in one experiment and positive responses at 2 non-consecutive concentrations in a second experiment (Table 7). ZOIN was not cytotoxic at any concentration evaluated in this study. The responses observed with ZOIN

TABLE 3

RESULTS OF THE INHIBITION OF METABOLIC COOPERATION ASSAYS WITH *o*-TOLUIDINE (TOL)

	Absolute cloning efficiency [a]	Relative survival [b]	Absolute 6-TG mutant recovery [a]	Relative enhancement of recovery % [c]
Expt. 1				
Solvent control	69.3	100.0	14.3	0
TPA	76.8	110.8	42.8	100.0
Chemical concentration (μg/ml)				
5	75.0	108.3	34.0	69.3
10	58.0	83.8	25.0	37.7
15	62.5	90.3	30.8	57.9
20	63.5	91.7	32.5	64.0
25	45.5	65.7	52.0	132.6 *
Expt. 2				
Solvent control	46.0	100.0	11.8	0
TPA	45.8	99.5	32.0	100.0
Chemical concentration (μg/ml)				
5	43.8	95.1	29.8	88.9
10	42.3	92.0	23.3	56.7
15	33.7	73.2	27.3	76.4
20	27.3	59.2	33.5	107.3 *
25	20.8	45.1	36.8	123.4 *
Expt. 3				
Solvent control	87.6 (5)	100.0	5.0 (10)	0
TPA	85.3 (3)	97.4	62.2 (10)	100.0
Chemical concentration (μg/ml)				
0.5	79.3	90.5	4.4 (8)	(−)
1.0	81.5 (2)	92.8	2.1 (7)	(−)
2.5	84.7 (3)	96.7	3.8 (6)	0.1
5.0	82.0	93.6	8.9 (7)	6.8
15.0	83.8	95.6	7.7 (6)	4.7

See Table 2 for legend.

were weak with relative recovery efficiency enhancement ranging from 10 to 16%. ZOIN was considered as weakly positive (or suspect) in this study.

DES produced no positive responses in this study (Table 8). The concentrations evaluated ranged from 0.1 to 1 μg/ml. Cytotoxicity was observed only at 1 μg/ml. DES was considered as negative in this study.

HMPA and BEN produced no positive responses in this study (Tables 9 and 10). The concentrations evaluated ranged from 100 to 500 μg/ml. Cytotoxicity was not observed at any of the concentrations evaluated in this study. HMPA and BEN were considered negative in this study.

CAP produced only one positive response in this study (Table 11). The concentrations evaluated ranged from 100 to 500 μg/ml. Cytotoxicity was not observed at any concentration evaluated in this study. CAP was considered negative in this study.

The results with all chemicals evaluated in this study are summarized in Table 12. DEHP, TOL and ACN were considered positive in the assay. SAF, PB and ZOIN were considered weakly positive (or suspect), and DES, HMPA, BEN, and CAP were considered negative in the assay.

TABLE 4

RESULTS OF THE INHIBITION OF METABOLIC COOPERATION ASSAYS WITH ACRYLONITRILE (ACN)

	Absolute cloning efficiency [a]	Relative survival % [b]	Absolute 6-TG mutant recovery [a]	Relative enhancement of recovery % [c]
Expt. 1				
Solvent control	49.3 (3)	100.0	14.0	0
TPA	48.3 (3)	98.0	35.3	100.0
Chemical concentration (μg/ml)				
10	48.5	98.3	7.8	(−)
20	52.0 (2)	105.4	18.3	20.0
30	42.3 (3)	85.8	15.5	7.0
40	48.0 (1)	97.3	16.8	13.0
50	48.5	98.3	18.3 (3)	20.4
Expt. 2				
Solvent control	97.3	100.0	11.3	0
TPA	96.0	98.7	50.8	100.0
Chemical concentration (μg/ml)				
10	93.8	96.4	7.0	(−)
20	91.5	94.1	11.5	0.5
30	101.5	104.3	18.7 (3)	18.7
40	87.8	90.2	15.5	10.6
50	86.8	89.2	28.5	43.6
Expt. 3				
Solvent control	59.8 (5)	100.0	3.9 (10)	0
TPA	57.0 (5)	95.3	30.0 (10)	100.0
Chemical concentration (μg/ml)				
30	56.3	94.1	5.7 (8)	6.9
40	54.3	90.7	5.3 (8)	2.3
50	52.0	87.0	6.3 (7)	9.2
60	55.0	92.0	8.1 (8)	16.1

See Table 2 for legend.

TABLE 5

RESULTS OF THE INHIBITION OF METABOLIC COOPERATION ASSAYS WITH SAFROLE (SAF)

	Absolute cloning efficiency [a]	Relative survival % [b]	Absolute 6-TG mutant recovery [a]	Relative enhancement of recovery % [c]
Expt. 1				
Solvent control	68.3	100.0	7.8	0
TPA	69.3	102.2	34.8	100.0
Chemical concentration (μg/ml)				
10	60.5	88.6	9.0	4.6
20	64.5	94.5	8.3	1.8
40	61.3	89.7	8.0	0.8
60	63.3	92.7	11.3	12.9
80	51.0	74.7	11.3	12.9
Expt. 2				
Solvent control	77.5	100.0	19.5	0
TPA	68.5	88.4	44.5	100.0
Chemical concentration (μg/ml)				
10	71.8	92.6	12.3	(−)
20	61.8	79.7	11.5	(−)
40	60.0	77.4	18.3	(−)
60	56.3	72.6	24.0	18.0
80	42.3	54.5	18.5	(−) *
Solvent control	87.6 (5)	100.0	5.0 (10)	0
TPA	85.3 (3)	97.4	62.2 (10)	100.0
Chemical concentration (μg/ml)				
10	74.3 (3)	84.8	1.0 (8)	(−)
20	74.0	84.5	1.7 (7)	(−)
40	80.5	91.9	5.7 (7)	0.1
60	81.3	92.8	3.3 (6)	(−)
80	69.0	78.8	4.3 (6)	(−)

See Table 2 for legend.

TABLE 6

RESULTS OF THE INHIBITION OF METABOLIC COOPERATION ASSAYS WITH PHENOBARBITAL (PB)

	Absolute cloning efficiency [a]	Relative survival % [b]	Absolute 6-TG mutant recovery [a]	Relative enhancement of recovery % [c]
Expt. 1				
Solvent control	34.8	100.0	8.3	0
TPA	38.5	113.2	33.3	100.0
Chemical concentration (μg/ml)				
100	34.8	102.2	7.0	(−)
200	31.5	92.6	8.0	(−)
300	36.0	105.9	10.3	7.9
400	35.3	103.7	12.3	15.9
500	31.0	91.2	14.3	23.9
Expt. 2				
Solvent control	37.5	100.0	9.3	0
TPA	36.8	98.0	28.0	100.0
Chemical concentration (μg/ml)				
100	30.8	82.0	5.8	(−)
200	32.5	86.7	7.8	(−)
300	29.3	78.0	8.5	(−)
400	27.5	73.3	9.8	2.6
500	27.5	73.3	13.5	12.0
Expt. 3				
Solvent control	59.8 (5)	100.0	3.9 (10)	0
TPA	57.0 (5)	95.3	30.0 (10)	100.0
Chemical concentration (μg/ml)				
100	59.8	100.0	2.6 (8)	(−)
200	58.0 (3)	97.0	2.1 (8)	(−)
300	50.5	84.4	3.9 (8)	0.0
400	58.0	97.0	5.3 (8)	5.4
500	54.0	90.3	6.4 (8)	9.6

See Table 2 for legend.

TABLE 7

RESULTS OF THE INHIBITION OF METABOLIC COOPERATION ASSAYS WITH BENZOIN (ZOIN)

	Absolute cloning efficiency [a]	Relative survival % [b]	Absolute 6-TG mutant recovery [a]	Relative enhancement of recovery % [c]
Expt. 1				
Solvent control	41.8	100.0	7.8	0
TPA	40.8	97.6	34.3	100.0
Chemical concentration (μg/ml)				
10	38.3	91.6	8.5 (2)	2.5
20	35.0	83.7	8.5	2.5
30	33.8	80.9	10.5	10.1
40	31.3	74.9	8.8	3.8
50	34.8	83.3	12.0	15.8
Expt. 2				
Solvent control	93.3	100.0	12.5	0
TPA	94.0	100.8	56.0	100.0
Chemical concentration (μg/ml)				
10	82.8	88.7	8.8	(−)
20	95.8	102.7	18.5	13.7
30	86.5	92.7	19.0	15.0
40	93.0	99.7	18.0	12.7
50	88.0	94.3	14.3	4.1
Expt. 3				
Solvent control	59.8 (5)	100.0	3.9 (10)	0
TPA	57.0 (5)	95.3	30.0 (10)	100.0
Chemical concentration (μg/ml)				
20	60.5	101.2	3.4 (8)	(−)
30	59.0	98.7	5.8 (8)	7.1
40	54.3 (3)	90.8	4.5 (8)	2.3
50	56.5	94.5	4.5 (6)	2.3
60	55.5	92.8	5.1 (8)	4.6

See Table 2 for legend.

TABLE 8

RESULTS OF THE INHIBITION OF METABOLIC COOPERATION ASSAYS WITH DIETHYLSTILBESTROL (DES)

	Avsolute cloning efficiency [a]	Relative survival % [b]	Absolute 6-TG mutant recovery [a]	Relative enhancement of recovery % [c]
Expt. 1				
Solvent control	43.3	100.0	14.3	0
TPA	39.8	91.9	38.3	100.0
Chemical concentration (μg/ml)				
0.10	37.3	86.1	6.0	(−)
0.25	40.0	92.5	7.0	(−)
0.50	42.5	98.3	8.0	(−)
0.75	39.5	91.3	16.0	7.4
1.00	27.5	63.6	11.8	(−) *
Expt. 2				
Solvent control	89.8	100.0	17.8	0
TPA	91.5	101.9	57.8	100.0
Chemical concentration (μg/ml)				
0.10	93.8	104.5	7.0	(−)
0.25	95.5	106.4	10.3	(−)
0.50	95.8	106.7	15.0	(−)
0.75	81.5	90.8	16.3	(−)
1.00	53.5	59.6	13.5	(−) *

See Table 2 for legend.

TABLE 9

RESULTS OF THE INHIBITION OF METABOLIC COOPERATION ASSAYS WITH HEXAMETHYLPHOSPHORAMIDE (HMPA)

	Absolute cloning efficiency [a]	Relative survival % [b]	Absolute 6-TG recovery [a]	Relative mutantenhancement of recovery % [c]
Expt. 1				
Solvent control	67.6	100.0	8.8	0
TPA	59.6	88.2	41.2	100.0
Chemical concentration (μg/ml)				
100	55.6	82.2	6.8	(−)
200	62.2	92.0	6.0	(−)
300	60.6	89.6	8.6	(−)
400	59.2	87.6	7.4	(−)
500	56.2	83.1	7.8	(−)
Expt. 2				
Solvent control	58.8	100.0	14.8	0
TPA	59.8	101.6	42.5	100.0
Chemical concentration (μg/ml)				
100	61.0	103.7	9.3	(−)
200	(58.8	100.0	10.8	(−)
300	61.0	103.7	10.8	(−)
400	62.3	105.9	8.0	(−)
500	55.3	94.0	14.5	(−)

See Table 2 for legend.

TABLE 10

RESULTS OF THE INHIBITION OF METABOLIC COOPERATION ASSAYS WITH BENZENE (BEN)

	Absolute cloning efficiency [a]	Relative survival % [b]	Absolute 6-TG mutant recovery [a]	Relative enhancement of recovery % [c]
Expt. 1				
Solvent control	73.3	100.0	16.8	0
TPA	74.3	101.4	55.3	100.0
Chemical concentration (μg/ml)				
100	68.0	92.8	9.8	(−)
200	73.0	99.7	8.3	(−)
300	70.0	95.6	11.5	(−)
400	51.0	69.6	10.8	(−)
500	57.8	78.8	9.7 (3)	(−)
Expt. 2				
Solvent control	64.0	100.0	10.3	0
TPA	60.3	94.1	45.5	100.0
Chemical concentration (μg/ml)				
100	63.3	98.8	6.0 (3)	(−)
200	56.0	87.5	7.3	(−)
300	53.5	83.6	7.8	(−)
400	56.3	87.9	10.5	0.7
500	57.0	89.1	10.8	1.5
Expt. 3				
Solvent control	87.6 (5)	100.0	5.0 (10)	0
TPA	85.3 (3)	97.4	62.2 (10)	100.0
Chemical concentration (μg/ml)				
100	87.8	100.2	1.9 (8)	(−)
200	82.3	93.9	2.7 (7)	(−)
300	82.5	94.2	3.4 (7)	(−)
400	88.7 (3)	101.3	4.8 (8)	(−)
500	77.0	87.9	4.0 (7)	(−)

See Table 2 for legend.

TABLE 11

RESULTS OF THE INHIBITION OF METABOLIC COOPERATION ASSAYS WITH CAPROLACTAM (CAP)

	Absolute cloning efficiency [a]	Relative survival % [b]	Absolute 6-TG mutant recovery [a]	Relative enhancement of recovery % [c]
Expt. 1				
Solvent control	38.0	100.0	8.8	0
TPA	33.3	87.5	33.0	100.0
Chemical concentration (μg/ml)				
100	34.8	91.6	3.8	(−)
200	30.8	81.1	6.3	(−)
300	34.5	90.8	5.7 (3)	(−)
400	32.8	86.3	9.3	2.4
500	35.0	92.1	11.8	12.7
Expt. 2				
Solvent control	29.3	100.0	8.0	0
TPA	34.5	117.7	31.5	100.0
Chemical concentration (μg/ml)				
100	34.5	117.7	5.0	(−)
200	33.8	115.4	5.8	(−)
300	31.8	108.5	7.8	(−)
400	32.8	111.9	9.3	5.5
500	32.5	110.9	10.3	9.9
Expt. 3				
Solvent control	59.8 (5)	100.0	3.9 (10)	0
TPA	57.0 (5)	95.3	30.0 (10)	100.0
Chemical concentration (μg/ml)				
100	55.5	92.8	2.4 (8)	(−)
200	56.8	94.9	4.0 (8)	0.4
300	64.3	107.4	3.3 (7)	(−)
400	56.0	93.6	3.5 (8)	(−)
500	64.0	107.0	4.3 (8)	1.3

See Table 2 for legend.

TABLE 12

SUMMARY OF RESULTS: CHINESE HAMSTER V79 INHIBITION OF METABOLIC COOPERATION ASSAY

Compound	assay response	Carcinogenicity
DEHP	+	+
TOL	+	+
ACN	+	+
SAF	+/−	+
PB	+/−	+
ZOIN	+/−	−
DES	−	+
HMPA	−	+
BEN	−	+
CAP	−	−

+, positive response.
+/−, weakly positive or suspect response.
−, negative response.

Discussion

Ambient environmental toxicants may have important chronic human health effects on populations through their ability to alter cellular regulation. One such cellular alteration that can be quantitatively measured is the inhibition of metabolic cooperation, which produces a transient loss of the normal cellular control mechanisms mediated by cell-to-cell communication. Although inhibition of metabolic cooperation is a fairly specific event, thought to occur through relatively few mechanisms (Trosko, personal communication), the loss of cell-to-cell communication may cause major changes in the control of cellular differentiation and function (Loewenstein, 1979). Changes in cellular differentiation have been implicated in the expression of cancer, birth defects, neurological diseases, and reproductive disorders.

In our laboratory, 3 chemicals, DEHP, TOL and ACN were considered positive in the inhibition of metabolic cooperation assay. DEHP has previously been reported positive (Malcolm and Mills, 1984). Our findings with TOL are in agreement with that of Scott et al. (this volume). Our findings with ACN are in agreement with Umeda et al. (this volume).

Three chemicals, SAF, PB and ZOIN, were considered weakly positive because they produced a positive response in only 1 of 3 experiments and therefore did not meet the criteria for a positive chemical. The lack of cytotoxicity and the relative dose response suggests that these chemicals may be weakly positive with variable responses due to experimental variation. Therefore, this may explain the conflicting reports of Umeda et al. (1980) and Trosko et al. (1981) for PB. ZOIN also produced a positive response in one experiment. In a second experiment, two nonconsecutive concentrations showed activity; however, a chemical must produce activity at consecutive doses to be considered positive by the criteria used in this study. This finding suggests that ZOIN may have some activity as an epigenetic chemical and may need to be considered for additional evaluation.

DES, HMPA and BEN produced negative responses in our assays. This may reflect the capabilities of the V79 cells to activate these chemicals. If an external metabolic activation system or the active derivative(s) of each chemical was used, the assay may have been more responsive. CAP also produced a negative response in our assays. Additional methodology utilizing other metabolically competent cell types needs to be developed to permit the assessment of possibly false negatives in the inhibition of metabolic cooperation assay with V79 cells due to the metabolizing capacity of these cells.

The strengths of the Chinese hamster V79 inhibition of metabolic cooperation assay include: (a) it appears to be responsive to many classes of chemicals (Tables 1–7), (b) it does not require supplementary metabolic activation for most chemicals, (c) it can readily be quantified, (d) it can evaluate chemicals at concentrations which produce little or no cytotoxic effects, and (e) it can be completed in 1 week. The current limitations of the assay include: (a) the correlation of inhibition of metabolic cooperation to human diseases has not been demonstrated, (b) the adaptability of the assay to utilize metabolic activation systems has not been shown, and (c) the methodology for studying gases and chemicals that volatilize at ambient temperatures has not been adapted to this assay.

In summary, the Chinese hamster V79 inhibition of metabolic cooperation assay showed positive responses with DEHP, TOL and ACN, sus-

pect (weak) responses with SAF, PB and ZOIN, and negative responses with DES, HMPA, BEN and CAP. Due to the current research and development studies with this relatively new assay, it is possible that future improvements in sensitivity may permit the confirmation of activity with those chemicals that we have reported as weak (or suspect) and the detection of activity with those chemicals which we have reported as negative in this study. The results from these experiments and others (Table 1) suggest that this assay for epigenetic chemicals could complement the bacterial tests in the evaluation of environmental toxicants. However, additional research and development coupled with validation studies are needed to fully assess the assay's potential in this area.

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Assays for inhibition of metabolic cooperation between mammalian cells in culture

J.K. Scott, H. Davidson and A.J. Nelmes

Research and Development, Gallaher Ltd., Belfast (Northern Ireland)

Summary

Toluidine inhibited metabolic cooperation between mammalian cells in culture as measured by mutant rescue. Hexamethylphosphoramide, safrole, benzene and caprolactam did not.

Metabolic cooperation is the transfer between contacting cells of relatively large molecules (Azarnia and Lowenstein, 1976). Over the past 4 years, a correlation has emerged between a chemical's ability to inhibit metabolic cooperation in vitro (Trosko et al., 1982) and its capacity to promote tumours in vivo. Furthermore, two inhibitors of tumour promotion, stimulate metabolic cooperation (Elias et al., 1980; Fitzgerald and Murray, 1980). The inhibition of metabolic cooperation may therefore be relevant to carcinogenesis.

Several carcinogens, not detected by the Ames mutagenicity screen, have inhibited metabolic cooperation (Trosko et al., 1983). The aim of part I of the IPCS CSTT programme was to identify in vitro eukaryotic assays that could complement the Ames test in detecting potential carcinogens. A metabolic cooperation system, based on cultured Chinese hamster cells, was used to this end. The first 5 chemicals of the study list were tested. These were *o*-toluidine (TOL), benzene (BEN), hexamethylphosphoramide (HMPA), safrole (SAF) and caprolactam (CAP).

Materials and methods

Chemicals

Dimethyl sulphoxide (DMSO), research grade, was obtained from Serva Feinbiochemica. 12-*O*-Tetradecanoyl-phorbol-13-acetate (TPA) and 4-β-phorbol, and 8-azaguanine (AzG) came from Sigma. Dihydroteleocidin B was a gift from T. Sugimura, National Cancer Centre Research Institute (Japan).

Test compounds were dissolved in DMSO. Aliquots were stored at $-20\,^{\circ}C$, and used only once. AzG stock solutions, 1.2 mg/ml in 0.1 M NaOH, were stored at $4\,^{\circ}C$, for not more than 2 weeks.

Cell culture

Tissue culture materials were obtained from Gibco (Europe). V79/4-K1 and V79-M13 Chinese hamster cells were supplied by R.F. Newbold, Institute of Cancer Research (Great Britain).

V79/4-K1 cells were cultured in Dulbecco's modified Eagles medium (DMEM), supplemented with foetal calf serum (10% v/v) and penicillin–streptomycin (50 units/ml). V79-M13 cells were maintained in the same medium but containing AzG (30 μg/ml). V79/4-K1 cells were AzG-sensitive, V79-M13 cells were AzG-resistant. Incubation was at $37\,^{\circ}C$ in a humidified atmosphere, containing carbon dioxide (10% v/v).

Cytotoxicity assays

(a) V79/4-K1 cells were plated at 8×10^5/20 ml medium/9-cm plate. Plates were incubated for 2 h, then exposed for 22 h to a dose range of the

test compound, added at 0.1 ml/plate, 2 plates/dose. Cultures were suspended in trypsin (0.25% w/v in phosphate-buffered saline; 2 ml/plate/dose), and cells counted manually, using a haemocytometer. Toxicity was demonstrated by a reduction in cell density relative to the nontoxic solvent control cultures. The assay was performed 3 times per test chemical.

(b) Cytotoxicity was also determined as plating efficiency, V79-M13 cells were plated at 3×10^2 cells/20 ml medium/9-cm plate. After 2 h, cultures were treated with a dose range of the test compound, at 0.1 ml/plate, 3 plates/dose. Incubation continued for 14 days, then colonies were orcein–giemsa stained and counted.

Colonies/plate was expressed as a percentage of the solvent control results, i.e. as a plating efficiency. Toxicity was demonstrated as a reduction in plating efficiency relative to the solvent control. The assay was done 3 times per test chemical.

Metabolic cooperation assays

V79/4-K1 and V79-M13 cells were coplated at 8×10^5 and 3×10^2 cells/20 ml medium/9-cm plate, respectively. After 2 h, cocultures were treated with a series of doses of the test agent, at 0.1 ml/plate, 6 plates/dose. 24 h post plating, plates were made to 30 μg/ml AzG. 14 days after plating, colonies were orcein–giemsa stained and counted.

TPA (325 nM) and dihydroteleocidin B (465 pM) were used as positive controls, 4-β-phorbol (325 nM) as a negative control and DMSO (0.5% v/v) as the solvent control. The assay was done 3 times per test chemical.

Metabolic cooperation and cytotoxicity were measured simultaneously. No exogenous metabolic activation system was used.

Results

Results are summarised in Tables 1–5.

Cytotoxicity

Results from method (a) are not shown, but were submitted with the raw data. Assays (a) and (b) gave similar results for a given test chemical, with the possible exceptions of SAF and HMPA. In some experiments cell counts were more sensitive than plating efficiency to SAF toxicity, and vice versa for HMPA. This may reflect differences in cytostatic and cytocidal properties of these test chemicals.

The maximum nontoxic dose of each chemical was defined using both sets of toxicity data. Solubility was used in place of toxicity for benzene, as it became visibly insoluble at 565 μM, before it expressed toxicity. Insolubility and toxicity are artefacts in interpreting metabolic cooperation results. Metabolic cooperation results were therefore only interpreted up to the maximum nontoxic (or soluble) doses, given in Table 6.

TABLE 1

o-TOLUIDINE

Concentration (μM)	Inhibition of metabolic cooperation (mean colonies/dish ± standard error)			Cytotoxicity (% survival relative to solvent control, mean ± standard error)		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
4700	219 ± 10	198 ± 5	278 ± 13	76 ± 1	38 ± 2	72 ± 7
1570	140 ± 4	91 ± 3	121 ± 7	102 ± 3	86 ± 6	95 ± 5
470	107 ± 4	75 ± 9	112 ± 11	98 ± 4	89 ± 4	101 ± 6
157	95 ± 4	NT	75 ± 6	100 ± 7	NT	113 ± 6
47	80 ± 5	NT	83 ± 10	100 ± 4	NT	116 ± 6
16	56 ± 4	NT	85 ± 5	106 ± 2	NT	109 ± 6
Controls						
TPA	244 ± 6	171 ± 4	216 ± 6	NT	NT	NT
Dihydroteleocidin B	NT	224 ± 5	NT	NT	NT	NT
Phorbol	75 ± 2	52 ± 7	51 ± 3	NT	NT	NT
DMSO	NT	NT	NT	100 ± 1	100 ± 10	100 ± 6

TABLE 2

CAPROLACTAM

Concentration (μM)	Inhibition of metabolic cooperation (mean colonies/dish ± standard error)			Cytotoxicity (% survival relative to solvent control, mean ± standard error)		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
18000	NT	NT	NT	NT	43 ± 7	NT
3600	163 ± 9	45 ± 6	57 ± 3	91 ± 5	89 ± 7	92 ± 3
1800	157 ± 6	39 ± 2	59 ± 3	95 ± 3	100 ± 11	100 ± 5
600	162 ± 7	47 ± 4	75 ± 7	92 ± 4	109 ± 7	103 ± 1
180	165 ± 8	34 ± 2	73 ± 4	92 ± 5	109 ± 2	107 ± 7
60	147 ± 2	39 ± 3	62 ± 5	80 ± 3	91 ± 4	116 ± 5
18	148 ± 4	40 ± 4	63 ± 5	86 ± 2	NT	113 ± 8
Controls						
TPA	286 ± 6	139 ± 6	155 ± 8	NT	NT	NT
Phorbol	152 ± 7	40 ± 3	58 ± 5	NT	NT	NT
DMSO	NT	NT	NT	100 ± 4	100 ± 2	100 ± 14

Inhibition of metabolic cooperation

At the cell density used, cocultured V79/4-K1 and V79-M13 cells metabolically cooperate. Cooperation exposed the otherwise AzG-resistant V79-M13 cells to a lethal AzG metabolite produced in the AzG-sensitive V79/4-K1 cells. Metabolic cooperation ultimately caused a reduction in the number of AzG-resistant (AzGr) colonies per coculture plate. Conversely, a chemical which inhibited metabolic cooperation would increase the number of AzGr colonies/coculture plate. Table 1 shows that at nontoxic doses TOL appeared to inhibit metabolic cooperation. To control for de novo mutation to AzG-resistance 'cooperation' assays were performed without V79-M13 cells. In these assays TOL did not induce AzG-resistance. The effect of TOL on the cocultures must therefore have been a genuine inhibition of metabolic cooperation.

The other 4 test chemicals did not inhibit metabolic cooperation. It is difficult to compare SAF and BEN with TOL, since their maximum interpretable doses were so different (Table 6).

TABLE 3

BENZENE

Concentration (μM)	Inhibition of metabolic cooperation (mean colonies/dish ± standard error)			Cytotoxicity (% survival relative to solvent control, mean ± standard error)		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
56500	NT	91 ± 3	NT	NT	78 ± 2	NT
18840	NT	80 ± 5	NT	NT	62 ± 5	NT
5650	62 ± 6	66 ± 6	74 ± 4	71 ± 12	62 ± 6	88 ± 7
1884	59 ± 4	85 ± 4	85 ± 7	102 ± 5	95 ± 2	72 ± 6
565	65 ± 3	101 ± 8	109 ± 6	128 ± 6	116 ± 5	112 ± 4
188	64 ± 2	96 ± 5	100 ± 5	110 ± 7	120 ± 8	109 ± 10
57	72 ± 5	NT	90 ± 3	129 ± 1	NT	113 ± 8
19	64 ± 3	NT	74 ± 5	116 ± 5	NT	119 ± 4
Controls						
TPA	143 ± 3	192 ± 4	192 ± 13	NT	NT	NT
Phorbol	72 ± 4	94 ± 6	81 ± 7	NT	NT	NT
DMSO	NT	NT	NT	100 ± 9	100 ± 2	100 ± 10

TABLE 4

SAFROLE

Concentration (μM)	Inhibition of metabolic cooperation (mean colonies/dish ± standard error)			Cytotoxicity (% survival relative to solvent control, mean ± standard error)		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
340	48 ± 2	52 ± 5	45 ± 3	49 ± 3	60 ± 16	51 ± 6
110	60 ± 4	63 ± 4	48 ± 5	90 ± 5	87 ± 13	106 ± 9
34	60 ± 5	57 ± 3	43 ± 4	104 ± 9	118 ± 2	115 ± 4
11	66 ± 5	57 ± 4	54 ± 3	111 ± 1	93 ± 13	98 ± 4
3	71 ± 3	62 ± 4	61 ± 4	111 ± 3	107 ± 2	128 ± 11
1	71 ± 3	64 ± 3	45 ± 4	111 ± 2	87 ± 7	126 ± 13
Controls						
TPA	132 ± 3	155 ± 5	106 ± 5	NT	NT	NT
Dihydroteleocidin B	NT	167 ± 2	NT	NT	NT	NT
Phorbol	70 ± 4	73 ± 3	41 ± 5	NT	NT	NT
DMSO	NT	NT	NT	100 ± 6	100 ± 9	100 ± 4

TABLE 5

HEXAMETHYLPHOSPHORAMIDE

Concentration (μM)	Inhibition of metabolic cooperation (mean colonies/dish ± standard error)			Cytotoxicity (% survival relative to solvent control, mean ± standard error)		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
28 500	NT	28 ± 2	NT	NT	50 ± 4	NT
9500	NT	23 ± 2	21 ± 3	NT	96 ± 0	77 ± 8
2850	55 ± 3	27 ± 4	22 ± 3	91 ± 8	117 ± 11	96 ± 13
950	57 ± 4	51 ± 12	25 ± 4	118 ± 5	109 ± 7	119 ± 9
285	44 ± 2	36 ± 5	22 ± 3	100 ± 8	117 ± 3	81 ± 9
95	43 ± 2	NT	20 ± 3	99 ± 8	NT	104 ± 6
29	42 ± 4	NT	15 ± 2	89 ± 5	NT	98 ± 2
10	49 ± 4	NT	NT	107 ± 4	NT	NT
Controls						
TPA	82 ± 3	175 ± 4	115 ± 8	NT	NT	NT
Phorbol	36 ± 2	35 ± 6	13 ± 2	NT	NT	NT
DMSO	NT	NT	NT	100 ± 9	100 ± 6	100 ± 9

TABLE 6

MAXIMUM INTERPRETABLE DOSES FOR METABOLIC COOPERATION ASSAY

Chemical	Maximum nontoxic or soluble dose (μM)
SAF	100
BEN	500
TOL	3000
CAP	4000
HMPA	10000

Discussion

The conclusions are summarised in Table 7. Because of the correlation between inhibition of metabolic cooperation and tumour promotion, TOL is now a suspected tumour promoter. Its weak carcinogenicity might reflect an ability to promote tumours initiated by its own weak genotoxicity (Ashby, 1981).

TOL and SAF both induced mitotic aneuploidy in yeast (Parry and Sharp, 1981), another property

TABLE 7

CONCLUSIONS

Chemical	Inhibition of metabolic cooperation
TOL	Positive
CAP	Negative
HMPA	Negative
BEN	Negative
SAF	Negative

associated with tumour promoters. Only TOL inhibited metabolic cooperation in this study. Tumour promotion is a multistage process (Parry et al., 1981). The aneuploidy and cooperation systems may map onto different aspects of this process, since the incomplete promoter, mezerein, did not induce aneuploidy (Parry et al., 1981) but inhibited metabolic cooperation (Scott and Davidson, 1983; Trosko et al., 1982; Newbold and Amos, 1981). SAF might also be an incomplete tumour promoter, able to induce aneuploidy, but not to inhibit metabolic cooperation.

Phenobarbitone was part of the IPCS CSTT programme, but not among the 5 chemical series tested in this study. It inhibited metabolic cooperation (Trosko et al., 1982) as expected for a known tumour promoter (Stevens and Pocine, 1983).

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Assays for inhibition of metabolic cooperation by a microassay method

Makoto Umeda [1,2], Kouichi Noda [1] and Kumiko Tanaka [2]

[1] *Tissue Culture Laboratory, Yokohama City University School of Medicine, Yokohama (Japan) and* [2] *Tokyo Metropolitan Institute of Medical Science, Tokyo (Japan)*

Summary

The effect of 10 compounds supplied by the IPCS committee on metabolic cooperation was examined by the microassay method using Chinese hamster V79 cells and 6-thioguanine-resistant cloned cells. Among 10 compounds, 2 compounds were active in the assay. Acrylonitrile apparently and safrole weakly inhibited metabolic cooperation in a dose-dependent manner. Benzoin, caprolactam, diethylhexylphthalate and *o*-toluidine showed very weak inhibition, but these effects were not significant. The other 4 compounds, benzene, diethylstilbestrol, hexamethylphosphoramide and phenobarbital, showed no effect on metabolic cooperation.

Many tumor promoters have been reported to be positive in an assay for inhibition of metabolic cooperation. The principle of the assay is based on a phenomenon that recovery of 6-thioguanine(6TG)-resistant cells cocultivated with 6TG-sensitive cells in 6TG-containing medium increased by addition of compounds that inhibit metabolic cooperation (Yotti et al., 1979). To examine inhibition of metabolic cooperation by many compounds, we developed a microassay method using 24-multiwell plates. By the employment of this microassay method, results can be obtained after 5 days, and the scale of one experiment becomes small. Therefore, reproducibility can be easily checked by repeated experiments.

For this report, 10 compounds supplied by the committee were examined by this microassay method to determine if they inhibit metabolic cooperation. We found that acrylonitrile had inhibitory activity and safrole had very weak activity.

Materials and methods

Materials

Wild-type V79 cells ($6TG^s$) and a clone (T2-14) of 6TG-resistant cells ($6TG^r$), derived from V79 cells, were used. The frozen-stocked cells were cultured in Eagle's MEM + 10% fetal bovine serum in a 5% CO_2 incubator at 37 °C and used within a month. 6TG was dissolved in 1/25 M NaOH at 1 mg/ml, filter-sterilized and stored at 4 °C. 12-*O*-Tetradecanoyl-phorbol-13-acetate (TPA) was obtained from Consolidated Midland Corp., New York; and the 10 compounds were supplied by the committee of the program. They were dissolved in DMSO at 200 times the concentration desired. Aliquots were stored in ampules at −20 °C, and used one at a time.

Assay for metabolic cooperation

To examine inhibition of metabolic cooperation by test compounds, 50 T2-14 cells and 1×10^4 V79 cells were cocultivated in each well of 24-multiwell plates in 0.5 ml of control medium for 24 h. Then medium was changed to the one containing 10 μg/ml of 6TG and various concentrations of the test compounds. In separate plates, 50 T2-14 cells were seeded in each well and treated with the experimental media to examine toxic effect of test compounds on cells. Examined concentrations of

compounds were from nontoxic to toxic, which were determined by preliminary toxicity experiments. After cultivation for another 4 days, cultures were fixed and stained. Colonies consisting of over 20 cells were scored by the use of a stereomicroscope. 6 wells were used for each experimental group and each control group. Data were expressed as percentages of mean number of colonies in experimental cultures to that in culture in which T2-14 cells were seeded and treated with 6TG alone. At least 3 Expts. were performed for each compound, and the results were expressed as the average ±SE (%).

Results

Microassay method

For experiments of metabolic cooperation, 2 types of cells must be distributed evenly on the culture surface. When microplates were used, careful and gentle handling and little shaking was critically important before cell attachment; otherwise inoculated cells tended to gather to the center of wells and attach. In addition, colonies were still small after 5 days of cultivation. With the aid of a stereomicroscope, however, colonies could be counted distinctly without any difficulty. Under the experimental condition, activity of a typical tumor promoter, TPA, on the inhibition of metabolic cooperation was preliminary tested and shown to be dose-dependent (Fig. 1), and the result was quite similar to that reported previously (Umeda et al., 1980). Thus, the method could be validated to give a result comparable to that obtained by the conventional method.

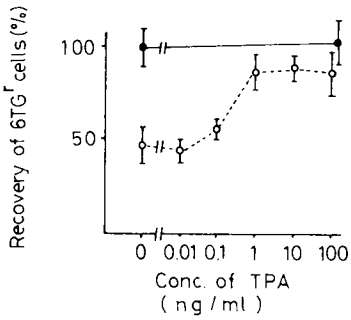

Fig. 1. Dose-dependent inhibition of metabolic cooperation by TPA: 50 T2-14 cells or 50 T2-14 cells and 2×10^4 V79 cells were cultivated for 4 h. Then, the cultures were treated with 10 μg/ml of 6TG and various concentrations of TPA as indicated. After cultivation for 5 days, colonies were counted and recovery of 6TG^r cells was determined as described in the text. 6 wells were used to determine each point; mean values from coculture O------O and from T2-14 cells alone ●———●. Bars indicate SD(%).

Test for 10 compounds

Inhibitory activity of 10 compounds supplied by the committee was examined by the microassay method together with a positive control, TPA. Experiments were repeated more than 3 times to confirm reproducibility. Under the experimental condition, average recovery of 6TG^r cells was around 20% in control cultures, and around 60% in cultures treated with 0.1 μg/ml of TPA. Recovery obtained with the 10 compounds did not exhibit as high rate as that of TPA.

Among the 10 compounds, acrylonitrile inhibited metabolic cooperation slightly and dose-dependently (Table 1). Average recovery of 6TG^r cells was 22% in the control, 33% at 1 mM and 39% at 2 mM. The values were statistically significant, and we considered this compound was positive in the assay.

Every experiment with safrole showed a dose-dependent increase in recovery of 6TG^r cells, but the rate was very slight (Table 9). We considered this compound was plus/minus as to effect on metabolic cooperation.

Very slight increase in recovery of 6TG^r cells

TABLE 1

EFFECT OF ACRYLONITRILE ON THE RECOVERY OF 6TG^r CELLS UNDER CONDITIONS OF METABOLIC COOPERATION [a]

Concentration (mM)	Presence of 6TG^s cells	
	−	+
0	100	22± 3
0.2	102± 6	26± 2
0.5	91± 3	31± 6
1.0	93± 2	33± 3 [b]
2.0	87± 5	39± 4 [b]
5.0	64±17	34±10
TPA 0.1 μg/ml	92± 4	62± 8

[a] The result was the average ± SE(%) of recovered 6TG^r cells in 6 repeated experiments.

[b] $p < 0.05$.

TABLE 2

EFFECT OF BENZENE ON THE RECOVERY OF 6TG^r CELLS UNDER CONDITIONS OF METABOLIC COOPERATION [a]

Concentration (mM)	Presence of 6TG^s cells	
	–	+
0	100	15 ± 2
0.1	–	15 ± 1
0.2	–	13 ± 2
0.5	102 ± 1	13 ± 3
1.0	94 ± 8	14 ± 2
2.0	37 ± 18	9 ± 4

[a] The result was the average ± SE(%) of recovered 6TG^r cells in 3 repeated experiments.

TABLE 3

EFFECT OF BENZOIN ON THE RECOVERY OF 6TG^r CELLS UNDER CONDITIONS OF METABOLIC COOPERATION [a]

Concentration (mM)	Presence of 6TG^s cells	
	–	+
0	100	19 ± 3
0.02	110 ± 6	25 ± 4
0.05	96 ± 5	24 ± 4
0.1	103 ± 5	21 ± 2
0.2	72 ± 8	20 ± 3
0.5	0	3 ± 1
TPA 0.1 μg/ml	100 ± 6	64 ± 7

[a] The result was the average ± SE(%) of recovered 6TG^r cells in 4 repeated experiments.

TABLE 4

EFFECT OF CAPROLACTAM ON THE RECOVERY OF 6TG^r CELLS UNDER CONDITIONS OF METABOLIC COOPERATION [a]

Concentration (mM)	Presence of 6TG^s cells	
	–	+
0	100	19 ± 1
0.5	95	21 ± 1
1.0	87 ± 6	17 ± 2
2.0	94 ± 7	24 ± 0
5.0	89 ± 6	21 ± 2
10	88	21
20	78	18
TPA 0.1 μg/ml	82	50

[a] The result was the average ± SE(%) of recovered 6TG^r cells in 3 repeated experiments.

TABLE 5

EFFECT OF DIETHYLHEXYLPHTHALATE ON THE RECOVERY OF 6TG^r CELLS UNDER CONDITIONS OF METABOLIC COOPERATION [a]

Concentration (mM)	Presence of 6TG^s cells	
	–	+
0	100	22 ± 3
0.01	100	27 ± 4
0.02	96 ± 3	24 ± 1
0.05	99 ± 1	28 ± 2
0.1	93 ± 8	28 ± 3
0.2	76 ± 18	25 ± 2
0.5	6	19

[a] The result was the average ± SE(%) of recovered 6TG^r cells in 3 repeated experiments.

TABLE 6

EFFECT OF DIETHYLSTILBESTROL ON THE RECOVERY OF 6TG^r CELLS UNDER CONDITIONS OF METABOLIC COOPERATION [a]

Concentration (mM)	Presence of 6TG^s cells	
	–	+
0	100	18 ± 2
2	–	16
5	–	16 ± 2
10	102	18 ± 3
20	70 ± 5	8 ± 2
50	0	0

[a] The result was the average ± SE(%) of recovered 6TG^r cells in 3 repeated experiments.

TABLE 7

EFFECT OF HEXAMETHYLPHOSPHORAMIDE ON THE RECOVERY OF 6TG^r CELLS UNDER CONDITIONS OF METABOLIC COOPERATION [a]

Concentration (mM)	Presence of 6TG^s cells	
	–	+
0	100	19 ± 1
0.5	87	21
1.0	95 ± 4	17 ± 1
2.0	91 ± 4	19 ± 1
5.0	93 ± 8	21 ± 1
10	85	18
20	86	24
TPA 0.1 μg/ml	82	50

[a] The result was the average ± SE(%) of recovered 6TG^r cells in 3 repeated experiments.

TABLE 8

EFFECT OF PHENOBARBITAL ON THE RECOVERY OF 6TG^r CELLS UNDER CONDITIONS OF METABOLIC COOPERATION [a]

Concentration (mM)	Presence of 6TG^s cells	
	−	+
0	100	22 ± 4
0.5	101	19 ± 1
1.0	101 ± 6	20 ± 4
2.0	105 ± 6	20 ± 4
5.0	37 ± 22	4 ± 3
10	0	0
TPA 0.1 μg/ml	97	61

[a] The result was the average ± SE(%) of recovered 6TG^r cells in 3 repeated experiments.

TABLE 9

EFFECT OF SAFROL ON THE RECOVERY OF 6TG^r CELLS UNDER CONDITIONS OF METABOLIC COOPERATION [a]

Concentration (mM)	Presence of 6TG^s cells	
	−	+
0	100	18 ± 1
0.1	–	17 ± 1
0.2	–	19 ± 2
0.5	100 ± 1	24 ± 1 [b]
1.0	92 ± 2	23 ± 2
2.0	33 ± 3	17 ± 3

[a] The result was the average ± SE(%) of recovered 6TG^r cells in 3 repeated experiments.

[b] $p < 0.05$.

TABLE 10

EFFECT OF *o*-TOLUIDINE ON THE RECOVERY OF 6TG^r CELLS UNDER CONDITIONS OF METABOLIC COOPERATION [a]

Concentration (mM)	Presence of 6TG^s cells	
	−	+
0	100	15 ± 2
0.5	–	17 ± 2
1.0	–	17 ± 2
2.0	100 ± 1	19 ± 3
5.0	73 ± 10	21 ± 4
10	8	12 ± 5

[a] The result was the average ± SE(%) of recovered 6TG^r cells in 5 repeated experiments.

was observed on average with benzoin, caprolactam, diethylhexylphthalate and *o*-toluidine (Tables 3, 4, 5 and 10). Statistical analysis of these results was shown to be insignificant. Therefore, we judged that these compounds had little effect on metabolic cooperation. The other 4 compounds apparently showed no effect on metabolic cooperation (Tables 2, 6, 7 and 8).

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Tests with the Syrian hamster embryo cell transformation assay

J. Carl Barrett and Patricia W. Lamb

Environmental Carcinogenesis Group, Laboratory of Pulmonary Function and Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC (U.S.A.)

Summary

The abilities of 8 carcinogens and 2 noncarcinogens to induce morphological transformation of Syrian hamster embryo cells were examined. 7 of the 8 carcinogens were active, including hexamethylphosphoramide, safrole, *o*-toluidine, benzene, diethylhexylphthalate, diethylstilbestrol and acrylonitrile. Caprolactam, a noncarcinogen, was also active at high doses. Phenobarbital, a carcinogen, and benzoin, a noncarcinogen, were both inactive. These results indicate a good correlation between induction of cell transformation in this system and carcinogenicity. Furthermore, the results agreed with published findings with the compounds when available indicating good reproducibility of the system.

The Syrian hamster embryo cell transformation assay developed 20 years ago by Berwald and Sachs (1963, 1965) was the first cellular system for the study of chemical carcinogen induced neoplastic transformation. Since that time this system has been used as a short-term screen for testing potential carcinogens and for mechanistic studies of oncogenesis (Barrett et al., 1984). The basis of this assay is the quantitation of morphologically altered colonies of cells which form 7–8 days after exposure to chemical carcinogens. Morphologically-transformed cells are not neoplastically transformed at this time but rise give to cells that can form tumors when injected into syngeneic hamsters or nude mice (Barrett and Ts'o, 1978a). Thus, the assay measures the induction of preneoplastic cells. Syrian hamster embryo cells maintain a stable diploid karyotype in culture and the frequency of spontaneous morphological transformation is very low ($< 0.01\%$). Following treatment with a carcinogen such as benzo[*a*]pyrene (BaP), a high frequency ($> 1\%$) of the surviving cells are morphologically transformed. The induction of cell transformation with BaP and other carcinogens is dose-dependent in a manner consistent with a "one-hit" process (Gart et al., 1979), is not due to selection of pre-existing cells, is unrelated to cytotoxicity (DiPaolo et al., 1971a), and is dependent on the number of cells at risk (DiPaolo et al., 1971b).

The carcinogens chosen for the International Program for Chemical Safety (IPCS) study were ones that were missed in other assays possibly due to unusual metabolic activation requirements or because it was postulated that they were carcinogenic by nonmutagenic mechanisms (J. Ashby, this volume). Syrian hamster embryo cells in culture retain the capacity to metabolize a wide variety of chemical carcinogens (Barrett and Ts'o, 1978b). Therefore, exogenous metabolic activation systems often are not required for a positive response in this system and all the assays in our laboratory for this study were done without exogenous metabolic activation. The Syrian hamster embryo cell transformation assay has been shown previously to detect a number of chemicals reported to be nonmutagenic (Barrett et al., 1983, 1984). Therefore, it was of considerable interest to determine if this assay could detect the possible "nonmutagenic" carcinogens included in this IPCS study.

Materials and methods

Cells, growth medium and chemicals

All chemicals, obtained from J. Ashby or the NCI Chemical Respository (BaP), were dissolved in DMSO and added to the culture medium.

Syrian-hamster-embryo cell cultures were established from 13-day gestation fetuses collected aseptically by Caesarean section from inbred Syrian hamsters, strain LSH/ssLAK (Lakeview Hamster Colony, Newfield, NJ). Pools of primary cultures from litters mates were stored in liquid nitrogen. Secondary cultures were initiated from the frozen stocks and all experiments were performed with tertiary or later cultures. All cultures were routinely tested by Microbiological Associates (Bethesda, MD) and found free of mycoplasma contamination. The cell culture medium used was IBR Dulbecco's modified Eagle's reinforced medium, (Biolabs, Northbrook, IL) with 0.37% (w/v) $NaHCO_3$, 10%–20% fetal bovine serum (Gibco, Grand Island, NY), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were grown in a humidified atmosphere of 12% CO_2 in air at 37 °C and were transferred by gentle trypsinization with 0.1% trypsin (1 : 250, Gibco) for 5 min at 37 °C.

Cell transformation assay

The in vitro transformation procedure used was a modification of the earlier work of Berwald and Sachs (1965), DiPaolo et al. (1971a, b), and Pienta (1980). Target cells (250 cells) were seeded on a layer of 2×10^4 lethally irradiated (5000 R) ho-

TABLE 1

MORPHOLOGICAL TRANSFORMATION OF SYRIAN HAMSTER EMBRYO CELLS IN THE ABSENCE OF EXOGENOUS METABOLIC ACTIVATION

Chemical	Dose (μg/ml)	Relative survival	Morphologically transformed colonies per total colonies	Transformed colonies (%)
Control (same experiment)		1.0	1/1763	0.06
Control (concurrent)		1.0	2/14214	0.014
Control (historical)		1.0	2/34829	0.006
HMPA	0.1	1.0	3/1020	0.29
	1.0	1.1	4/1100	0.36
	10.0	1.1	4/1057	0.37
SAF	0.001	0.98	0/284	–
	0.01	0.94	0/274	–
	0.1	0.93	0/135	–
	1.0	1.0	0/860	–
	10	0.83	8/1397	0.57
TOL	0.1	1.0	0/261	–
	1.0	1.0	5/1918	0.26
	10	0.97	1/1851	0.05
	100	0.96	1/2123	0.05
	500	1.2	0/336	–
CAP	0.01	0.97	0/201	–
	0.1	0.99	0/215	–
	1.0	1.08	0/293	–
	10	1.0	4/1385	0.28
	100	0.99	3/1370	0.22

TABLE 1 (continued)

Chemical	Dose (μg/ml)	Relative survival	Morphologically transformed colonies per total colonies	Transformed colonies (%)
ZOIN	0.01	1.1	0/309	–
	0.1	0.97	0/283	–
	1.0	0.98	0/285	–
	10	1.1	0/334	–
	100	0.54	0/568	–
BEN	0.01	1.0	0/293	–
	0.1	1.1	0/307	–
	1.0	0.96	0/280	–
	10	0.91	7/561	1.2
	100	0.94	4/619	0.65
DEHP	0.01	1.0	0/148	–
	0.1	1.0	0/303	–
	1.0	1.0	1/689	0.15
	10	0.91	2/772	0.26
	100	0.91	6/686	0.87
DES	0.01	1.0	0/305	–
	0.1	1.0	1/900	0.1
	1.0	1.0	5/1380	0.36
ACN	0.01	1.0	4/1149	0.35
	0.1	0.98	1/1087	0.09
	1.0	0.95	2/1084	0.18
	10	1.0	0/1197	–
	100	0.13	0/660	–
PB	0.1	0.97	0/284	–
	1.0	0.99	0/145	–
	10	0.98	0/286	–
	100	0.91	0/764	–

mologous feeder cells in 60-mm dishes (No. 3002; Falcon Plastics, Oxnard, CA) in complete medium with 20% serum. To minimize photodecomposition of the chemicals, all work with the compounds was done under illumination by yellow lamps (Sylvania F 40GO). The chemicals were dissolved in DMSO (Fisher Scientific, Pittsburgh, PA). Desired concentrations were obtained by diluting with culture medium such that the final DMSO concentration did not exceed 0.1% (v/v). Following addition of the chemical, the cultures were incubated in the dark in a 37° humidified incubator with 12% CO_2 in air. 7 days later, the cultures were fixed in absolute methanol (Fisher), stained with 10% aqueous Giemsa stain (Fisher), and scored for cloning efficiency and morphological transformation by criteria described previously (Barrett and Ts'o, 1978a).

Results

The results of the assay of the 10 compounds are presented in Table 1 and summarized in Table 2. One transformed colony was observed in the control (DMSO-treated) cultures among 1763 colonies examined (0.06%). These controls were run simultaneously with the test compounds. Data for control cultures from experiments run concurrently in the laboratory for purposes other than this study are also included in Table 1, as well as controls run in the laboratory over the past two years. The frequency of spontaneous transformation in the concurrent controls (0.014%) is similar to the historical controls (0.006%) and to the previously reported spontaneous frequency of 0.01% by Barrett and Ts'o (1978c).

The test compounds were assayed generally at

TABLE 2

SUMMARY OF RESULTS

Compound	SHE cell transformation	Carcinogenicity
HMPA	+	+
SAF	+	+
TOL	+	+
CAP	+	−
ZOIN	−	−
BEN	+	+
DEHP	+	+
DES	+	+
ACN	+	+
PB	−	+

0.01, 0.1, 1.0, 10 and 100 μg/ml. The compounds were assayed 2–3 times, although the repeat assays were performed at doses selected from the initial experiments. The data from all experiments were combined and are presented in Table 1. A compound was considered positive if it induced more than 4 morphologically transformed colonies per 2000 surviving colonies and negative if no transformed colonies per 1000 surviving colonies were observed. All compounds scored as positive yielded morphologically transformed colonies in at least 2 of 2 or 3 assays.

As summarized in Table 2, hexamethylphosphoramide (HMPA), safrole (SAF), *o*-toluidine (TOL), caprolactam (CAP), benzene (BEN), diethylhexylphthalate (DEHP), diethylstilbestrol (DES), and acrylonitrile (ACN), were found positive in our study. Benzoin (ZOIN) and phenobarbital (PB) were negative in all assays.

Discussion

One of the difficulties with the Syrian hamster embryo (SHE) cell transformation assay is that it is not a selective assay; hence one must score morphological changes in each surviving colony, normal and transformed. When the frequency of transformed colonies is low, one must examine many colonies to obtain positive results. However, if the spontaneous frequency of transformed colonies is very low, the induction of transformation can be demonstrated with only a small number of positive colonies in the treated group, as shown by Pienta et al. (1977). This allows establishment of the qualitative response of the SHE cells to carcinogens with only 2 or 3 experiments, each requiring 8–10 days. This approach often does not yield quantitative dose–response results due to the low number of transformation events scored, although dose–response studies with a variety of chemicals and physical carcinogens have been reported with SHE cells. Considerably more experiments are required for this information (Barrett et al., 1984). For example, the qualitative induction of cell transformation at a level significantly above spontaneous levels requires ~ 1000 colonies per dose, whereas dose–response studies require 10 000 or more colonies per dose based on our studies (Barrett et al., 1984). Thus, the purpose of a SHE cell-transformation study has to be considered when deciding on the extent of that study. The purpose of the present study was to determine the qualitative response of the 10 test compounds and therefore, only 2–3 assays per compound were performed. Time restraints prevented additional studies and the results should not be considered more than qualitative. The lack of a dose–response with some of the compounds should not be considered conclusive or significant.

Several of the compounds in this study have been previously assayed by others in the Syrian hamster embryo cell-transformation system. Pienta (1980) reported positive results with SAF, TOL and DES and negative results with CAP and PB. Our findings agree with Pienta's with the exception of CAP, which we found to be positive at high doses. Amacher and Zelljadt (1983) reported that benzene transforms SHE cells and we have confirmed these results in an independent study of this compound (T. Tsutsui and J.C. Barrett, unpublished). DEHP was reported positive in SHE cells by Tomita et al. (1982), while no prior studies of HMPA, benzoin or acrylonitrile are known to us.

Sanner (this volume) also tested the same 10 compounds for the IPCS study in the SHE cell-transformation assay. His results agreed with our findings for 8 of the 10 compounds. The 2 exceptions were DES, which he reported negative, and PB, which he reported positive. As mentioned, our findings confirmed the earlier work of Pienta so

additional studies will be necessary to conclusively establish the response of these 2 compounds. Our laboratory has studied DES for a number of years and has reproducibly demonstrated that DES induces morphological transformation in numerous assays (Barrett et al., 1981; McLachlan et al., 1982; Tsutsui et al., 1983). In addition, we have reported that DES induces neoplastic transformation of the SHE cells (McLachlan et al., 1982).

Tanner has modified the SHE cell assay by replenishing the cultures with fresh medium and test compound 2 days prior to fixation of the cells. This is the major difference in the protocols used in his study compared to Pienta's or our studies. Sanner's modification results in an enhanced frequency of transformation in both the control cultures as well as the carcinogen-treated cultures. The activity of DES in our assays is often weak, although statistically greater than control values (McLachlan et al., 1982). We have never observed a transformation frequency of $> 1\%$ with DES. The high spontaneous transformation frequency in Sanner's assay may have precluded his detection of the activity of DES. We have also demonstrated that DES is metabolized by SHE cells via a peroxidase-mediated reaction, possibly by prostaglandin synthetase (Degen et al., 1982), and we have suggested that DES metabolism may be important in its cell transformation (McLachlan et al., 1982). Pienta's and our laboratories used hamsters from the Lakeview Hamster Colony, while Sanner used a different strain of hamsters. Strain differences in DES metabolism may account for the differences in response in DES-induced cell transformation. Further studies will be needed to test this possibility.

Our data with phenobarbital is limited, but we detected no activity in our study; this is consistent with the results reported by Pienta (1980). We have no explanation for the positive results observed by Sanner.

Comparing the reported carcinogenicity in vivo of the 10 compounds in this study to their activity in our cell-transformation assays, we find very good agreement. 7 of the 8 carcinogens in the study were found to induce cell transformation including DES, benzene and DEHP, which were negative in many of the mutagenicity assays. The only carcinogen in this study that was not active in our assay was phenobarbital (PB). Since PB is primarily a liver carcinogen and promoter, its lack of activity may be related to its target cell specificity and/or its mechanism of action. One of the noncarcinogens in this study (benzoin) was negative in our assay, while the other (caprolactam) was positive at high concentrations. Caprolactam was also positive in some of the other assays in this international study, including clastogenicity in human lymphocytes and somatic mutations in Drosophila. Further studies of the activity and metabolism of caprolactam are needed.

DES, DEHP and benzene were found to induce aneuploidy in yeast and transformation of SHE cells, but were inactive in the other tests of the IPCS study. Our laboratory (Barrett et al., 1983; Tsutsui et al., 1983) has previously proposed aneuploidy induction as a possible mechanism for cell transformation and these findings are consistent with our hypothesis.

Different results were obtained with the cell transformation assays that use established mouse cell lines. We have previously suggested that different cell-transformation assays are intrinsically different (Barrett and Thomassen, 1984). The SHE cell assay involves the use of normal, diploid cells and one measures the induction of preneoplastic cells by carcinogens. On the other hand, cell lines have been shown to be preneoplastic and one measures the induction of a later stage in cell transformation with these assays. Differences in results, especially with compounds that induce aneuploidy, are not surprising when one considers that the target cell in one assay is diploid, while in the other assay the target cell is already aneuploid.

The performance of the SHE cell transformation assay in this international study is judged to be very good. In particular, the assay demonstrated the activity of certain carcinogens not active in other assays. Thus, the system appears to be important for the detection of carcinogens and for studies of their mechanism of action.

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Assays for enhanced DNA viral transformation of primary Syrian hamster embryo (SHE) cells

G.G. Hatch and T.M. Anderson

In Vitro Toxicology Division, Northrop Services Inc., Research Triangle Park, NC (U.S.A.)

Summary

The chemicals hexamethylphosphoramide, *o*-toluidine, safrole, caprolactam, diethylhexylphthalate and diethylstilbestrol were assayed for their capacity to enhance the adenovirus (SA7) transformation of Syrian hamster embryo (SHE) cells. The SHE–SA7 system reflects the capacity of a chemical to damage cellular DNA and provides a rapid mammalian embryo cell-transformation bioassay system for screening suspected genotoxic potential of environmental chemicals. All chemicals were tested at toxic to nontoxic doses. Replicate transformation assays were completed on each compound, and the test results were analyzed according to a previously defined set of response criteria for the SHE–SA7 bioassay. The animal carcinogens, hexamethylphosphoramide, *o*-toluidine, diethylhexylphthalate and diethylstilbestrol produced activity when tested without exogenous metabolic activation in the SHE–SA7 transformation assay. Benzene and safrole produced negative results. The putative negative control, caprolactam, which does not produce tumors in rodent tumor bioassays, did not produce activity in this in vitro test. Those test chemicals producing a response in the SHE–SA7 transformation system may have carcinogenic potential since positive results in this mammalian cell bioassay system closely correlate with the carcinogenic potential of many diverse compounds and structurally similar agents.

Short-term in vitro tests for genetic toxicity offer a means for screening large numbers of structurally diverse compounds in a relatively short time and at relatively low cost. A short-term in vitro assay for mutagens and carcinogens has been developed (Casto, 1973b, 1981a; Casto et al., 1973, 1974, 1976; Hatch et al., 1982c, 1983a) that determines the ability of pretreatment by various chemicals of many diverse classes to enhance the adenovirus (SA7) transformation of primary Syrian hamster embryo (SHE) cells (SHE–SA7 bioassay) as well as complex mixtures (Casto et al., 1981) or multiple agents (Hatch and Anderson, in press).

In brief, experimental data suggest that discrete changes in cell DNA resulting from chemical treatment can be successfully detected by observing a quantitative increase in viral transformation. Such increases in DNA viral transformation have been demonstrated in hamster, rat and human cells treated with a variety of carcinogenic agents (Casto 1981a; Hatch et al., 1983a). The viral enhancement assay reflects the capacity of a chemical to damage cell DNA by either direct or indirect means and provides a rapid mammalian cell bioassay system for screening of suspect genotoxic environmental chemicals. Concurrently, it has been shown that carcinogen treatment results in an increased incorporation of SA7 viral DNA into cellular DNA (Casto et al., 1979). In addition, a quantitative relationship between chemical–DNA adduct formation and biological effect as measured by enhanced viral transformation frequency has been demonstrated (Theall et al., 1982). Positive test results in this system are generally concordant with those obtained in bacterial and mammalian cell mutagenesis assays (Casto, 1981b;

Hatch et al., 1982b) and in in vivo carcinogenesis assays (Casto, 1981a, b; Hatch et al., 1983a, Hatch et al., in preparation).

To fully utilize the SHE–SA7 transformation bioassay as a reliable system for screening potential genotoxic agents, it is necessary to consistently reproduce experimental results both within and between laboratories. Recently, the protocol for chemical enhancement of SA7 adenovirus transformation was standardized, and the variance of the system determined, by 2 independent laboratories using a set of 9 model compounds known to produce positive and negative responses in the test system (Hatch and Anderson, 1983; Schechtman et al., in preparation). In addition, the ability of this bioassay system to successfully produce similar experimental data on coded chemicals both within and between laboratories utilizing a wide variety of structurally diverse compounds in widespread industrial and pharmacological use has been documented (Hatch et al., in preparation).

To further optimize cell-culture systems for detecting all types of potential genotoxic agents, suitable methodologies must also be developed that will permit the detection of different physical forms of test agents. We have previously reported the successful development of quantitative methods for treating mammalian cell cultures with volatile chlorinated organic liquids and gases (Hatch et al., 1982c, 1983a), ethylene oxide (Hatch et al., 1982b) and formaldehyde (Hatch et al., 1983b) utilizing minimal volumes of buffered media, rocker platforms, and closed treatment chambers. These techniques allow for the evaluation of the genotoxic effects of these agents in diverse in vitro systems including the SHE–SA7 viral enhancement bioassay.

In the present study, the SHE–SA7 viral enhancement assay was utilized to evaluate 7 of the 10 compounds selected by the International program on Chemical Safety for collaboratorive studies of short-term assays for chemical carcinogens. All compounds were added to culture medium using established protocols, except for benzene treatments, which utilized the methodology developed for exposing cells to the vapor phase of selected chemicals in closed treatment chambers. After replicate SHE–SA7 bioassays were completed, the test results on each compound were compared and analyzed with reference to a previously defined set of response criteria.

Materials and methods

Test chemicals

Test chemicals were supplied by John Ashby, Imperial Chemical Industries, North Macclesfield, Cheshire, U.K., and stored at −20 °C. Some replicate experiments utilized the following sources of chemicals: hexamethylphosphoramide (Radian Corporation, Austin, TX), and benzene (Aldrich Gold Label [99 + % pure], Aldrich Chemical Co., Milwaukee, WI).

Preparation of test chemicals

For treatment in culture medium, the test compounds were dissolved in dimethyl sulfoxide or directly in modified Dulbecco's medium (MDM) containing 0.11 g% $NaHCO_3$ and supplemented with 10% fetal bovine serum (FBS). Serial 2-fold dilutions were made until 5 or more treatment concentrations were obtained. The positive control, benzo[*a*]pyrene [BP], was dissolved in dimethyl sulfoxide and added to complete culture medium to produce final treatment concentrations of 0.004, 0.002 and 0.001 mM. Working stocks of all test chemicals were prepared and used for chemical treatment within 2 h.

For treatment in modular chambers, 5 vol. of benzene were rapidly volatilized at 37 °C within 4.6 l treatment chambers containing cultured cells to give the desired treatment concentrations. The chemical, glass pipettes, and petri dishes were prechilled to −20 °C. Each treatment volume was mechanically dispensed with a glass pipette into an open glass petri dish. Each dish was quickly placed in an exposure chamber with the cultured cells, and the chambers were rapidly sealed and the chemical volatilized at 37 °C. The positive control for the volatile treatment protocol was 1,2-dichloroethane (DCE) (Aldrich Chemical Co., Milwaukee, WI). DCE is a known animal carcinogen in rodent tumor bioassays (Infante and Tsongas, 1982) and enhances SA7 virus transformation (Hatch et al., 1983a). Treatment concentrations of 160, 80, and 40 ppm of DCE were volatilized within individual treatment chambers as described above.

Cell cultures

Primary Syrian hamster embryo (SHE) cell cultures were prepared by trypsinization of eviscerated and decapitated embryos (Charles River Breeding Laboratories, Wilmington, MA) after approximately 14 days of gestation. Cells were resuspended in MDM (Grand Island Biological Co., Grand Island, NY) containing 0.22 g% $NaHCO_3$ and supplemented with 10% FBS (Sterile Systems, Logan, UT). Approximately 5×10^6 cells in 5 ml of medium were seeded into 60-mm glass petri dishes (Corning Glass, Corning, NY) and incubated at 37° in a 5% CO_2 atmosphere. Total cell counts after 3 days were approximately 5×10^6 cells per dish.

Transforming virus

A continuous line of African Green monkey kidney (Vero) cells (ATCC : CCL-81) was used for the preparation and titration of Simian Agent 7 (SA7) transforming adenovirus. The cells were cultured in Eagle's minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, NY) containing 10% FBS and 0.22 g% $NaHCO_3$.

Vero cells were cultured to yield approximately 1.5×10^7 cells/100-mm dish and then infected with SA7 virus (3–5 plaque-forming units per cell) in 0.5 ml of MEM with 2% FBS. After a 3-h adsorption at 37°C, 5 ml of MEM with 5% FBS and 0.22 g% $NaHCO_3$ were added to each dish. After 72–96 h, cytopathic effects were complete, and the cells were harvested and the virus released by 4 cycles of freeze-thawing. Virus stocks were titered for focus-forming and plaque-forming units and utilized in virus transformation experiments.

Chemical treatment in culture medium

Primary hamster embryo cells were cultured 72–96 h and refed with fresh medium (3 ml/dish of MDM, 10% FBS, 0.22 g% $NaHCO_3$) 6–8 h prior to chemical treatment. Medium from replicate dishes of SHE cells was removed and replaced with medium containing each treatment concentration of test chemical or positive control. Chemical treatment was maintained for approximately 20 h, after which the cells were assayed for viability and enhancement of viral transformation.

Chemical treatment by volatile treatment protocol in modular chambers

The methodology developed for exposing cells to the vapors of volatile chemicals has been previously described (Hatch et al., 1982c, 1983a). In brief, primary SHE cells were cultured 72–96 h and refed with fresh medium (3 ml/dish of MDM, 10% FBS, 0.22 g% $NaHCO_3$) 6–8 h prior to treatment. This medium was replaced with 1 ml of medium supplemented with 7.5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid, Leon Labs, St. Louis, MO) buffer in place of sodium bicarbonate when cells were transferred to closed treatment chambers.

Polycarbonate treatment chambers of 4.6-l volume (Billups-Rothenberg, Flow Labs, McLean VA) were utilized for chemical treatment. The chambers were opened for insertion of dishes of cultured cells, and 10 ml of sterile water were added to the bottom of each chamber to ensure adequate humidification and to prevent desiccation of the small volumes of cell-culture medium. Treatment dishes containing appropriate volumes of test chemicals or positive control were placed in the chambers, which were rapidly resealed with springsteel clamps. The sealed chambers were placed on rocker platforms in a 37°C incubator. The chambers were tilted to provide sufficient inclination (10°) and rocked 4 times per minute so that a portion of the cell monolayer was always in contact with test vapor. Treatment was maintained for 20 h, after which the vapors were exhausted through charcoal under negative pressure, and the cells were assayed for viability and enhancement of viral transformation. Individual chambers were used for exposure to each concentration of volatilized chemical.

Preliminary cytotoxicity dose determination study

To determine the concentration of test chemical to be used in the virus transformation assay, replicate 60-mm dishes of primary SHE cells (approximately 5×10^6 cells per dish) were treated with 5 concentrations of sample contained in 3 ml of complete medium. The final concentration of chemical ranged from 1000 to 1.6 μg/ml in 5-fold dilutions. After a 20-h treatment period, the chemicals were removed, and the cultures trypsinized from the dishes. The cells were centrifuged at

$800 \times g$ for 5 min, resuspended in complete medium to give 10^6 cells per ml, diluted to 3.3×10^3 cells/ml, and 0.2 ml (666 cells) were plated onto 5 dishes. 3 ml of medium (MDM, 10% FBS, 0.11 g% $NaHCO_3$) were added and replaced with medium containing 0.22 g% $NaHCO_3$ after 72 h of incubation at 37 °C. Cells were incubated for 8 days at 37 °C in 5% CO_2, fixed in buffered 10% formalin, and stained with 0.02% crystal violet.

The stained colonies formed from cells that survived chemical treatment were enumerated on a Biotran III Colony Counter (New Brunswick Scientific Co., Inc., Edison, NJ). Relative plating efficiencies were determined for control and chemically treated cells. The treatment concentration that produced $\geq 50\%$ cytotoxicity was selected as the highest treatment dose for the subsequent viral enhancement experiment, and 4 or more additional treatment concentrations at 2-fold decrements were selected. Doses were modified for replicate virus enhancement experiments as further cytotoxicity information was obtained.

Virus transformation experiments

Complete adenovirus transformation procedures have been previously described (Casto, 1968, 1973; Casto et al., 1973, 1974). Following 20 h of chemical treatment, SHE cells were rinsed and inoculated with 3×10^7 plaque-forming units of SA7 virus in 0.2 ml of medium (MEM, 2% FBS). After 3 h of adsorption, the cultures were trypsinized, centrifuged, and resuspended in complete medium to 10^6 cells/ml.

Additional cultures of SHE cells from the same embryo pools were treated with the following concentrations of positive control: benzo[*a*]pyrene (BP) at 0.004, 0.002 and 0.001 mM or 1,2-dichloroethane (DCE) at 160, 80 and 40 ppm.

For viral transformation assays, 2×10^5 cells per test concentration were seeded onto each of ten 60-mm petri dishes. After 48 h, this medium was changed to medium with low calcium (0.1 mM) (Freeman et al., 1967) and 5 days after cell transfer, was changed to low calcium medium supplemented with 0.3% agar. The cells were maintained by the weekly addition of 3 ml of fresh agar medium to each dish, formalin fixed, and Giemsa stained after 21–25 days. The adenovirus foci appeared as darkly stained areas of piled-up cells against a lightly stained background of normal cells. The SA7 transformed foci had a unique cytomorphology and were easily distinguished from areas of normal cell clusters.

For determination of cytotoxicity following test article exposure, the suspension (10^6 cells/ml) of treated, virus-inoculated cells were serially diluted ($1:15 \times 1:20$) to give 333 cells per 0.1 ml; 0.2 ml were then placed into each of 5 dishes, fed with 3 ml of medium, changed with fresh medium after 48 h, and fixed and stained after 8–10 days of incubation at 37 °C in 5% CO_2. Plating efficiencies of control cells usually ranged from 5 to 15%.

Data collection and interpretation

The number of colonies per dish in the cell survival assay and the number of transformed foci per dish in the transformation assay were determined. The absolute plating efficiencies of treated and control cells were determined by dividing the number of colonies observed on 5 dishes by 3330 [the number of dishes (5) $\times$ the number of cells plated per dish (666)] and expressed as a percent. The plating efficiency of the treated cells divided by that of the solvent control cells was used to determine the surviving fraction at each treatment concentration. The number of foci observed on 10 plates was divided by 2 and by the surviving fraction at that dose to obtain the transformation frequency (viral foci per 10^6 surviving cells). Detailed methods for determining the frequency of transformation and enhancement ratios from actual transformation experiments have been published (Casto et al., 1973, 1976). Similar methods for calculation have been used for the determination of mutation frequency (Chu, 1971) and for the demonstration of enhancement of SV40 transformation by DNA-base analogues (Todaro and Green, 1964) in mammalian cells.

For determination of the enhancement ratio, the transformation frequency obtained from virus and chemically treated cells was divided by the transformation frequency obtained from cells inoculated with virus only. Statistical significance was determined from a table of critical ratios (Casto et al., 1973) constructed from Poisson distribution tables of significance developed by Lorenz (1962). The total number (N) of foci (control plus treated) for use with the table was taken

from the actual number counted and not from values corrected for toxicity. An increase in transformation frequency was considered significant at the 5% confidence level if the enhancement ratio exceeded the respective value in the table for N number of foci. Enhancement ratios uncorrected for cytotoxicity were calculated to indicate those treatment doses producing significant absolute increases in the number of viral foci relative to controls. Those dose levels producing enhancement independent of cytotoxicity were identified.

TABLE 1

RESPONSE CRITERIA FOR SHE-SA7 TRANSFORMATION BIOASSAY

Classificiation	Response criteria
Positive	Statistically significant [a] dose-dependent enhancement of viral transformation [b] at two or more treatment concentrations (where survival $\geq 10\%$), with a significant absolute increase [c] in viral foci at one or more treatment concentrations.
Strong suspect	Statistically significant [a] enhancement of viral transformation [b] at two or more concentrations.
Weak suspect	Statistically significant [a] enhancement of viral transformation [b] at one concentration.
Negative	No enhancement at any of 4–5 concentrations producing from 50 to 100% survival.
No response	No enhancement or toxicity produced at any concentration tested.
Null	An experiment that fails to meet technical acceptance criteria.

[a] Statistical significance is determined using a table of critical ratios (Casto et al., 1973) constructed from Poisson distribution tables (Lorenz, 1962). Increased transformation is considered statistically significant at the 5% confidence level, if the enhancement ratio (see footnotes b and c) exceeds the appropriate value obtained form the table of critical ratios.

[b] Enhancement is expressed as the ratio between transformation frequency of cells treated with chemical and virus and the transformation frequency of virus-treated control cells. Transformation frequencies are corrected for cytotoxicity by dividing the number of viral foci observed by the surviving fraction of chemically treated cells.

[c] Enhancement is expressed as the ratio between the absolute number of viral foci observed on cells treated with chemical and virus and the number of foci observed on virus-treated control cells. No correction is made for chemically induced cytotoxicity.

Response criteria for the SHE–SA7 viral enhancement bioassay

Working response criteria for the SHE–SA7 bioassay (Table 1) were developed prior to testing to provide a means for evaluating the activity of the test compounds in individual experiments. These criteria provide a definition of positive, negative, and several intermediate suspect responses in the bioassay system. The results of individual experiments were then evaluated to give an overall classification of positive, suspect, or negative activity for each test chemical in the SHE–SA7 system.

Results

Tables 2 and 3 present results of replicate experiments on the 7 compounds and 2 positive controls tested in the SHE–SA7 system. The compounds were tested at 5 or more 2-fold serial dilutions extending over an approximately 16–64-fold dose range. Dose ranges for transformation experiments were selected from preliminary cytotoxicity screens (data not shown). For brevity, some 100% lethal dose levels have been omitted. All compounds were tested from 100% lethal to nonlethal doses except where significant lethality could not be demonstrated utilizing elevated concentrations of chemical.

A positive control, the polycyclic hydrocarbon benzo[*a*]pyrene, was included in each bioassay to assure adequate sensitivity and metabolic capability of individual pools of primary SHE cells and consistently enhanced viral transformation at 0.004, 0.002 and 0.001 mM. The representative experiments shown (Table 2) demonstrate enhancement independent of cytotoxicity for significance at all dose levels in the first experiment and at 0.001 mM in a replicate experiment.

In the volatile treatment protocol, 1,2-dichloroethane was tested as a positive control and produced enhancement of viral transformation independent of cytotoxicity at treatment concentrations of 80 and 40 ppm in replicate experiments (Table 3).

Hexamethylphosphoramide (Table 2) produced significant cytotoxicity and enhanced viral transformation at the highest treatment dose of 112 mM in both experiments.

TABLE 2

ENHANCEMENT OF ADENOVIRUS TRANSFORMATION OF CHEMICALLY PRETREATED HAMSTER EMBRYO CELLS

Test chemical	Concentration [a] (mM)	Expt. 1			Expt. 2		
		Surviving fraction [b] (%)	SA7 foci [c]	Enhancement ratio [d]	Surviving fraction [b] (%)	SA7 foci [c]	Enhancement ratio [d]
Hexamethylphosphoramide	112 [e]	41	31	3.8	31	17	2.5
	84	NT [f]	NT	NT	78	18	1.0
M. wt. 179.24	56	81	23	1.4	77	19	1.1
CAS 680-31-9	28	85	29	1.7	100	14	0.6
	14	91	24	1.3	94	30	1.4
	0	100	20	1.0	100	22	1.0
o-Toluidine	19	NT	NT	NT	29	19	1.4
M. wt. 107.17	17	56	11	1.0	34	20	1.2
CAS 95-53-4	15	65	43 [g]	3.3	37	27	1.5
	13	51	23	2.2	51	37	1.5
	11	123	36	1.5	90	56	1.3
	9	93	39 [g]	2.1	92	35	0.8
	0	100	20	1.0	100	49	1.0
Diethylhexylphthalate	2.6	15	2	0.5	97	74 [g]	1.6
	1.3	50	16	1.2	100	76 [g]	1.6
M. wt. 390.56	0.6	95	25	0.9	103	55	1.1
CAS 117-81-7	0.3	112	42	1.4	94	42	0.9
	0.2	97	43	1.6	106	45	0.9
	0	100	28	1.0	100	48	1.0
Diethylstilbestrol	0.113 [e]	50	39	2.8	89	57	1.3
	0.056	58	26	1.6	76	57	1.6
M. wt. 268.38	0.028	76	37	1.7	83	35	0.9
CAS 000056531	0.014	108	34	1.1	99	43	0.9
	0.007	102	38	1.3	120	53	0.9
	0	100	28	1.0	100	48	1.0
Benzo[*a*]pyrene (positive control)	0.004	18	47 [g]	9.9	15	32	5.0
	0.002	64	63 [g]	3.8	66	58	2.1
M. wt. 252.32	0.001	77	58 [g]	2.9	83	76 [g]	2.2
CAS 000050328	0	100	26	1.0	100	42	1.0

[a] Test chemical was dissolved in solvent, added to cell culture medium, and added to replicate dishes of SHE cells for 20 h. Cells were rinsed and SA7 virus was adsorbed for 3 h. Cells were then transferred for survival (500–700 cells/dish) and transformation (200000–300000 cells/dish) assays.

[b] Determined from plates receiving 500–700 cells. The number of colonies from virally and chemically treated cells was divided by the number of colonies from virus-inoculated control cells to give the surviving faction. Plating efficiency of control was typically 5–15%.

[c] Number of foci from 2×10^6 plated cells.

[d] Enhancement ratio was determined by dividing the transformation frequency (TF) of treated cells (TF = SA7 foci × reciprocal of the surviving fraction) by that obtained from control cells. Underlined figures are significant at the 5% confidence level.

[e] Higher test doses were 100% toxic.

[f] NT, not tested.

[g] Absolute number of foci is significantly higher than control foci without correcting for cytotoxicity.

o-Toluidine (Table 2) was tested over a narrow dose range because preliminary cytotoxicity experiments indicated a sharp toxicity breakpoint. Enhancement of virus transformation was observed at 15, 13 and 9 mM in Expt. 1; this enhancement was independent of cytotoxicity at

TABLE 3

EVALUATION OF ADENOVIRUS TRANSFORMATION IN CHEMICALLY PRETREATED HAMSTER EMBRYO CELLS

Test chemical	Concentration [a] (mM)	Expt. 1			Expt. 2		
		Surviving fraction [b] (%)	SA7 foci [c]	Enhancement ratio [d]	Surviving fraction [b] (%)	SA7 foci [c]	Enhancement ratio [d]
Benzene	160 [e,f]	NT [g]	NT	NT	70	13	0.7
M. wt. 78.12	120	NT	NT	NT	83	18	0.8
CAS 71-43-2	100	97	32	1.5	NT	NT	NT
	80	NT	NT	NT	75	19	0.9
	50	103	34	1.5	NT	NT	NT
	40	NT	NT	NT	73	30	1.5
	0	100	22	1.0	100	27	1.0
Safrole	0.86 [e]	NT	NT	NT	14	1	0.2
M. wt. 162.20	0.62	114	20	0.4	86	39	1.0
CAS 94-59-7	0.43	NT	NT	NT	91	29	0.7
	0.31	83	31	0.9	91	42	1.1
	0.22	NT	NT	NT	112	38	0.8
	0.15	94	29	0.7	NT	NT	NT
	0	100	42	1.0	100	43	1.0
Caprolactam	44 [e]	NT	NT	NT	66	50	1.5
M. wt. 113.18	22	NT	NT	NT	82	49	1.2
CAS 105-60-2	11	NT	NT	NT	108	52	1.0
	8.8	101	34	0.8	NT	NT	NT
	5.3	NT	NT	NT	101	39	0.8
	4.4	89	22	0.6	NT	NT	NT
	2.2	93	36	0.9	NT	NT	NT
	0	100	42	1.0	100	43	1.0
1,2-Dichloro-	160 [f]	6	2	1.6	0	0	0
ethane	80	85	57 [h]	3.1	32	60 [h]	7.0
(positive control)	40	80	106 [h]	6.0	63	85 [h]	5.0
M. wt. 98.96	0	100	22	1.0	100	27	1.0
CAS 000107062							

[a] Test chemical was dissolved in solvent, added to cell culture medium, and added to replicate dishes of SHE cells for 20 h. Cells were rinsed and SA7 virus was adsorbed for 3 h. Cells were then transferred for survival (500–700 cells/dish) and transformation (200000–300000 cells/dish) assays.

[b] Determined from plates receiving 500–700 cells. The number of colonies from virally and chemically treated cells was divided by the number of colonies from virus-inoculated control cells to give the surviving fraction. Plating efficiency of control was typically 5–15%.

[c] Number of foci from 2×10^6 plated cells.

[d] Enhancement ratio was determined by dividing the transformation frequency (TF) of treated cells (TF = SA7 foci × reciprocal of the surviving fraction) by that obtained from control cells. Underlined figures are significant at the 5% confidence level.

[e] Higher test doses were 100% toxic.

[f] Treatment concentrations are represented in parts-per-million of volatilized chemical in individual treatment chambers.

[g] NT, not tested.

[h] Absolute number of foci is significantly higher than control foci without correcting for cytotoxicity.

15 and 9 mM. In Expt. 2, a similar dose range was employed and similar cytotoxicity was produced, but no activity was detected using an alternate pool of primary embryo cells.

Diethylhexylphthalate (Table 2) was tested from toxic to nontoxic doses in Expt. 1, but no enhancement of virus transformation was observed. A second experiment detected activity at the two

TABLE 4

RESPONSE OF TEST AND POSITIVE CONTROL COMPOUNDS IN SHE–SA7 VIRUS ENHANCEMENT BIOASSAY

Chemical [a]	Expt. 1	Expt. 2	Overall [b]
Hexamethylphosphoramide	Weak suspect	Weak suspect	Suspect
o-Toluidine	Positive	Negative	Suspect
Benzene	Negative	Negative	Negative
Safrole	Negative	Negative	Negative
Caprolactam	No response	Negative	Negative
Diethylhexylphthalate	Negative	Strong suspect	Suspect
Diethylstilbestrol	Suspect	Weak suspect	Suspect
Benzo[*a*]pyrene (control)	Positive	Positive	Positive
1,2-Dichloroethane (control)	Positive	Positive	Positive

[a] Chemicals are presented in the order recommended for testing.

[b] The overall activity of each compound as determined from replicate experiments is classified as positive, suspect, or negative.

highest doses of 2.6 and 1.3 mM that was independent of cytotoxicity for significance.

Diethylstilbestrol (Table 2) produced significant cytotoxicity and enhanced viral transformation at 0.113 and 0.028 mM in Expt. 1. Similar activity was produced at 0.056 mM in a second experiment.

Benzene (Table 3) demonstrated a narrow toxicity breakpoint (no doses producing moderate cytotoxicity) in Expt. 1, and no enhancement of virus transformation was observed. In a second experiment, some intermediate doses were tested that produced slight toxicity but no enhancement.

Safrole (Table 3) did not produce moderate toxicity or enhancement at any dose tested in Expt. 1. Intermediate doses were tested in a second experiment, but no enhancement of virus transformation was produced even at toxic doses.

Caprolactam (Table 3) did not demonstrate cytotoxicity or enhancement of virus transformation at doses up to 8.8 mM in Expt. 1. Significantly higher doses were tested in Expt. 2, and although some cytotoxicity was produced, no enhancement of virus transformation was observed.

Table 4 summarizes the individual test responses and overall classification for each of the 7 test compounds and the positive controls.

Discussion

Hexamethylphosphoramide, *o*-toluidine, diethylhexylphthalate and diethylstilbestrol produced suspect activity in the SHE–SA7 transformation assay. Benzene and safrole produced negative results. The putative negative control, caprolactam, which does not produce tumors in rodent tumor bioassays, did not produce any activity in the two experiments run.

The protocol utilized in this study, i.e. a 20-h chemical pretreatment of primary embryo cells (with no exogenous metabolic activation added) followed by inoculation with SA7 virus, was selected because it successfully detects the majority of carcinogenic chemicals in the historial data base of this system.

For example, data from bioassays of approximately 130 chemicals from 29 different chemical classes have been published using viral transformation enhancement assays. Unpublished or preliminary data exist for more than 100 other chemicals. These classes include alcohols and phenols, aliphatic amines, alkyl sulfates and sultones, aromatic amines, aryl halides, carbohydrates and derivatives, hydroxylamines, mycotoxins, polycyclic hydrocarbons, epoxides, aldehydes, organophosphates, phthalimides, hydrazines, inorganic metals, steroid hormones and chlorinated hydrocarbons (Casto et al., 1980; Casto, 1981a, b; Hatch et al., 1982a, b, c, 1983a, b). The last 4 classes are generally negative in standard bacterial (Salmonella) assays (McCann et al., 1975; McCann and Ames, 1976).

Detection of many diverse chemicals may be due to the inherent sensitivity of the system and

the use of primary embryo cells that contain considerable metabolic capacity including cytochrome P450 oxidases (Kouri et al., 1974).

Although primary hamster embryo cells are metabolically active, a very small number of compounds require the use of exogenous metabolic activation systems such as the addition of whole hepatocytes or subcellular S9 fractions from hamsters treated with the suspect chemical similar to those developed for other bacterial and mammalian mutagenesis and transformation assays. These compounds include urethane, thiourea, ethylenethiourea, dimethylnitrosamine (DMN), diethylnitrosamine (DEN) and dioxane, which are negative without exogenous activation added in the SHE–SA7 transformation system.

For example, urethane and DMN were positive in the SHE–SA7 transformation assay when appropriate subcellular (S9) (Casto and Hatch, 1977) or cellular (hepatocytes) (Hatch, unpublished) activation components were added. Similarly DiPaolo et al. (1972) have shown that urethane and DEN were negative for direct transformation in vitro utilizing a colony assay in primary hamster cells, but were positive when administered via the transplacental route.

In addition to the metabolic activation problems encountered with a few chemicals, a small number of carcinogenic chemicals are optimally detected in the SHE–SA7 transformation system when virus is added before chemical treatment or after a short (i.e. 2 h) treatment time (Casto, 1981a; Hatch et al., 1983a). For these reasons the 2 carcinogens, benzene and safrole, not detected in these first tests should be retested utilizing alternate protocols with varying treatment times and addition of exogenous activation systems. This was not done in this study.

It should be noted that benzene, although negative in the experiments reported herein, has previously produced positive activity in some pools of hamster embryo cells (unpublished). This apparent inconsistency is best explained by differences in metabolic activation capacity and/or sensitivity of the respective target cells of different primary embryo cell pools. Since benzene is a weak carcinogen and generally leukemogenic, critical target cells may be present in low numbers in cell populations that are predominantly fibroblasts.

In conclusion, 4 carcinogens were detected in the SHE–SA7 viral transformation assay. 2 compounds not detected in the initial tests, benzene and safrole, are judged to require the use of additional chemical treatment times and/or addition of exogenous activation. The negative control, caprolactam, produced no activity.

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Assays for morphological transformation and mutation to ouabain resistance of Balb/c-3T3 cells in culture

Edwin J. Matthews, T. DelBalzo and John O. Rundell

Department of Molecular Toxicology, Litton Bionetics Inc., Kensington, MD (U.S.A.)

Summary

8 rodent carcinogens [acrylonitrile (ACN), benzene (BEN), diethylstilbestrol (DES), diethylhexylphthalate (DEHP), hexamethylphosphoramide (HMPA), phenobarbital (PHEN), safrole (SAF), and *o*-toluidine (TOL)] and 2 noncarcinogens [benzoin (ZOIN) and caprolactam (CAP)] were tested for their ability to induce morphological transformants and ouabain-resistant variants of Balb/c-3T3 cells. In the absence of a provision for exogenous metabolic activation, none of the 10 chemicals tested induced morphological transformation in these cells. In contrast, when the 3T3 target cells were cocultured with primary rat-liver cells, 8 of the 10 test chemicals induced positive responses: ACN, BEN, CAP, DES, PHEN, SAF, TOL and ZOIN were active under conditions of rat-liver cell-mediated exogenous metabolic activation. DEHP and HMPA were inactive.

The 10 test chemicals were also evaluated for their ability to induce ouabain-resistant variants of Balb/c-3T3 cells. The treatments with ACN, BEN, SAF and TOL resulted in the appearance of statistically significant numbers of Oua^r variants. Treatments with ZOIN, CAP and DEHP resulted in at least 2-fold increases in the frequencies of the appearance of Oua^r variants but these frequencies did not attain statistical significance relative to the control values. DES, HMPA and PHEN were inactive.

Balb/c-3T3 cells have been widely applied to the routine testing of chemicals for their in vitro transforming potential. Although a number of 3T3 cell clones have been described, few are useful in the context of morphological transformation and all of those that are presently available were originally isolated by T. Kakunaga (1973). Most chemical screening studies have been conducted using a derivative of Kakunaga's clone 1–13.

Clone 1–13 of Balb/c-3T3 cells multiply in culture until a monolayer is achieved and then they cease further division; i.e., they exhibit density-dependent inhibition of replication or "contact inhibition." These cells, if injected into Balb/c or athymic mice, do not produce tumors (Kakunaga, 1973; Rundell et al., 1983). However, clone 1–13 cell populations treated in vitro with chemical carcinogens may give rise to cellular foci of differential growth and morphology superimposed on the normal-appearing cell monolayer. When these foci of transformed cells were isolated, and the cells grown to larger numbers and transplanted into appropriate host animals, sarcomatous tumors developed (Kakunaga, 1973; Rundell et al., 1983). Thus, the appearance of foci of morphologically altered cells is associated with the process of the cellular acquisition of malignant properties. This observation forms the basis for the use of these cells in chemical screening. The Balb/c-3T3 cell transformation assay has been shown to be insensitive to test chemicals such as nitrosamines and certain alkylating procarcinogens that require metabolic activation (Rundell et al., 1983). To overcome this limitation, we have developed an exogenous metabolic activation system for these cells, which is based on the use of primary rat-liver

cells in an effector cell–target cell coculture system (Matthews and Rundell, 1984). The application of this system has resulted in a significant increase in the sensitivity of clone 1–13 cells to treatments with a variety of chemicals representative of diverse chemical classes.

Clone 1–13 cells have also been used in screening studies designed to measure chemical mutagenic potential. Treatments of these cells with chemical mutagens followed by expression and selection using the cardiac glycoside ouabain resulted in the appearance of ouabain-resistant (Oua^r) variants thought to arise by a mutation in the gene controlling the synthesis of cell membrane Na^+/K^+ adenosine triphosphatase (Corsaro and Migeon, 1978). The possible significance of this is the observation that many carcinogens have been found to induce somatic cell gene mutations at a number of genetic loci, including the locus controlling the expression of ouabain resistance. However, there is much evidence that transformation to malignancy is a multistep process wherein the carcinogen-induced primary genetic lesion may be insufficient to cause the direct expression of malignant potential (Boutwell, 1974).

These observations support the premise that screening systems which combine tests measuring in vitro transformation with specific-locus mutation tests in the same target-cell population might yield data materially more useful in estimations of the carcinogenic potential of test chemicals; i.e., combined endpoint tests (measuring cell transformation and point mutation) may have application to questions of the mechanism of action of test chemicals. Accordingly, we have studied the ability of the test chemicals acrylonitrile (ACN), benzene (BEN), benzoin (ZOIN), caprolactam (CAP), diethylhexylphthalate (DEHP), diethylstilbestrol (DES), hexamethylphosphoramide (HMPA), phenobarbital (PHEN), safrole (SAF), and *o*-toluidine (TOL) to induce Balb/c-3T3 clone 1–13 cell morphological transformation and mutation to ouabain resistance in the presence and absence of exogenous metabolic activation. The results of these studies are the subject of this paper.

Materials and methods

Cells and cultures

Clone 1–13 of Balb/c-3T3 mouse embryo cells was obtained from T. Kakunaga of the National Cancer Institute. A subclone, C-14, was established in our laboratory and was used for these experiments. 3T3 cell stock cultures were cryopreserved in liquid nitrogen. Cells for use in the assays were grown and passaged biweekly in Eagle's minimal essential medium (EMEM) (Grand Island Biological Co., Grand Island, NY) supplemented with selected fetal bovine serum (FBS) (Armor Pharmaceuticals, Kankakee, IL), L-glutamine, penicillin, streptomycin and gentamycin (HEM, Rockville, MD). Cell incubations were conducted in a water-saturated 5% CO_2, 95% air atmosphere at $36.5 \pm 0.5\,^{\circ}C$.

Chemicals

The 10 test chemicals were supplied by John Ashby, Imperial Chemical Industries, Nr. Macclesfield, Cheshire, U.K., and were stored at $-20\,^{\circ}C$ until use. Supplies of 4 of the test chemicals were exhausted during the course of these studies and were replaced with commercial materials as follows: (1) benzene, Fisher Scientific Company, Catalog No. B-411 spectranalyzed Lot 703368; (2) hexamethylphosphoramide, Aldrich Chemical Co., Catalog No. H1, 160-2, 99% pure, Lot 0420PH; (3) caprolactam, Aldrich Chemical Co., Catalog No. 24059-1 99% pure, Lot 3807AJ, and (4) diethylhexylphthalate, Aldrich Chemical Co., Catalog No. D20, 115-4 98% pure, Lot 4711PJ.

Nonactivation transformation assay

Dose selections for the nonactivation Balb/c-3T3 cell transformation assays were made by choosing 15 dose levels of each chemical starting at the solubility limit or 10 mg/ml (10 μl/ml for liquids) and decreasing in 2-fold dilution steps. Each concentration was applied to 3 culture dishes seeded 24 h earlier with 200 3T3 cells per dish. After 72 h, the cells were washed and the incubations were continued for a total of 7–8 days, after which the surviving colonies were fixed with methanol, stained with 10% aqueous Giemsa and counted. Relative cell survivals were determined and doses that caused cell survivals over the 10–100% range were chosen for use in the transformation assays. The transformation assay experimental design used in these studies was adapted from that reported by Kakunaga (1973) and in-

volved treating 24-h-old cultures seeded with 3×10^4 cells with 5 preselected doses of the test chemical, a positive control and a solvent control (Rundell et al., 1983). The dishes were incubated for 72 h and then the cells were washed and incubation continued for approximately 24 days with twice-weekly refeedings. The assays were terminated by fixation with methanol and staining with 10% aqueous Giemsa. Transformed foci were scored microscopically and consisted of foci of densely piled-up cells with a disoriented criss-cross pattern that was invasive into a uniformly stained, continguous monolayer of cells.

Activation transformation assay

Primary rat-liver cells (RLC) were prepared from the livers of 300–400 g male Fischer-344 rats by the method of William's (1977). RLCs thus obtained were lethally X-irradiated (~ 5000 rad) and then plated at 1×10^5 viable cells per culture into 60-mm dishes plated 24 h earlier with 200 or 5×10^4 3T3 cells. The RLC-3T3 cocultures were incubated for 3 h to allow for RLC attachment and then treated with the test chemicals for a total of 48 h. Beginning 1–2 days after the test chemical treatment had been completed, the cocultures were treated with 0.05 μg/ml 12-*O*-tetradecenoylphorbol-13-acetate (TPA). The TPA posttreatments were continued biweekly for a total of 3 weeks and were discontinued one feeding interval prior to the termination of the assay.

Ouabain resistance (Oua^r) mutation assay

Doses for use in the Oua^{r}mutation assay were selected by use of a clonal cell survival assay that was similar to that previously described for the transformation assay; however, the nonactivation assay and S9-mediated activation assays employed test chemical exposure periods of 24 and 4 h, respectively. The S9 preparations used in the experiments were obtained from Aroclor-induced Sprague–Dawley rat-liver homogenates and were prescreened for their low intrinsic toxicity to 3T3 cells and their capacity to convert DMN to cytotoxic and mutagenic intermediates for the 3T3 target cells. The cofactors used included 236 μg/ml NADP and 1552 μg/ml isocitrate (core mix). Chemical treatments of 24-h-old 3T3 cultures (5×10^5/100 mm dish) were for 24 h (4 h for the S9 activation assays) using preselected test chemical doses. After the treatments, the test chemicals were removed and the cultures were refed and maintained for a 5–6-day expression period. At the end of the expression period, $5–20 \times 10^6$ cells were trypsinized, counted and replated at 0.25×10^6/60-mm dish (25 dishes per treatment condition and 75 or more dishes for the solvent control). Selection media containing 2 mM ouabain was added 1, 8 and 16 days after replating and the resultant Oua^r colonies were fixed, stained and counted at 19–21 days post plating. Each mutation assay included a survival assay conducted at the time of the treatments and a cloning efficiency assay conducted at the time of replating for selection.

Data collection and analysis

The data collected from individual 3T3 colony formation, nonactivation transformation, activation transformation and Oua^r mutagenesis assays were recorded on individual data forms. These forms included records of the materials used, dose preparation schemes, and number of 3T3 colonies, transformed foci, or Oua^r variants observed for the individual assays. Data analysis was complicated by the skewing observed in the distribution of foci/dish and Oua^r mutants/dish. This skewing was compensated for by converting the data to the $\log_{10}$ equivalent value prior to calculating the mean and standard error (SE) of the observations for the individual treatment conditions. Statistical comparisons of the various treatments against the concomitant controls were conducted using Bailey's (1959) modification of Student's *t*-test. Responses at one or more test chemical doses which attained at least the 95% confidence level were regarded as positive. All other categories of response were evaluated as negative.

Results

Nonactivation 3T3 cell transformation

A summary of the 3T3 cell transformation activities of the 10 organic chemicals tested is shown in Table 1 and the raw data is presented in Table 3. All chemicals were tested at doses yielding 10 to 100% relative cell survival as determined by a clonal survival assay (see Materials and Methods).

TABLE 1

SUMMARY OF THE TRANSFORMATION ACTIVITY OF THE 10 TEST CHEMICALS IN Balb/c-3T3 CELLS [a]

Chemical	Acronym	Assay response evaluation [b]		
		−RLC [c]	+RLC [d]	SUM [e]
Acrylonitrile	ACN	−	+	+[w f]
Benzene	BEN	−	+	+[w]
Benzoin	ZOIN	−	+	+[w]
Caprolactam	CAP	−	++	++[w]
Diethylhexylphthalate	DEHP	−	−	−
Diethylstilbestrol	DES	−, −	+, ++	++[w]
Hexamethylphosphoramide	HMPA	−	−	−
Phenobarbitol	PHEN	−	+	+[w]
Safrole	SAF	−	++, −	++[w]
o-Toluidine	TOL	−	++	++[w]
Dimethylnitrosamine	DMN	−	++	++[w]
3-Methylcholanthrene	MCA	++	++	++

[a] The results from individual transformation experiments with Balb/c-3T3 are in Table 3.

[b] Test chemical assay responses were evaluated statistically for significance using Bailey's modification of Student's *t*-test (1959): A strong positive response (++) indicates that $p < 0.01$ for 1 or more test chemical doses; a moderate positive response (+) indicates that $0.01 < p < 0.05$ at 1 or more test chemical doses; and a negative response (−) indicates that $p > 0.05$.

[c] RLC, primary Fischer 344 rat-liver cells prepared according to Williams (1977) and X-irradiated (see Materials and Methods) prior to use in the cocultures with Balb/c-3T3 cells.

[d] RLC denotes a RLC activation transformation assay where TPA (0.05–0.1 μg/ml) was used to enhance transformation activity (see Materials and Methods).

[e] SUM denotes the overall evaluation of the test chemical activities in the activation and nonactivation transformation assays.

[f] +[w] or ++[w] denotes a positive response observed only in the presence of exogenous activation.

TABLE 2

SUMMARY OF THE OUABAIN MUTAGENIC ACTIVITY OF THE 10 CHEMICALS IN Balb/c-3T3 CELLS [a]

Chemical	Acronym	Assay response evaluation [b]		
		−(S9) [c]	+(S9)	Sum
Acrylonitrile	ACN	ND	+	+
Benzene	BEN	ND	++	++
Benzoin	ZOIN	ND	?	?
Caprolactam	CAP	ND	?	?
Diethylhexylphthalate	DEHP	ND	?	?
Diethylstilbestrol	DES	−	−	−
Hexamethylphosphoramide	HMPA	ND	−	−
Phenobarbitol	PHEN	ND	−	−
Safrole	SAF	ND	++	++
o-Toluidine	TOL	ND	+	+
Dimethylnitrosamine	DMN	−	++	++

[a] The results from individual ouabain-resistance mutagenesis experiments using an exogenous S9 activation system are found in Table 4.

[b] Test chemical assay responses were evaluated for significance by examining the increase of absolute (ABS) and cloning efficiency (CE) corrected Oua[r] mutation frequencies relative to solvent controls. When the absolute Oua[r] mutant frequency for a test chemical dose exceeded the solvent control the statistical significance of this increase was determined using a modified *t*-test (Bailey, 1975). A *strong positive* response (++) occurred when ABS and CE adjusted Oua[r] frequencies were increased > 2-fold and $p < 0.01$; a *positive* response occurred when ABS and CE adjusted Oua[r] frequencies were increased > 2-fold and $0.05 \leq p \leq 0.01$; a *questionable* (?) response occurred when ABS and CE adjusted Oua[r] frequencies were increased > 2-fold, but $p < 0.05$; and a negative response (−) occurred when ABS and CE adjusted Oua[r] frequencies were increased < 2-fold and $p > 0.05$.

[c] Data for the nonactivation condition was available for DMN and DES only. ND, not done.

None of the test chemical treatments resulted in significant increases in the number of transformed foci. False negative responses are apparent in this data set suggesting that the 3T3 target cells were unable to metabolize the test chemicals to their proximate forms.

RLC activation transformation

Data obtained using an RLC-mediated metabolic activation system are summarized in Table 1 and the raw data is compiled in Table 3. In contrast to the nonactivation assay results, the treatments with 8 of the test chemicals resulted in statistically significant increases in the appearance of transformed 3T3 cell foci. The doses yielding the maximum observed activities for these chemicals ranged from 0.012 mM to 56.5 mM, including: (1) 0.012 mM (3.1 μg/ml) for DES; (2) 0.13 mM (8.8 nl/ml) for ACN; (3) 0.68 mM (100 nl/ml) for SAF; (4) 1.41 mM (150 nl/ml) for TOL; (5) 1.88 mM (400 μg/ml) for ZOIN; (6) 2.87 mM (667 μg/ml) for PHEN; (7) 44.2 mM (5000 μg/ml) for CAP; and (8) 56.5 mM (5000 nl/ml) for BEN (see Table 3). These doses were all within the limits of apparent solubility of the respective chemicals in the culture media and all were lower than the optimal positive control DMN concentration of 107 mM (8.0 μl/ml). DEHP and HMPA were tested at relative cell survivals equivalent to those employed for the aforementioned test chemicals, but the treatments with these test chemicals did not result in statistically significant responses in the presence of RLC in the assays. Treatments with DEHP resulted in significant detoxification; i.e., the 20% survival dose shifted from 16.0 nl/ml to over 20.0 μl/ml when tested in the absence and in the presence of RLC, respectively (see Table 3).

The procarcinogen DMN was used as the positive control in these assays and has been shown to be nontransforming and nonmutagenic to 3T3 cells in the absence of an exogenous metabolic activation system. Treatments of 3T3-RLC cocultures with DMN resulted in an increase in transformation activity which usually attained the 90–95% confidence level. Posttreatments of the DMN-treated 3T3-RLC cocultures with the potent in vivo tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA, 0.05 μg/ml) resulted in an enhancement of the response of the 3T3 target cells to the treatments with the procarcinogen. Treatments of 3T3-RLC cocultures also resulted in the recovery of a high frequency of Oua^r variants showing that the RLC mediated the formation of DMN mutagenic intermediates (Matthews and Rundell, 1984). The use of TPA treatments to enhance transformation activity has been reported with transformable C3H/10T$\frac{1}{2}$ cells (Frazelle et al., 1982; Mondal et al., 1976), and 3T3 cells (Shimomura et al., 1983). In both 3T3 and C3H/10T$\frac{1}{2}$ cells, the TPA posttreatments alone failed to transform the cells in the absence of pretreatments with a mutagen; the treatments with TPA were most effective when the TPA treatments were begun 1–3 days after the completion of the initial exposure to the mutagens. Structural analogs of TPA without in vivo tumor-promoting activity failed to enhance transformation activity in both cell systems.

Ouabain resistance (Oua^r) mutagenesis

More than 30 chemicals have been tested for their mutagenic activity at the Oua^r locus in 3T3 cells in this laboratory. In an ongoing study of nonactivation and S9 activation Oua^r mutagenesis assay designs using model mutagens, the use of an S9 activation system has been shown to increase the sensitivity of the assays to treatments with a variety of promutagens. With the exceptions of DES and DMN, the 10 chemicals tested in the present study were evaluated only in the presence of an S9 activation system. Under S9 activation conditions, 4 test chemicals induced statistically significant increases in the frequency of the appearance of Oua^r variants (see Table 2 for a summary of these data). The maximum active doses for these chemicals were: (1) 0.76 mM (50 nl/ml) for ACN; (2) 3.38 mM (0.5 μl/ml) for SAF; (3) 18.8 mM (2.0 μl/ml) for TOL; and (4) 56.5 mM (5.0 μl/ml) for BEN. These 4 chemicals also induced positive transformation responses in 3T3 cells. Clear dosimetric responses were not observed for these activities possibly because of an effect of the toxicity of the treatments on expression time. 3 chemicals (ZOIN, CAP and DEHP) induced greater than a 2-fold increase in the appearance of Oua^r mutants at one or more doses. However, these activities were not statistically sig-

TABLE 3

RESULTS FROM INDIVIDUAL TRANSFORMATION EXPERIMENTS WITH Balb/c-3T3 CELLS

Chemical (acronym) trial	± RLC [a]	Culture vessel [b]	Relative cell survival [c]		Relative transformation activity		
			Dose (nl/ml or μg/ml)	% Survival	Dose (nl/ml or μg/ml)	Foci/vessel [d] $\bar{x} \pm SE$ (n)	p [e]
Acrylonitrile (ACN, 1000 nl/ml ≅ 15.2 mM)							
1	− RLC	C.F.	0.0	100	0.0	0.00 ± 0.00 (26)	Control
			4.7	62	5.1	0.00 ± 0.00 (14)	N.S.
			9.4	86	10.2	0.04 ± 0.04 (18)	$0.3 < p < 0.4$
			18.8	8	12.8	0.00 ± 0.00 (15)	N.S.
			37.5	0	16.0	0.04 ± 0.04 (17)	$0.3 < p < 0.4$
			75.0	0	20.0	0.00 ± 0.00 (17)	N.S.
2	+ RLC	C.F.	0.0	100	0.0	1.09 ± 0.12 (21)	Control
			8.8	95	8.8	2.35 ± 0.15 (18)	$0.01 < p < 0.02$ *
			16.7	22	16.7	0.99 ± 0.15 (18)	N.S.
			25.0	0	25.0	0.29 ± 0.09 (18)	N.S.
Benzene (BEN, 1000 nl/ml ≅ 11.3 mM)							
1	− RLC	C.F.	0.0	100	0.0	0.29 ± 0.15 (40)	Control
			1500.0	98	500.0	0.09 ± 0.09 (8)	N.S.
			3000.0	59	2000.0	0.09 ± 0.07 (20)	N.S.
			6000.0	0	4000.0	0.08 ± 0.05 (18)	N.S.
2	+ RLC	C.F.	0.0	100	0.0	1.09 ± 0.12 (21)	Control
			1667.0	103	1667.0	1.43 ± 0.14 (18)	$0.3 < p < 0.4$
			3333.0	59	3333.0	2.26 ± 0.19 (18)	$0.02 < p < 0.05$*
			5000.0	0.3	5000.0	2.62 ± 0.21 (14)	$0.01 < p < 0.02$*
Benzoin (ZOIN, 100 μg/ml ≅ 4.71 mM)							
1	− RLC	D	0.0	100	0.0	0.00 ± 0.00 (19)	Control
			15.6	85	9.2	0.00 ± 0.00 (15)	N.S.
			31.3	81	36.7	0.00 ± 0.00 (10)	N.S.
			62.5	50	73.3	0.00 ± 0.00 (4)	N.S.
			125.0	15	110.0	0.00 ± 0.00 (16)	N.S.
			250.0	0.5	165.0	0.00 ± 0.00 (7)	N.S.
2	+ RLC	D	0.0	100	0.0	0.55 ± 0.08 (39)	Control
			200.0	92	200.0	0.75 ± 0.15 (20)	$0.4 < p < 0.5$
			400.0	75	400.0	1.25 ± 0.17 (17)	$0.02 < p < 0.05$*
			600.0	77	600.0	1.24 ± 0.18 (20)	$0.05 < p < 0.1$
Caprolactam (CAP, 1000 μg/ml ≅ 8.84 mM)							
1	− RLC	D	0.0	100	0.0	0.00 ± 0.00 (19)	Control
			250.0	98	396.0	0.00 ± 0.00 (15)	N.S.
			500.0	100	1583.0	0.04 ± 0.04 (17)	$0.3 < p < 0.4$
			1000.0	78	3167.0	0.04 ± 0.04 (17)	$0.3 < p < 0.4$
			2000.0	69	4750.0	0.00 ± 0.00 (12)	N.S.
			4000.0	31	5938.0	0.10 ± 0.07 (14)	$0.1 < p < 0.2$
2	+ RLC	D	0.0	100	0.0	0.55 ± 0.08 (39)	Control
			2500.0	96	2500.0	2.52 ± 0.12 (20)	$p < 0.01$*
			5000.0	79	5000.0	4.99 ± 0.12 (20)	$p < 0.01$*
			7500.0	32	7500.0	2.32 ± 0.16 (20)	$p < 0.01$*

TABLE 3 (continued)

Chemical (acronym) trial	± RLC [a]	Culture vessel [b]	Relative cell survival [c]		Relative transformation activity		
			Dose (nl/ml or μg/ml)	% Survival	Dose (nl/ml or μg/ml)	Foci/vessel [d] $\bar{x} \pm SE$ (n)	p [e]
Diethylhexylphthalate (DEHP, 1000 nl/ml ≅ 2.51 mM)							
1	− RLC	C.F.	0.0	100	0.0	0.37 ± 0.11 (20)	Control
			3.91	77	0.9	0.29 ± 0.10 (19)	N.S.
			7.81	75	3.5	0.57 ± 0.19 (19)	$0.5 < p < 0.6$
			15.6	30	7.0	0.19 ± 0.13 (12)	N.S.
			31.3	3	14.0	0.09 ± 0.06 (17)	N.S.
			62.5	1	21.0	0.19 ± 0.10 (12)	N.S.
2	+ RLC	C.F.	0.0	100	0.0	1.09 ± 0.12 (21)	Control
			10000.0	62	10000.0	0.08 ± 0.05 (18)	N.S.
			25000.0	46	25000.0	0.04 ± 0.04 (18)	N.S.
			50000.0	26	50000.0	0.08 ± 0.05 (18)	N.S.
Diethylstilbestrol (DES, 1000 μg/ml ≅ 3.73 mM)							
1	− RLC	D	0.0	100	0.0	0.00 ± 0.00 (19)	Control
			1.6	81	1.1	0.05 ± 0.05 (14)	$0.3 < p < 0.4$
			3.1	8	2.3	0.00 ± 0.00 (9)	N.S.
			6.3	14	4.5	0.04 ± 0.04 (18)	$0.3 < p < 0.4$
			12.5	9	9.0	0.04 ± 0.04 (16)	$0.3 < p < 0.4$
			25.0	0	18.0	0.00 ± 0.00 (18)	N.S.
2	− RLC	D	0.0	100	0.0	0.12 ± 0.05 (30)	Control
			3.1	69	0.5	0.05 ± 0.05 (15)	N.S.
			6.3	51	4.5	0.22 ± 0.10 (18)	$0.4 < p < 0.5$
			12.5	31	9.0	0.04 ± 0.04 (17)	N.S.
			25.0	0	18.0	0.26 ± 0.14 (20)	$0.4 < p < 0.5$
3	+ RLC	D	0.0	100	0.0	0.25 ± 0.13 (11)	Control
			6.3	?	6.3	0.82 ± 0.15 (15)	$p = 0.05$
			12.5	?	12.5	0.06 ± 0.06 (12)	N.S.
			25.0	?	25.0	0.29 ± 0.11 (11)	$0.8 < p < 0.9$
4	+ RLC	D	0.0	100	0.0	0.55 ± 0.08 (39)	Control
			1.0	92	1.0	1.24 ± 0.14 (19)	$0.02 < p < 0.05$*
			3.1	79	3.1	3.08 ± 0.15 (20)	$p < 0.01$*
			10.0	20	10.0	0.26 ± 0.10 (20)	N.S.
Hexamethylphosphoramide (HMPA, 1000 nl/ml ≅ 3.75 nl/ml)							
1	− RLC	C.F.	0.0	100	0.0	0.00 ± 0.00 (22)	Control
			500.0	94	667.0	0.00 ± 0.00 (0)	N.S.
			1000.0	97	1334.0	0.21 ± 0.15 (11)	$0.1 < p < 0.2$
			2000.0	65	2667.0	0.00 ± 0.00 (10)	N.S.
			4000.0	22	4000.0	0.00 ± 0.00 (4)	N.S.
			8000.0	0	5000.0	0.13 ± 0.09 (11)	$0.1 < p < 0.2$
2	+ RLC	D	0.0	100	0.0	0.55 ± 0.08 (39)	Control
			3750.0	81	3750.0	0.63 ± 0.11 (20)	$0.6 < p < 0.7$
			7500.0	47	7500.0	0.61 ± 0.10 (20)	$0.7 < p < 0.8$
			15000.0	6	15000.0	0.35 ± 0.09 (20)	N.S.
Phenobarbitol (PHEN, 1000 μg/ml ≅ 4.30 mM)							
1	− RLC	D	0.0	100	0.0	0.00 ± 0.00 (19)	Control
			250.0	86	117.0	0.00 ± 0.00 (14)	N.S.

TABLE 3 (continued)

Chemical (acronym) trial	± RLC [a]	Culture vessel [b]	Relative cell survival [c]		Relative transformation activity		
			Dose (nl/ml or μg/ml)	% Survival	Dose (nl/ml or μg/ml)	Foci/vessel [d] $\bar{x} \pm SE$ (n)	p [e]
Phenobarbitol (*continued*)							
			500.0	64	467.0	0.00 ± 0.00 (11)	N.S.
			1000.0	41	933.0	0.00 ± 0.00 (12)	N.S.
			2000.0	3	1400.0	0.00 ± 0.00 (11)	N.S.
			4000.0	0	1700.0	0.00 ± 0.00 (11)	N.S.
2	+ RLC	D	0.0	100	0.0	0.55 ± 0.08 (39)	Control
			667.0	99	667.0	1.34 ± 0.14 (20)	$0.01 < p < 0.02$*
			1333.0	73	1333.0	0.89 ± 0.11 (20)	$0.1 < p < 0.2$
			2000.0	39	2000.0	0.64 ± 0.15 (20)	$0.7 < p < 0.8$
Safrole (SAF, 1000 nl/ml ≅ 6.76 mM)							
1	− RLC	C.F.	0.0	100	0.0	0.00 ± 0.00 (22)	Control
			31.0	81	10.0	0.00 ± 0.00 (16)	N.S.
			63.0	53	40.0	0.04 ± 0.04 (16)	$0.3 < p < 0.4$
			125.0	18	80.0	0.07 ± 0.07 (10)	$0.3 < p < 0.4$
			250.0	0	120.0	0.00 ± 0.00 (13)	N.S.
			500.0	0	180.0	0.13 ± 0.09 (13)	$0.1 < p < 0.2$
2	+ RLC	D	0.0	100	0.0	0.23 ± 0.13 (11)	Control
			125.0	NA	125.0	0.36 ± 0.13 (15)	$0.6 < p < 0.7$
			250.0	NA	250.0	0.07 ± 0.05 (20)	N.S.
			500.0	NA	500.0	0.08 ± 0.05 (19)	N.S.
3	+ RLC	D	0.0	100	0.0	0.55 ± 0.08 (39)	Control
			50.0	99	50.0	0.77 ± 0.15 (20)	$0.4 < p < 0.5$
			100.0	50	100.0	3.38 ± 0.18 (20)	$p < 0.01$*
			150.0	13	150.0	0.92 ± 0.12 (20)	$0.1 < p < 0.2$
o-Toluidine (TOL, 1000 nl/ml ≅ 9.41 mM)							
1	− RLC	C.F.	0.0	100	0.0	0.00 ± 0.00 (22)	Control
			31.0	75	14.0	0.00 ± 0.00 (4)	N.S.
			63.0	68	55.0	0.00 ± 0.00 (7)	N.S.
			125.0	30	110.0	0.00 ± 0.00 (12)	N.S.
			250.0	17	220.0	0.00 ± 0.00 (6)	N.S.
			500.0	0	330.0	0.04 ± 0.04 (16)	$0.3 < p < 0.4$
2	+ RLC	C.F.	0.0	100	0.0	1.09 ± 0.12 (21)	Control
			150.0	94	150.0	6.83 ± 0.11 (18)	$p < 0.01$*
			300.0	80	300.0	4.10 ± 0.10 (17)	$p < 0.01$*
			600.0	29	600.0	2.06 ± 0.15 (18)	$0.02 < p < 0.05$*
Dimethylnitrosamine (DMN, 1000 nl/ml ≅ 13.4)							
1	+ RLC	D	0.0	100	0.0	0.55 ± 0.08 (39)	Control
			8000.0	100	8000.0	4.40 ± 0.07 (20)	$p < 0.01$*
			12000.0	41	12000.0	0.79 ± 0.18 (19)	$0.4 < p < 0.5$
2	+ RLC	O.F.	0.0	100	0.0	1.09 ± 0.12 (21)	Control
			8000.0	82	8000.0	3.20 ± 0.14 (18)	$p < 0.01$*
			12000.0	31	12000.0	0.91 ± 0.12 (18)	N.S.
3	+ RLC	D	0.0	100	0.0	0.23 ± 0.13 (11)	Control
			7500.0	NA	7500.0	0.81 ± 0.17 (19)	$0.05 < p < 0.1$
			10000.0	NA	10000.0	1.22 ± 0.13 (20)	$p < 0.01$*
			12500.0	NA	12500.0	0.91 ± 0.15 (17)	$0.02 < p < 0.05$*

TABLE 3 (continued)

Chemical (acronym) trial	± RLC [a]	Culture vessel [b]	Relative cell survival [c]		Relative transformation activity		
			Dose (nl/ml or μg/ml)	% Survival	Dose (nl/ml or μg/ml)	Foci/vessel [d] $\bar{x} \pm$ SE (n)	p [e]
3-Methylcholanthrene (MCA), 1000 μg/ml ≅ 3.73 mM)							
1	− RLC	D	0.0	100	0.0	0.00 ± 0.00 (15)	Control
			5.0	NA	5.0	2.67 ± 0.12 (20)	$p < 0.01$*
2	− RLC	D	0.0	100	0.0	0.12 ± 0.05 (30)	Control
			5.0	NA	5.0	4.60 ± 0.12 (18)	$p < 0.01$*
3	− RLC	C.F.	0.0	100	0.0	0.00 ± 0.00 (22)	Control
			5.0	NA	5.0	0.81 ± 0.10 (26)	$p < 0.01$*
4	− RLC	C.F.	0.0	100	0.0	0.29 ± 0.15 (40)	Control
			5.0	NA	5.0	5.17 ± 0.16 (38)	$p < 0.01$*
5	− RLC	D	0.0	100	0.0	0.37 ± 0.11 (20)	Control
			2.5	NA	2.5	8.87 ± 0.20 (20)	$p < 0.01$*

[a] See footnotes c and d in Table 1.

[b] Closed 25-cm^2 tissue culture flasks (C.F.) were used for these test chemical treatments; D., 60-mm tissue culture dishes; O.F., loosely capped 25-cm^2 flasks.

[c] Relative cell survival. The solvent control was arbitrarily set at 100% and the reductions in cell survivals due to the treatments were calculated as a fraction of this value.

[d] The appearance of transformed foci of Balb/c-3T3 cells in control and chemical-treated cultures had a skewed distribution which fit a normal distribution when the data from individual vessels was converted to $\log_{10}$ equivalent value. The mean ($\bar{x}$) and standard error (SE) of the number of transformed foci listed in Table 3 are represented as the antilogs of the $\bar{x} \pm$ SE log transformed data. "n" is the number of vessels available for analysis at the completion of the assay.

[e] Test chemical responses in the assays were evaluated for statistical significance using the $\log_{10}$ transformed values and a modified t-test (Bailey, 1975) (see footnote b of Table 1 for an explanation and qualitative evaluation).

TABLE 4

RESULTS FROM INDIVIDUAL Oua^r MUTAGENESIS EXPERIMENTS USING Balb/c-3T3 CELLS WITH S9 ACTIVATION [a]

Chemical (acronym) nl or μg/ml	Relative cell survival [b]		Relative Oua^r activity			Oua^r frequencies		
	CFE (%)	CE (%)	Number of cultures	Number of Oua^r colonies	Frequency [c] $\bar{x} \pm$ S.E.	Absolute [d]	Adjusted [e]	p
Acrylonitrile (ACN)								
0	NA	44	71	4	0.040 ± 0.019	1.00	1.00	Control
50	NA	60	25	8	0.234 ± 0.073	5.85*	5.87*	$0.01 < p < 0.02$*
100	NA	60	22	5	0.120 ± 0.081	3.00*	2.17*	$0.3 < p < 0.4$
150	NA	28	20	4	0.149 ± 0.066	3.73*	4.24*	$0.1 < p < 0.2$
200	NA	23	15	0	0.000 ± 0.000	–	–	N.S.
Benzene (BEN)								
0	100	22	80	14	0.140 ± 0.030	1.00	1.00	Control
2500	90	23	25	34	1.075 ± 0.109	3.26*	3.15*	$p < 0.01$*
5000	16	NA	25	14	0.457 ± 0.080	7.68*	?	$p < 0.01$*
10000	0	0	0	0	–	–	–	N.S.
20000	0	0	0	0	–	–	–	N.S.

TABLE 4 (continued)

Chemical (acronym) nl or μg/ml	Relative cell survival [b]		Relative Oua^r activity			Oua^r frequencies		
	CFE (%)	CE (%)	Number of cultures	Number of Oua^r colonies	Frequency [c] $\bar{x} \pm$ S.E.	Absolute [d]	Adjusted [e]	p
Benzoin (ZOIN)								
0	100	34	79	0	$< 0.009 \pm 0.009$	1.00	1.00	Control
31	85	24	22	0	0.000 ± 0.000	–	–	N.S.
63	74	29	25	0	0.000 ± 0.000	–	–	N.S.
125	45	28	25	1	0.028 ± 0.028	> 2.20*	> 2.68*	$0.5 < p < 0.6$
250	33	37	25	0	0.000 ± 0.000	–	–	N.S.
Caprolactam (CAP)								
0	100	29	73	0	$< 0.009 \pm 0.009$	1.00	1.00	Control
1875	87	40	25	0	0.000 ± 0.000	–	–	N.S.
3750	95	41	24	3	0.077 ± 0.054	4.15*	3.91*	$0.2 < p < 0.3$
7500	83	37	23	0	0.000 ± 0.000	–	–	N.S.
15000	62	33	25	3	0.057 ± 0.057	5.60*	3.59*	$0.4 < p < 0.5$
Diethylhexylphthalate (DEHP)								
0	100	22	80	14	0.140 ± 0.030	1.00	1.00	Control
79	92	17	23	0	0.000 ± 0.000	–	–	N.S.
250	78	15	25	9	0.227 ± 0.084	1.62	2.46*	$0.3 < p < 0.4$
791	63	22	25	5	0.136 ± 0.061	0.97	1.00	N.S.
2000	29	35	22	8	0.215 ± 0.084	1.54	0.99	$0.4 < p < 0.5$
7910	16	27	12	3	0.189 ± 0.095	1.35	1.10	$0.6 < p < 0.7$
Diethylstilbestrol (DES)								
0	100	44	71	4	0.040 ± 0.019	1.00	1.00	Control
6.3	NA	56	24	0	0.000 ± 0.000	–	–	N.S.
12.5	NA	52	25	2	0.053 ± 0.039	1.33	1.12	$0.7 < p < 0.8$
25.0	NA	40	25	0	0.000 ± 0.000	–	–	N.S.
50.0	NA	22	25	1	0.026 ± 0.026	0.65	1.30	N.S.
Hexamethylphosphamide (HMPA)								
0	100	34	79	0	$< 0.009 \pm 0.009$	1.00	1.00	Control
2000	97	NA	0	–	–	–	–	N.S.
4000	99	NA	0	–	–	–	–	N.S.
8000	95	31	25	0	0.000 ± 0.000	–	–	N.S.
16000	81	33	25	0	0.000 ± 0.000	–	–	N.S.
0	100	29	73	0	$< 0.009 \pm 0.009$	1.00	1.00	Control
16000	78	34	25	0	0.000 ± 0.000	–	–	N.S.
24000	18	26	25	1	0.028 ± 0.028	> 2.04*	> 2.23*	$0.5 < p < 0.6$
32000	3	33	25	0	0.000 ± 0.000	–	–	N.S.
64000	0	0	0	0	–	–	–	N.S.
Phenobarbitol (PHEN)								
0	100	34	79	0	$< 0.009 \pm 0.009$	1.00	1.00	Control
1000	93	37	25	0	0.000 ± 0.000	–	–	N.S.
2000	76	33	25	0	0.000 ± 0.000	–	–	N.S.
4000	59	40	25	0	0.000 ± 0.000	–	–	N.S.
6000	27	35	24	0	0.000 ± 0.000	–	–	N.S.
Safrole (SAF)								
0	100	44	71	4	0.040 ± 0.019	1.00	1.00	Control
125	NA	37	25	1	0.028 ± 0.028	0.70	0.83	N.S.
250	NA	28	25	2	0.056 ± 0.39	1.40	2.14*	$0.7 < p < 0.8$
500	NA	13	11	7	0.475 ± 0.151	11.88*	40.65*	$p < 0.01$*

TABLE 4 (continued)

Chemical (acronym) nl or μg/ml	Relative cell [b] survival		Relative Oua^r activity			Oua^r frequencies		
	CFE (%)	CE (%)	Number of cultures	Number of Oua^r colonies	Frequency [c] $\bar{x} \pm$ S.E.	Absolute [d]	Adjusted [e]	p
Safrole (SAF) (*continued*)								
750	NA	21	14	1	0.051 ± 0.051	1.28	2.61*	$0.8 < p < 0.9$
1000	NA	26	24	0	0.000 ± 0.000	–	–	N.S.
o-Toluidine (TOL)								
0	100	29	73	0	< 0.009 ± 0.009	1.00	1.00	Control
250	88	32	23	6	0.183 ± 0.72	> 13.31*	> 12.17*	$0.02 < p < 0.05$*
500	95	22	25	0	0.000 ± 0.000	–	–	N.S.
1000	80	16	13	0	0.000 ± 0.000	–	–	N.S.
2000	65	27	24	7	0.189 ± 0.78	> 13.75*	> 14.94*	$0.02 < p < 0.05$*
4000	0	0	0	0	–	–	–	N.S.
Dimethylnitrosamine (DMN)								
0	100	44	71	4	0.040 ± 0.019	1.00	1.00	Control
5000	NA	13	25	215	3.507 ± 0.260	87.7	286.0	$p < 0.01$*
0	100	34	79	0	< 0.009 ± 0.009	1.00	1.00	Control
5000	24	10	25	200	4.800 ± 0.188	190.0	1275	$p < 0.01$*
Diethylstilbestrol (DES), nonactivation trial								
0	100	NA	40	0	< 0.03 ± 0.03	1.00	1.00	Control
0.5	88	NA	40	0	0.00 ± 0.00	–	–	N.S.
4.5	58	NA	40	0	0.00 ± 0.00	–	–	N.S.
9.0	42	NA	40	0	0.00 ± 0.00	–	–	N.S.
18.0	20	NA	40	0	0.00 ± 0.00	–	–	N.S.
MNNG								
1.0	20	NA	40	393	19.29 ± 0.47	643.0	?	$p < 0.01$*

[a] A summary and evaluation of the Oua^r mutagenesis activities of these test chemicals is presented in Table 2.

[b] CFE, relative colony forming efficiency (cell survivals due to the treatments). C.E., cloning efficiency (number of clonable cells at the time of replating for selection). The CFE values for the treatments were determined in a concomitant cell survival assay at the time the Balb/c-3T3 target cells were treated with the test chemicals. The C.E. values were determined using a colony-formation assay at the time the cells were trypsinized and replated for selection of Oua^r variants.

[c] The distribution of Oua^r variants in the selection cultures was log-normal in the positive controls, possibly due to sister-colony formation (respreading) during the selection period. A normal distribution of the variants was obtained when the data was converted to their $\log_{10}$ equivalent value. The frequency data presented in Table 4 represents the antilog of the logarithmic mean ($\bar{x}$) and standard error (SE) numbers of variants seen per culture for each condition.

[d] Calculated as the fold-increase (decrease) in the mean number of ouabain resistant variants per 1×10^6 ouabain-treated (selected) cells observed for each experimental condition in relation to the negative control values.

[e] Calculated as the ratio of the mean absolute number of ouabain resistant variants per 1×10^6 selected cells to the number of clonable cells for each experimental condition in relation to the negative control values.

nificant (see Table 4 for raw data); and, therefore, their mutagenic activity in these studies was classified as questionable. Finally, treatments with DES, HMPA and PHEN did not result in any evidence of mutagenic activity. Since these chemicals were tested at relative cell survivals shown to be optimal for detection of other model mutagens, it seems unlikely that additional trials would have resulted in evidence of significant mutagenic activity.

Discussion

The purpose of the IPCS collaborative study was to evaluate 10 well-characterized organic chemicals in a wide variety of in vitro assays for

the purpose of recommending a test battery for use in routine screening. To control for specificity in relation to the available rodent bioassay data, 2 noncarcinogens (ZOIN and CAP) were included. In this regard, 6 of the 8 carcinogens tested (ACN, BEN, DES, PHEN, SAF and TOL) induced significant levels of transformation of 3T3 cells relative to the solvent controls. 4 of the chemicals found to be active as 3T3 cell-transforming agents (ACN, BEN, SAF and TOL) also induced significant increases in the frequency of Oua^r mutants. However, DES and PHEN were not mutagenic, suggesting that the induction of morphological transformants by these chemicals may have resulted because of their induction of a specific type of lesion leading to the expression of the transformed phenotype while not causing point mutations at the Oua ^{r} locus. In this regard, DES has been reported to be an effective transforming agent for Syrian hamster embryo cells, but DES treatments of these cells did not result in mutations at the two genetic loci tested (Barrett et al., 1981).

The treatments with DEHP and with HMPA did not result in significant transforming or mutagenic activities. One explanation for these apparent false negative results may lie in differences in the in vivo vs. the in vitro metabolism of these materials. For example, as is shown in Table 3, both HMPA and DEHP were significantly detoxified when the treatments were conducted in the presence of RLC. Such detoxifications may not occur at the level of the specific target organs for these materials under the conditions of rodent bioassay.

An unexpected finding of this study was the induction of significant 3T3 cell transformation by the rodent noncarcinogens CAP and ZOIN under conditions of RLC-mediated activation. These cell-transformation activities were observed in the absence of evidence of significant mutagenic activity (compare Table 3 and Table 4) as were the activities of DES and PHEN. The mechanism(s) of the increased sensitivity of the cell transformation system in relation to that of the point mutation system in these cells is unresolved and awaits a characterization of the gene product(s) of cell transformation and of the genetic and/or epigenetic processes which control its expression in transformable cells.

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Assays for the induction of morphological transformation in C3H/10T½ cells in culture with and without S9-mediated metabolic activation

N. Lawrence and D.B. McGregor

Mutagenesis and Cellular Toxicology Branch, Inveresk Research International Limited, Musselburgh (Scotland)

Summary

10 compounds of known carcinogenic potential either in man or in animal studies were examined for their ability to induce morphological transformation in cultured embryonic mouse fibroblasts (C3H/10T½ Clone 8 cells). The tests were conducted in the presence and absence of a postmitochondrial supernatant fraction from the livers of Aroclor-1254-treated adult male rats and the co-factors required for mixed-function oxidase activity (S9 mix). Transformation was considered to have been induced when 2 or more Type II or Type III foci occurred within a single dose group.

Results were: (A) negative for benzene and phenobarbital; (B) weakly positive for acrylonitrile, benzoin, caprolactam, diethylstilbestrol, di(2-ethyl)hexylphthalate, hexamethylphosphoramide, safrole and *o*-toluidine.

As part of the International Programme on Chemical Safety (IPCS) Collaborative Study, 10 compounds of known carcinogenic potential in either man or animal studies, were examined for their ability to induce morphological transformation in cultured embryonic mouse fibroblasts (C3H/10T½ Clone 8 cells). The methods used were those developed by Heidelberger and his coworkers (Reznikoff et al., 1979). These cells normally exhibit a high degree of postconfluence inhibition of cell division, but following treatment with certain chemicals known to be carcinogens, alterations in growth patterns occur as a result of reduced or lost contact inhibition of growth. Cells that exhibit such altered growth patterns have a high probability of forming tumours when injected into immunosuppressed mice.

Materials and methods

Test chemicals

The 10 test compounds were obtained from Imperial Chemical Industries, PLC, Macclesfield, Cheshire, as were samples of benzo[*a*]pyrene (B(a)P) and 3-methylcholanthrene (3-MC), used as positive controls. Other control chemicals were: 4-nitroquinoline-*N*-oxide (4-NQO), obtained from ICN K and K Laboratories, New York; cyclophosphamide (CP), ethylmethanesulphonate (EMS) and 2-acetylaminofluorene (2-AAF), obtained from Koch-Light Laboratories Limited, Colnbrook, Bucks.; dimethylnitrosamine (DMN), obtained from Aldrich Chemical Company, Wembley, Middlesex. All chemicals were dissolved and diluted in dimethyl sulphoxide (DMSO), obtained from BDH Chemicals Limited, Poole, Dorset. Solutions of test chemicals formed 1% of the incubation media to which cells were exposed.

Cells and culture maintenance

C3H/10T½ Clone 8 cells, obtained from the American Type Culture Collection, were grown as monolayers in 75-cm^2 Nunc flasks in Eagle's basal medium (BME) containing sodium bicarbonate (1.68 g/l), heat-inactivated foetal calf serum (10% v/v) and gentamicin (25 μg/ml). Cells were

trypsinised every 10 days and new flasks seeded with 1.5×10^5 cells.

Activation system

Tests were performed both in the presence and absence of a postmitochondrial (9000 *g* for 10 min) supernatant fraction from the livers of Aroclor-1254-treated adult male rats (S9). Cofactors required for mixed-function oxidase activity were included (S9 mix) in the following composition: 1 part S9 + 9 parts cofactor solution containing 4 mM NADP, 25 mM glucose 6-phosphate, 8 mM $MgCl_2 \cdot 6H_2O$, 33 mM KCl, all dissolved in 0.05 M phosphate buffer, pH 7.4.

When required, S9 mix formed 10% of the incubation medium to which cells were exposed.

Dose ranging study

The objective of this test was to select doses for use in the more precise cytotoxicity test.

A saturated solution of each of the test compounds was prepared in DMSO and 4 (10-fold) dilutions prepared. Cells from a stock flask were harvested and suspended in culture medium, sedimented by centrifugation at 200 *g* for 5 min and resuspended in fresh culture medium at a density of 1×10^5 cells/ml. Samples (1 ml) of the suspension were pipetted into the wells of a Linbro Multi-well plate (Flow Laboratories, Irvine, Scotland), which was then incubated in a humid atmosphere of 5% CO_2 in air at 37 °C for 24 h. The cultures were divided into 2 groups, to one of which was added 100 μl S9 mix per well; 10-μl samples of test compound were added to duplicate wells of both groups. Vehicle control cultures received 10 μl of DMSO. After incubation for 24 h at 37 °C in a humid atmosphere of 5% CO_2 in air the cultures were fixed with methanol, stained with 2% Giemsa and examined for evidence of cellular damage.

Cytotoxicity test

The objective of this test was to establish the range of concentrations to be used in the cell transformation assay.

The cells were harvested and suspended in growth medium, sedimented by centrifugation at 200 *g* for 5 min and resuspended in fresh culture medium at a density of 100 cells/ml. Samples of 10 ml were then pipetted into 75-cm^2 plastic tissue-culture flasks (Nunc), pregassed with an atmosphere of 5% CO_2 in air. The flasks were incubated for 24 h at 37 °C.

The cultures were exposed to the chemical for 24 h after which the medium was changed. The medium was subsequently changed twice weekly for 3 weeks before the cells were fixed in methanol, stained with 2% Giemsa and toxicity assessed by comparing the numbers of colonies in treated cultures with those found in the vehicle controls. These data were used to select dose levels for the transformation assay.

Cell transformation assay

Cells from subconfluent stock cultures of C3H/10T$\frac{1}{2}$ cells (passage 10) were distributed among 75-cm^2 tissue-culture flasks as in the preliminary toxicity test. The flasks were then incubated for 24 h at 37 °C in an atmosphere of 5% CO_2 in air.

The cultures were exposed to the chemicals for 24 h at 37 °C in the presence and absence of S9 mix (10% v/v), after which the medium was changed. 13 flasks were prepared for each treatment. One flask was selected from each treatment group to provide the assessment of cell survival. In these flasks, the medium was changed twice weekly for 3 weeks, after which they were fixed in methanol and stained with 2% Giemsa. Survival was assessed by comparing the numbers of colonies in treated cultures with those found in the vehicle controls.

In the remaining 12 flasks from each treatment group, the medium was changed twice weekly until the cells reached confluence; thereafter, the medium was changed once weekly.

Quantification of transformation

After an 8-week incubation period, the cultures were fixed for 30 min in methanol and stained with 2% Giemsa. All flasks were examined with the use of an inverted microscope, giving magnification of 40 × and 100 ×, and any foci of growth graded as Types I, II or III colonies. Scoring was performed on coded flasks.

Type I colonies. Areas of cell crowding in which cells are smaller and may even form 2 layers.

Type II colonies. Massive piling up of cells maintaining essentially the normal morphology of the cell line.

Type III colonies. Multilayering, massive piling up and criss-cross arrangements of cells with little perinuclear cytoplasm but long pseudopodia.

The frequency of transformation in these cells is very low and, within an experiment, it is usual to find no Type II or Type III colonies at all in vehicle control groups. Consequently, the finding of 2 Type II or III foci within a group is unlikely to be a chance occurrence and is taken as evidence of transforming potential.

Results

Data are shown in Table 1 (with S9 mix) and Table 2 (without S9 mix). Dose levels chosen were based upon preliminary toxicity tests.

Vehicle control groups

No Type III transformed foci were identified in the vehicle control groups. In the total of 111 vehicle control flasks examined there were only 2 Type II foci in the absence of S9 mix and one Type II focus in the presence of S9 mix. This result is consistent with the historic control values obtained in this laboratory and reported in the literature with this cell line.

Positive control groups

In the presence of S9 mix, 2-AAF (20 and 40 μg/ml) and cyclophosphamide (2 and 3 μg/ml) induced significant numbers of transformed foci. DMN (20 μg/ml) and B(a)P (20 μg/ml) did not induce a significant number of transformed foci. In the absence of S9 mix, DMN (20 μg/ml) again proved negative, but B(a)P (10 and 20 μg/ml) induced a significant number of both Type II and III transformed foci. EMS (125 and 250 μg/ml) and 4-NQO (0.022 μg/ml) failed to produce a positive response in the absence of S9 mix, but 3-MC induced very large numbers of Type II and III transformed foci.

The loss of transforming potential of B(a)P in the presence of S9 mix is a particularly interesting observation.

HMPA

In the presence of S9 mix, 2 Type II transformed foci were recorded in the 280 μg HMPA/ml group, one Type II and one Type III focus in the 560 μg HMPA/ml group and one Type II colony in the 1100 μg HMPA/ml group. In the absence of S9 mix, no transformed foci were identified. The frequency of transformation and the distribution of foci among 5 flasks in the presence of S9 mix is suggestive of a weak transforming potential.

TOL

In the presence of S9 mix, no transformed foci were recorded at 150 μg *o*-toluidine/ml, one Type II focus was identified at 300 μg *o*-toluidine/ml and 3 Type II transformed foci observed at 600 μg *o*-toluidine/ml.

In the absence of S9 mix no transformed foci were recorded at any concentration of *o*-toluidine. In the presence of S9 mix the frequency of transformation and the distribution of transformed foci among 4 flasks is indicative of a weak cell-transforming potential.

BEN

No transformed foci occurred at any concentration of benzene in the presence of S9 mix. In the absence of S9 mix, one Type II colony was identified in the 50 μg benzene/ml group. No transformed foci were recorded in either the 100 or 200 μg/ml groups. This result does not represent a significant incidence of transformation.

SAF

No transformed foci were recorded at any concentration of safrole in the presence of S9 mix but the compound appeared to react with the plastic of the tissue-culture flasks causing the appearance of elliptical shaped "bubbles" on the floor of the flask which increased in a dose-dependent manner. This was only observed in the presence of S9 mix. In the absence of S9 mix, 2 Type II and 1 Type III foci were recorded in the 50 μg safrole/ml group, one Type II focus at 100 μg safrole/ml and one Type III colony in the 200 μg safrole/ml group. The distribution of these transformed foci among 4 flasks indicates a weak potential to induce cell transformation.

TABLE 1

RESULTS OF TRANSFORMATION ASSAY AND SURVIVAL ASSAY PERFORMED ON C3H/10T$\frac{1}{2}$ CELLS IN THE PRESENCE OF S9 MIX

Substance	Concentration (μg/ml)	Number of flasks	Number of flasks with foci					Proportion of flasks with Type II or III	Total number of foci					Number of Type II + III per flask	Response
			Type I	Type II	Type III	Type I or II or III	Type II or III		Type I	Type II	Type III	Total I + II + III	Total II + III		
DMSO	–	12	1	0	0	1	0	0	1	0	0	1	0	0	–
2-AAF	20	11	3	7	3	10	8	0.73	8	13	3	24	16	1.46	+
	30	12	0	1	0	1	1	0.08	0	1	0	1	1	0.08	?
HMPA	280	11	0	2	0	2	2	0.18	0	2	0	2	2	0.18	+
	560	11	3	1	1	5	2	0.18	3	1	1	5	2	0.18	+
	1100	12	3	1	0	4	1	0.08	3	1	0	4	1	0.08	?
o-Toluidine	150	11	2	0	0	2	0	0	2	0	0	2	0	0	–
	300	12	3	1	0	4	1	0.08	3	1	0	4	1	0.08	?
	600	12	1	3	0	4	3	0.25	1	3	0	4	3	0.25	+
DMSO	–	12	2	1	0	2	1	0.08	2	1	0	3	1	0.08	?
CP	2	12	5	4	2	8	5	0.42	6	5	2	13	7	0.58	+
	3	12	8	5	2	10	7	0.58	11	7	2	20	9	0.75	+
Benzene	80	12	2	0	0	2	0	0	2	0	0	2	0	0	–
	160	12	2	0	0	2	0	0	2	0	0	2	0	0	–
	320	6	0	0	0	0	0	0	0	0	0	0	0	0	–
Safrole	* 100	12	1	0	0	1	0	0	2	0	0	2	0	0	–
	* 200	11	1	0	0	1	0	0	1	0	0	1	0	0	–
	* 200	10	1	0	0	1	0	0	1	0	0	0	1	0	–
DMSO	–	** 5	1	0	0	1	0	0	1	0	0	1	0	0	–
2-AAF	20	11	5	3	0	8	3	0.27	7	3	0	10	3	0.27	+
	40	12	8	4	0	10	4	0.33	14	4	0	18	4	0.33	+
B(a)P	20	10	2	0	1	3	1	0.1	3	0	1	4	1	0.1	?
Caprol'm	3750	12	6	1	0	7	1	0.08	22	1	0	23	1	0.08	?
Acrylonitrile	8	12	3	1	0	4	1	0.08	5	1	0	6	1	0.08	?
	16	12	3	2	0	5	2	0.17	3	2	0	5	2	0.17	+
	32	10	6	1	0	7	1	0.1	11	1	0	12	1	0.1	?
DMSO	–	11	0	0	0	0	0	0	0	0	0	0	0	0	–
DMN	20	11	1	1	0	2	1	0.09	4	1	0	5	1	0.09	?
DEHP	250	11	1	1	0	2	1	0.09	1	2	0	3	2	0.18	?
	500	11	1	0	0	1	0	0	1	0	0	1	0	0	–
	1000	11	2	1	1	4	2	0.18	5	1	1	7	2	0.18	+
Benzoin	20	10	4	1	1	6	2	0.2	7	1	1	9	2	0.2	+
	40	12	2	0	0	2	0	0	3	0	0	3	0	0	–
	80	12	0	1	0	1	1	0.08	0	3	0	3	3	0.25	?

TABLE 1 (continued)

Substance	Concentration (μg/ml)	Number of flasks	Number of flasks with foci					Proportion of flasks with Type II or III	Total number of foci					Number of Type II + III per flask	Response
			Type I	Type II	Type III	Type I or II or III	Type II or III		Type I	Type II	Type III	Total I + II + III	Total II + III		
DMSO	–	11	8	0	0	8	0	0	11	0	0	11	0	0	–
B(a)P	10	° 0													
Pheno-barbital	500	6	3	0	0	3	0	0	3	0	0	3	0	0	–
	1000	10	2	1	0	3	1	0.1	2	1	0	3	1	0.1	?
	2000	10	3	1	0	4	1	0.1	3	1	0	4	1	0.1	?
DES	5	° ° 3	1	0	0	1	0	0	1	0	0	1	0	0	–
	10	° ° 5	3	0	0	3	0	0	3	0	0	3	0	0	–
	20	° ° 5	0	0	0	0	0	0	0	0	0	0	0	0	–

* In the presence of S9 mix safrole appeared to react with the plastic of the tissue-culture flasks causing the appearance of elliptical shaped 'bubbles' on the floor of the flask. The number of these 'bubbles' increased in a dose-dependent manner.

** 7 flasks (which were all in the same pile) were discarded due to anomalous cell growth. There appeared to be something at the bottom end of the flasks that was causing a general loss of contact inhibition. This phenomenon has been observed before and the flasks were also discarded on that occasion. There was no growth from a focus.

° Benzo(a)pyrene 10 μg/ml, all the flasks were lost due to contamination.

° ° A significant proportion of the flasks were contaminated. The remaining number of flasks were insufficient for a comprehensive analysis of morphological transformation.

CAP

In the presence of S9 mix, one Type II transformed focus was observed in the 3750 μg caprolactam/ml group. This result does not represent a significant incidence of transformation. In the absence of S9 mix, one Type II and one Type III colony were recorded in the 4570 μg caprolactam/ml group. The occurrence of these transformed colonies in different flasks from a total of 12 flasks is indicative of a weak cell-transforming potential.

ACN

In the presence of S9 mix, 1 Type II transformed colony was identified in the 8 μg acrylonitrile/ml group, 2 Type II foci recorded in the 16 μg acrylonitrile/ml group and one Type II focus observed in the 32 μg acrylonitrile/ml group. The frequency of transformation and the distribution of foci among 4 flasks is suggestive of a cell-transforming potential. No transformed foci occurred at any concentration of acrylonitrile in the absence of S9 mix.

DEHP

In the presence of S9 mix, 1 Type II transformed focus was recorded in the 250 μg DEHP/ml group, 1 Type II and 1 Type III colony in the 1000 μg DEHP/ml group. No transformed foci were observed in the 500 μg DEHP/ml group. The frequency of transformation and the distribution of transformed foci among 3 flasks is indicative of a very weak cell-transforming potential. In the absence of S9 mix, 1 Type II focus was identified in the 10 μg DEHP/ml group while 2 Type II foci were observed in the 40 μg DEHP/ml group. No transformed foci were recorded in the 20 μg DEHP/ml group. The frequency of transformation and the distribution of foci among 3 flasks is suggestive of a very weak cell-transforming potential.

ZOIN

In the presence of S9 mix, 1 Type II and 1 Type III focus were recorded in the 20 μg benzoin/ml group and 1 Type II focus in the 80 μg benzoin/ml group. No transformed foci were observed in the 40 μg benzoin/ml group. The frequency of transformation and the distribution of foci among 3 flasks is suggestive of a very weak cell-transforming potential. No transformed colonies were identified at any concentration of benzoin in the absence of S9 mix.

TABLE 2

RESULTS OF TRANSFORMATION ASSAY AND SURVIVAL ASSAY PERFORMED ON C3H/10T$\frac{1}{2}$ CELLS IN THE ABSENCE OF S9 MIX

Substance	Concentration (μg/ml)	Number of flasks	Number of flasks with foci					Proportion of flasks with type II or III	Total number of foci					Number of Type II + III per flask	Response
			Type I	Type II	Type III	Type I or II or III	Type II or III		Type I	Type II	Type III	Total I + II + III	Total II + III		
DMSO	–	12	1	0	0	1	0	0	1	0	0	1	0	0	–
3-MC	10	12	11	10	2	12	10	0.83	44	32	6	82	38	3.2	+
B(a)P	10	12	10	7	0	11	7	0.58	45	17	0	62	17	1.4	+
4-NQO	0.042	* 0													
DES	5	12	1	0	0	1	0	0	1	0	0	1	0	0	–
	10	12	2	2	0	4	2	0.17	2	2	0	4	2	0.17	+
	20	12	0	0	0	0	0	0	0	0	0	0	0	0	–
Pheno-	500	12	1	0	0	1	0	0	1	0	0	1	0	0	–
barbital	1000	12	3	0	0	3	0	0	5	0	0	5	0	0	–
	2000	12	0	1	0	1	1	0.08	0	1	0	1	1	0.08	?
DMSO	–	12	0	1	0	1	1	0.08	0	1	0	1	1	0.08	?
3-MC	10	12	10	9	3	11	9	0.75	26	15	5	46	20	1.7	+
DMN	20	12	2	0	0	2	0	0	2	0	0	2	0	0	–
4-NQO	0.022	12	3	0	0	3	0	0	3	0	0	3	0	0	–
DEHP	10	12	0	1	0	1	1	0.08	0	1	0	1	1	0.08	?
	20	12	1	0	0	1	0	0	1	0	0	1	0	0	–
	40	10	0	2	0	2	2	0.2	0	2	0	2	2	0.2	+
Benzoin	50	12	2	0	0	2	0	0	2	0	0	2	0	0	–
	100	12	1	0	0	1	0	0	1	0	0	1	0	0	–
	200	* 1	0	0	0	0	0	0	0	0	0	0	0	0	–
DMSO	–	12	3	0	0	3	0	0	7	0	0	7	0	0	–
3-MC	5	10	5	9	0	10	9	0.9	10	13	0	23	13	1.3	+
	10	12	10	3	3	12	5	0.42	14	5	5	24	10	0.8	+
EMS	125	11	4	0	0	4	0	0	4	0	0	4	0	0	–
	250	12	1	0	0	1	0	0	1	0	0	1	0	0	–
B(a)P	20	12	7	8	5	11	8	0.67	19	10	6	35	16	1.3	+
Caprol'm	4570	12	2	1	1	3	2	0.17	3	1	1	5	2	0.17	+
Acrylon-	10	12	6	0	0	6	0	0	7	0	0	7	0	0	–
itrile	20	12	0	0	0	0	0	0	0	0	0	0	0	0	–
	40	9	1	0	0	1	0	0	1	0	0	1	0	0	–
DMSO	–	12	1	1	0	2	1	0.08	1	1	0	1	1	0.08	?
3-MC	5	12	9	11	3	12	11	0.92	40	91	4	135	95	7.9	+
	10	11	7	11	3	11	11	1.0	26	23	4	53	27	2.5	+
Safrole	50	12	3	2	1	5	2	0.17	3	7	1	11	8	0.67	+
	100	12	3	1	0	4	1	0.08	4	1	0	5	1	0.08	?
	200	12	3	0	1	4	1	0.08	5	0	1	6	1	0.08	?

TABLE 2 (continued)

Substance	Concentration (μg/ml)	Number of flasks	Number of flasks with foci					Proportion of flasks with type II or III	Total number of foci					Number of Type II + III per flask	Response
			Type I	Type II	Type III	Type I or II or III	Type II or III		Type I	Type II	Type III	Total I + II + III	Total II + III		
Benzene	50	12	0	1	0	1	1	0.08	0	1	0	1	1	0.08	?
	100	11	1	0	0	1	0	0	1	0	0	1	0	0	–
	200	12	1	0	0	1	0	0	1	0	0	1	0	0	–
DMSO	–	12	0	0	0	0	0	0	0	0	0	0	0	0	–
3-MC	10	12	11	11	4	12	11	0.92	40	38	6	84	44	3.7	+
4-NQO	0.022	11	4	0	0	4	0	0	4	0	0	4	0	0	–
EMS	125	12	0	0	0	0	0	0	0	0	0	0	0	0	–
	250	12	1	1	0	2	1	0.08	1	1	0	2	1	0.08	?
HMPA	3260	11	1	0	0	1	0	0	1	0	0	1	0	0	–
o-Toluidine	150	12	4	0	0	4	0	0	5	0	0	5	0	0	–
	300	12	0	0	0	0	0	0	0	0	0	0	0	0	–
	600	12	0	0	0	0	0	0	0	0	0	0	0	0	–

* 4 = NQO at 0.042 μg/ml resulted in zero survival.
° Benzoin at 200 μg/ml was too toxic and only 1 flask survived treatment.

DES

In the presence of S9 mix, a significant proportion of the flasks were contaminated, leaving insufficient data for an accurate analysis of cell transformation. No transformed foci were recorded in the 20 μg/ml groups in the absence of S9 mix, but 2 Type II foci were identified in the 10 μg DES/ml group. This result is suggestive of a very weak cell-transforming potential.

PB

In the presence of S9 mix, 1 Type II focus was identified in each of the 1000 and 2000 μg/ml groups. No transformed foci were observed in the 500 μg phenobarbital/ml group. In the absence of S9 mix, 1 Type II focus was observed in the 2000 μg/ml group.

No transformed foci were identified in either the 500 or 1000 μg/ml groups. These results do not represent a significant incidence of transformation.

Conclusions

No significant numbers of transformed foci were detected at any of the tested concentrations of benzene or phenobarbital either in the presence or absence of S9 mix, so it is concluded that these compounds do not induce morphological cell transformations in C3H/10T½ cells.

None of the compounds tested induced a clear dose-related incidence of transformed foci; however, significant numbers of transformed foci were detected in the following groups (number of foci in parenthesis).

		Reported carcinogenic potential
In the presence of S9 mix		
HMPA	at 280 (2) + 560 μg/ml (2)	+
o-Toluidine	at 200 μg/ml (3)	+
DEHP	at 1000 μg/ml (2)	+
Acrylonitrile	at 16 μg/ml (2)	+
Benzoin	at 20 μg/ml (2)	–
In the absence of S9 mix		
Safrole	50 μg/ml (3)	+
Caprolactam	4570 μg/ml (2)	–
DEHP	40 μg/ml (2)	+
DES	10 μg/ml (2)	+

These incidences of cell transformation represent the lowest required to indicate a significant

response as set by our criteria. It is concluded, therefore, that HMPA, *o*-toluidine, DEHP, acrylonitrile and benzoin, in the presence of S9 mix, and safrole, caprolactam, DES and DEHP, in the absence of S9 mix, may exhibit a very weak potential for inducing morphological cell transformation in C3H/10T$\frac{1}{2}$ cells.

Since both "noncarcinogens" induced responses judged to be significant and the 2 nontransforming compounds are at least suspected carcinogens, the test system cannot be said to have performed well with this series of compounds.

Reference

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Tests with the C3H/10T½ clone 8 morphological transformation bioassay

Stephen Nesnow, Gaynelle Curtis [1] and Helen Garland

Carcinogenesis and Metabolism Branch, Genetic Toxicology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC (U.S.A.) and [1] Northrop Services, Inc., Environmental Sciences, Research Triangle Park, NC (U.S.A.)

Summary

Hexamethylphosphoramide, *o*-toluidine, benzene, safrole, and ϵ-caprolactam were evaluated for their ability to induce morphological transformation in C3H/10T½ CL8 mouse embryo fibroblasts. Three of these chemicals, hexamethylphosphoramide, *o*-toluidine and benzene were administered to the cells in sealed flasks to take advantage of their volatile properties. After a 24-h treatment period the cells were washed free of the chemicals and maintained through a series of fresh media changes for 6 weeks at which time the induction of Type 1, Type 2 and Type 3 morphological transformants were scored. Concurrent cytotoxicity determinations were also performed. In this group of experiments spontaneous transformation was not detected in any of the solvent control dishes or flasks representing a spontaneous transformation frequency of $< 0.005\%$. Statistically significant levels of morphological transformation were observed with *o*-toluidine and safrole while marginally statistically significant levels of transformation were observed with hexamethylphosphoramide and benzene. ϵ-Caprolactam was considered inactive in this test system. These data are discussed in light of other chemicals bioassayed in this laboratory in the C3H/10T½ CL8 morphological transformation bioassay.

Oncogenic transformation systems are valuable tools in the study of the effects of chemicals on mammalian cells. A review of these systems by the late Charles Heidelberger and his colleagues has recently been published (Heidelberger et al., 1983). The C3H/10T½ CL8 (C3H/10T½) mouse embryo fibroblast system developed by Reznikoff et al. (1973a,b) is one of the major transformation systems using continuous (permanent) cell lines. This system has been used in the study of metabolism (Nesnow and Heidelberger, 1976; Nesnow et al., 1981), DNA binding (Amstad et al., 1983; Theall et al., 1982), cocarcinogenesis (Nesnow et al., 1981), initiation (Mordan et al., 1982; 1983), promotion (Frazelle et al., 1983; Lillehaug and Djurhuss, 1982; Mondal et al., 1976), mutation (Landolph and Heidelberger, 1979), and cytogenetics (Gehly et al., 1982).

Many variations exist on the original protocol for the C3H/10T½ system. Treating cells at particular phases of the cell cycle produces increased cell-transformation responses with direct-alkylating agents (Bertram and Heidelberger, 1974; Grisham et al., 1980); reseeding treated cells produces similar results (Evans et al., 1981). In general, increases in the cells' sensitivity to chemicals are achieved by longer treatment times (Oshiro and Balwierz, 1982) and by treating colonies of cells (Nesnow et al., 1982). Short treatment times are useful when testing effects of several polycyclic aromatic hydrocarbons (Sarrif et al., 1981) while preinduction of the endogenous mixed-function oxidases increases responses with these compounds (Nesnow et al., 1981). Exogenous metabolic activation of these cells has been reported (Benedict et al., 1978; Poole and McGregor, 1982), but it has not become a standardized technique.

In our laboratory, we have utilized C3H/10T½

TABLE 1

SUMMARY OF CHEMICALS BIOASSAYED IN C3H/10T$_2^1$ CL8 CELLS [a]

Chemical	Result [b]	Minimum detectable concentration or highest concentration Tested (μg/ml)	Carcinogenicity [c]
Polycyclic aromatic hydrocarbons			
Benzo[*a*]pyrene	+	0.1	C
Cyclopenta[*cd*]pyrene	+	0.3	C
7,12-Dimethylbenz[*a*]anthracene	+	0.3	C
3-Methylcholanthrene	+	0.3	C
Benz[*a*]anthracene	−	6.8	C
5,6-Benzoflavone	−	9.0	?
7,8-Benzoflavone	−	6.8	?
1,2-Dihydronaphthalane	−	6.5	?
1,2-Naphthoquinone	−	0.47	?
Phenanthrene-9,10-quinone	−	0.62	?
Styrene	−	5.0	C
Alkylating agents			
N-Acetoxy-2-fluorenylacetamide	+	1.0	C
Cyclopenta[*cd*]pyrene-3,4-oxide	+	0.3	?
Ethylnitrosourea	+	150	C
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine	+	0.3	C
Propane sultone	+	40	C
β-Propiolactone	+	0.1	C
Cyclohexene oxide	−	5.0	?
Styrene oxide	−	6.0	C
Aromatic amines/azo dyes			
4-Dimethylaminoazobenzene	−	22.5	C
2-Fluorenylacetamide	−	10	C
Solvents			
Acetone	−	1%	?
Dimethyl sulfoxide	−	1%	?
Ethanol	−	9.3	?
Other chemicals			
Aflatoxin B_1	+	1.0	C
4-Nitroquinoline-*N*-oxide	+	0.003	C
Trichlorfon	+	3.0	?
Toxaphene	+/−	12.4	C
Acephate	−	30	?
Aroclor-1254	−	9.8	?
4-Chloro-*o*-toluidine	−	106	C
Chlordimeform	−	100	?
Cyclohexene	−	4.1	?
p,p'-DDT	−	11	C
2,7-Dinitrofluoren-9-one	−	30	?
Hexachlorobenzene	−	14	C
Nickel sulfate	−	100	?
Phenobarbital	−	25.4	?
Pregnenolone-16α-carbonitrile	−	34	?
Trifluralin	−	1.0	?
2,4,7-Trinitrofluoren-9-one	−	1.0	?
Urethane	−	1000	C
Vinyl carbamate	−	1000	C

[a] Bioassays performed in the author's laboratory.

[b] +, a statistically significant dose-related response of Type II and Type III foci; −, no response at the highest concentration tested.

[c] C, known carcinogen.

cells to study the effects of environmental chemicals on mammalian cells. During the course of these studies, we bioassayed a wide variety of chemicals including polycyclic aromatic hydrocarbons, alkylating agents, aromatic amines, azo dyes, solvents, pesticides, mycotoxins, carbamates and nitroaromatics. The results of these studies are summarized in Table 1.

In the present study, we evaluated the effects of hexamethylphosphoramide (HMPA), *o*-toluidine, benzene, safrole, and caprolactam on oncogenic transformation of C3H/10T½ cells. The original protocol of Reznikoff et al. (1973a) was used with minor variation.

Materials and methods

Cells and culture conditions

The mouse embryo fibroblast cell line C3H/10T ½ CL8, derived by Reznikoff et al. (1973b), was used in these experiments. Cell cultures were incubated in humidified incubators (Hotpack Co., Philadelphia, PA) at 37 °C in an atmosphere of 5% CO_2 in air. All cultures were grown in Eagle's basal medium with Earle's salts and L-glutamine supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, NY). Cell cultures were routinely checked for Mycoplasma infection and were found to be Mycoplasma free.

Oncogenic transformation assay

C3H/10T½ cells were seeded into 60-mm Petri dishes (1000 cells in 5 ml of medium per dish, 24 dishes per experimental point). After 24 h, dishes were treated with various concentrations of safrole or caprolactam in 50 µl of dimethyl sulfoxide (DMSO) for 24 h according to the procedure of Reznikoff et al. (1973a). Dishes were unsealed during the 24-h treatment period.

For the treatment of volatiles, C3H/10T½ cells were seeded into 25-cm^2 flasks (1000 cells in 5 ml of medium per flask, 20 flasks per experimental point). After 24 h, flasks were treated with various concentrations of HMPA, *o*-toluidine or benzene in 50 µl of DMSO (Reznikoff et al., 1973a). Flasks were sealed during the 24-h treatment period.

After the 24-h treatment period, the medium was removed from the dishes or flasks and the cells received fresh complete medium containing 25 µg/ml of Garamycin sulfate (Schering Corp., Kenilworth, NJ). The medium was changed weekly until the cells reached confluency. After confluency was reached, the fetal calf serum concentration was reduced to 5% and weekly medium changes were continued.

At the end of 6 weeks, the cells were washed with a 0.85% NaCl solution, fixed with methanol, stained with Giemsa, and scored for oncogenic transformation by the following criteria: Type I, Type II and Type III foci.

Positive control cultures were treated with benzo[*a*]pyrene (B(a)P) (1 µg/ml); negative cultures were treated with DMSO (1%). The protocol was the same as that used for experimental cultures. Cytotoxicity assays were run concurrently with transformation assays. The protocol was the same as that used for experimental cultures with the following exceptions: dishes or flasks were seeded with 200 cells, there were 6 dishes or flasks per chemical concentration tested, and both were stained 10–12 days after seeding.

Statistical analysis

In general, the data did not indicate dose-related responses for the 5 chemicals tested. For this reason, Fisher's Exact Test was used as an alternative to the multiple logistic model previously used in similar experiments (Nesnow et al., 1981).

Results

The results of the bioassays of HMPA, *o*-toluidine, benzene, safrole and caprolactam are shown in Tables 2–6. Benzo[*a*]pyrene was used to treat positive control cultures in each experiment; it transformed cells in 46–90% of these cultures. In total, we observed foci in 52 of the 84 B(a)P-treated cultures (62%). We observed 43 Type II and 38 Type III foci in these cultures for an average of 0.85 foci per dish/flask. Spontaneous transformation was not detected in any of the 84 dishes/flasks containing DMSO-treated negative control cultures; this represents a spontaneous transformation frequency of $< 0.005\%$. Our laboratory's historial spontaneous transformation frequency is 0.001%.

The chemicals we tested are of low molecular

TABLE 2

EFFECT OF HMPA ON CYTOTOXICITY AND ONCOGENIC TRANSFORMATION IN C3H/10T$\frac{1}{2}$ CELLS [a]

Concentration (μg/ml)	Survival (% control)	Number of flasks scored	Type I foci	Type II foci	Type III foci	% Flasks with Type II and Type III foci
0	100	20	0	0	0	0
250	117	20	1	0	0	0
500	118	20	0	0	0	0
750	132	20	0	1	0	5
1000	138	20	0	1	2	15 [b]
B(a)P						
1	32	20	0	4	10	55

[a] C3H/10T$\frac{1}{2}$ cells exposed to chemical in sealed flasks.
[b] $p = 0.12$, Fisher's Exact Test.

TABLE 3

EFFECT OF *o*-TOLUIDINE ON CYTOTOXICITY AND ONCOGENIC TRANSFORMATION IN C3H/10T$\frac{1}{2}$ CELLS [a]

Concentration (μg/ml)	Survival (% control)	Number of flasks scored	Type I foci	Type II foci	Type III foci	% Flasks with Type II and Type III foci
0	100	20	0	0	0	0
250	108	20	0	0	0	0
500	86	20	0	4	1	25 [b]
750	71	20	0	1	2	10
1000	52	20	0	1	0	5
B(a)P						
1	59	20	3	22	12	90

[a] C3H/10T$\frac{1}{2}$ cells exposed to chemical in sealed flasks.
[b] $p = 0.02$, Fisher's Exact Test.

TABLE 4

EFFECT OF BENZENE ON CYTOTOXICITY AND ONCOGENIC TRANSFORMATION IN C3H/10T$\frac{1}{2}$ CELLS [a]

Concentration (μg/ml)	Survival (% control)	Number of flasks scored	Type I foci	Type II foci	Type III foci	% Flasks with Type II and Type III foci
0	100	20	0	0	0	0
250	103	20	0	1	0	5
500	92	20	0	0	0	0
750	116	20	0	1	0	5
1000	101	20	0	2	1	15 [b]
B(a)P						
1	11	20	1	7	12	60

[a] C3H/10T$\frac{1}{2}$ cells exposed to chemical in sealed flasks.
[b] $p = 0.12$, Fisher's Exact Test.

TABLE 5

EFFECT OF SAFROLE ON CYTOTOXICITY AND ONCOGENIC TRANSFORMATION IN C3H/10T$\frac{1}{2}$ CELLS [a]

Concentration (μg/ml)	Survival (% control)	Number of dishes scored	Type I foci	Type II foci	Type III foci	% Dishes with Type II and Type III foci
0	100	24	0	0	0	0
10	90	24	0	0	0	0
25	54	24	0	0	0	0
50	40	24	0	1	1	8
100	35	24	1	6	0	17 [b]
200	0	24	1	1	0	4
B(a)P						
1	31	24	1	10	4	46

[a] C3H/10T$\frac{1}{2}$ cells exposed to chemical in open dishes.
[b] $p = 0.06$, Fisher's Exact Test.

TABLE 6

EFFECT OF CAPROLACTAM ON CYTOTOXICITY AND ONCOGENIC TRANSFORMATION IN C3H/10T$\frac{1}{2}$ CELLS [a]

Concentration (μg/ml)	Survival (% control)	Number of dishes scored	Type I foci	Type II foci	Type III foci	% Dishes with Type II and Type III foci
0	100	24	0	0	0	0
200	102	24	0	0	0	0
400	107	24	0	0	0	0
600	101	24	0	0	0	0
800	90	24	0	0	0	0
1000	93	24	0	1	0	4
B(a)P						
1	31	24	1	10	4	46

[a] C3H/10T$\frac{1}{2}$ cells exposed to chemical in open dishes.

weight. To remain within reasonable physiological concentrations, 1000 μg/ml was selected as the highest concentration tested in the absence of cytotoxicity. Therefore, HMPA, benzene and caprolactam were not tested at concentrations greater than 1000 μg/ml.

Marginally statistically significant levels of transformation ($p = 0.12$) were observed with HMPA and benzene tested at 1000 μg/ml (Tables 2, 4); tests with *o*-toluidine and safrole (Tables 3, 5) produced significant levels of transformation at 500 μg/ml ($p = 0.02$) and 100 μg/ml ($p = 0.06$), respectively. Caprolactam was considered inactive.

Discussion

In this study, tests on benzene and HMPA produced marginal results; *o*-toluidine and safrole induced significant transformation. Caprolactam was inactive. Examination of these chemicals at more closely spaced dose levels might have produced additional evidence of dose-related responses, especially if response curves were steep.

C3H/10T$\frac{1}{2}$ cells contain microsomal cytochrome P-450 and epoxide hydrolase enzymes required for the metabolic activation of many chemical carcinogens (Nesnow et al., 1981). Recent results in our laboratory indicate that the active

conjugating systems UDPGA–glucuronyl transferase and PAPS–sulfotransferase are present in these cells. It is therefore not surprising that C3H/10T½ cells can activate safrole and *o*-toluidine into forms which then are able to transform them. Additional investigations are needed to confirm the observed oncogenic transformation of C3H/10T½ cells by these two chemicals and to probe the mechanisms of metabolic activation.

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This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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Tests with the Syrian hamster embryo (SHE) cell transformation assay

Tore Sanner and Edgar Rivedal

Laboratory for Environmental and Occupational Cancer and The Norwegian Cancer Society, NHIK, The Norwegian Radium Hospital, Montebello, Oslo (Norway)

Summary

Of the 10 compounds tested, 6 gave positive results in the Syrian hamster embryo cell transformation test. These were hexamethylphosphoramide, *o*-toluidine, benzene, acrylonitrile, diethylhexylphthalate and phenobarbital. Two compounds were negative when tested. These were benzoin and diethylstilbestrol. Two compounds, safrole and caprolactam, could not be evaluated, and further studies are necessary. Of the 8 substances that could be evaluated, 6 of the 7 carcinogens gave positive results. The only carcinogen that did not respond in the test was diethylstilbestrol. The noncarcinogen benzoin gave no response.

Transformation of mammalian cells by chemicals was first observed by Berwald and Sachs (1963, 1965) using Syrian hamster embryo (SHE) cells. The potential of these cells in testing of chemical carcinogens was explored later by DiPaolo and coworkers (DiPaolo et al., 1969, 1971; DiPaolo and Casto, 1977) using morphological transformation of colonies as endpoint. Pienta et al. (1977) introduced the use of cryopreserved cells in the test. This represented an important development because it made possible the use of the same preparation of cells in a large number of experiments. Recently, the sensitivity of the test has been increased by introducing a new step in the assay where the media and the test chemicals are renewed 1–3 days prior to scoring of the colonies (Rivedal and Sanner, 1982). With this modified procedure, a significant transformation frequency can be obtained with as little as 0.005 μg/ml of benzo[*a*]pyrene.

Pienta (1980) has reported that of 83 carcinogens and 28 noncarcinogens tested in the standard SHE cell bioassay, 84% of the carcinogens were detected, and all the noncarcinogens except 4-nitro-*o*-phenylenediamine gave negative results. When the false negative compounds were retested with extra metabolic activation systems such as hamster liver S9 or hamster hepatocytes, 8 of the 10 false negative compounds gave positive results. It was concluded that the standard SHE cell-transformation assay responded to most classes of carcinogens except aromatic amines, aminoazodyes and nitrosamines, which require metabolic activation by exogenous microsomal enzymes.

In the present collaborative study, hexamethylphosphoramide (HMPA), *o*-toluidine (TOL), safrole (SAF), benzene (BEN), caprolactam (CAP), acrylonitrile (ACN), diethylhexylphthalate (DEHP), benzoin (ZOIN), diethylstilbestrol (DES), and phenobarbital (PB) were tested in 2–5 experiments. All tests were performed without the addition of exogenous metabolic activation systems.

Materials and methods

Cell cultures

Primary cell cultures of Syrian hamster (Wright, Chelmsford, Essex, U.K.) embryos at 14 days of gestation, were prepared and cryopreserved in liquid nitrogen as described by Pienta et al. (1977). Mass cultures were grown in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 10% CO_2 atmosphere. Ampules with cryopreserved cells were used as stock cultures in the transformation assay.

Transformation assay

A feeder layer of 6×10^4 lethally X-irradiated SHE cells (4.500 rad) was seeded in 3 ml complete medium (DMEM supplemented with 20% FBS, 2 μg/ml insulin, no antibiotics) on 60-mm petri dishes (Costar, MA). The next day 150 target cells in 1 ml medium were added. After 24 h, the chemical to be studied was added in 4 ml medium, and the cells were incubated for 6 days. The medium was then removed, the dishes rinsed with Dulbecco's phosphate-buffered saline (PBS) (Flow Laboratories, Ayrshire, U.K.), and the chemical was added in 6 ml complete medium. 1 day later, the dishes were washed with PBS, the colonies fixed with methanol and stained with Giemsa before counting and examination. All the chemicals were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration was always less than 0.2%. It has been shown that this concentration of DMSO does not affect the results.

Quantification of transformation

Morphological transformation is defined as altered colony morphology consisting of criss-crossing and piling up of cells. A test was considered positive when a transformation frequency higher than 1% was obtained. To judge a substance positive in the assay, it was required that the test substance should give transformation frequencies higher than 1% in at least two independent experiments.

Results and discussion

The results are summarized in Table 1. In Table 2 are shown the individual results obtained in the control experiments, for the tested compounds, and for the positive control, benzo[*a*]pyrene.

In most previous experiments (Pienta et al., 1977; Pienta, 1980; Rivedal and Sanner, 1980, 1981) no morphologically-transformed colonies were observed in the control experiments. With renewal of medium and test chemical prior to scoring of the colonies, however, we find transformed colonies also in the control experiments. The appearance of transformed colonies in the control dishes is probably due to the increase in the sensitivity. Thus, with benzo[*a*]pyrene a transformation frequency of 1% or higher is obtained with concentrations as low as 0.005 μg/ml, while in the experiments by Pienta et al. (1977), a concentration of 10 μg/ml was needed to obtain a similar transformation frequency. The sensitivity and the frequency of transformation in the control dishes depend on the cell preparation, the batch and concentration of serum, as well as on whether the exchange of medium is made 1, 2 or 3 days prior to scoring of the colonies. In our experiments, we try to adjust these factors to give high sensitivity with low numbers of transformed colonies in the control. It is apparent from Table 2 that the total number of transformed colonies in the control experiments (usually 9 dishes) averages 1 (transformation frequency: 0.25%), but in some experiments may go up to 2 (transformation frequency: 0.5%). The average cloning efficiency was 30%, varying from 27 to 32%. In most experiments, the cloning efficiency at the highest concentration of test chemical was reduced by less than 30%.

The dose–response curves obtained for the different chemicals are given in Fig. 1. Each segment of the figure shows the individual data for a different experiment. All the curves are corrected with regard to the average transformation frequency in the control (0.25%). Based on whether at least one point in the dose response curve gave a transformation frequency greater than 1%, the evaluation of the different experiments in term of + or − are indicated. To consider a compound positive in the

TABLE 1

ACTIVITY OF THE 10 COMPOUNDS IN THE SHE CELL TRANSFORMATION TEST

Chemical	Conclusion (+, −, ?)
Hexamethylphosphoramide (HMPA)	+
o-Toluidine (TOL)	+
Safrole (SAF)	?
Benzene (BEN)	+
Caprolactam (CAP)	?
Acrylonitrile (ACN)	+
Diethylhexylphthalate (DEHP)	+
Benzoin (ZOIN)	−
Diethylstilbestrol (DES)	−
Phenobarbital (PB)	+

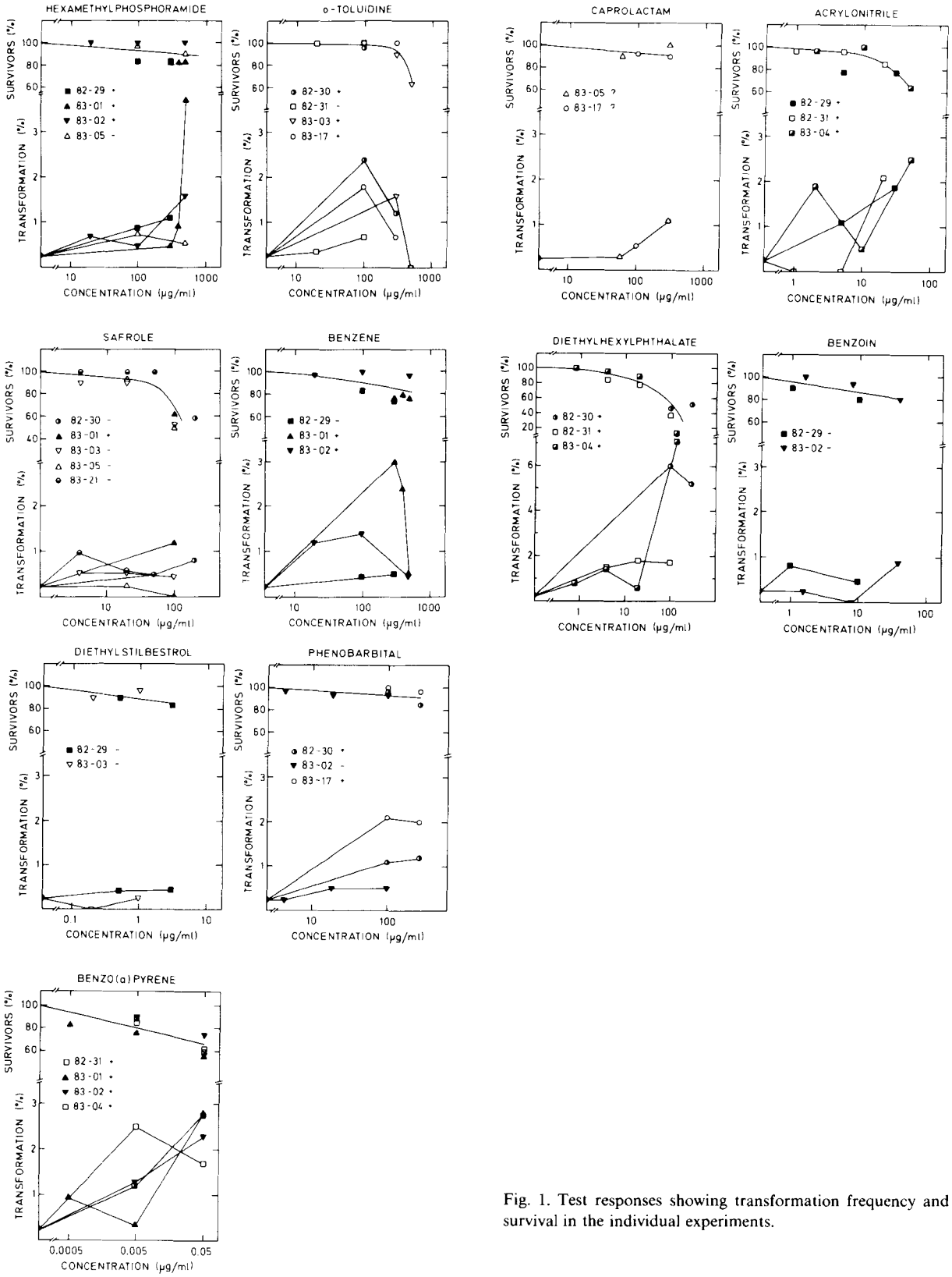

Fig. 1. Test responses showing transformation frequency and survival in the individual experiments.

TABLE 2

INDIVIDUAL RESULTS FROM THE SHE CELL TRANSFORMATION TEST

Chemical	Expt.	Concentration (μg/ml)	Total colonies	Cloning efficiency (%)	Total transformed colonies	Transformation frequency (%)
Control	82–29	–	225	30	0	0.0
	82–30	–	410	27	1	0.24
	82–31	–	283	27	0	0.0
	83–01	–	392	29	2	0.51
	83–02	–	418	31	1	0.24
	83–03	–	405	30	2	0.49
	83–04	–	378	28	1	0.26
	83–05	–	372	31	1	0.27
	83–17	–	405	30	1	0.25
	83–21	–	432	32	1	0.23
Control	Mean			30 ± 1.7		0.25 ± 0.17
Hexamethylphosphoramide	82–29	100	228	25	2	0.88
		300	182	25	2	1.10
	83–01	300	216	24	1	0.46
		400	108	24	1	0.92
		500	216	24	8	3.7
	83–02	20	432	32	3	0.69
		100	418	31	2	0.48
		500	432	32	7	1.6
	83–05	100	405	30	3	0.74
		500	378	28	2	0.53
o-Toluidine	82–30	100	252	26	6	2.4
		300	258	27	3	1.2
	82–31	20	294	28	1	0.34
		100	294	28	2	0.68
	83–03	300	122	27	2	1.6
		500	86	19	0	0.0
	83–17	100	446	33	8	1.8
		300	446	33	3	0.67
Benzene	82–29	100	222	25	1	0.45
		300	198	22	1	0.51
	83–01	300	165	22	5	3.0
		400	207	23	5	2.4
		500	198	22	1	0.51
	83–02	20	405	30	5	1.2
		100	418	31	6	1.4
		500	405	30	2	0.49
Safrole	82–30	50	198	27	1	0.51
		200	120	16	1	0.83
	83–01	100	243	18	3	1.2
	83–03	4	365	27	2	0.55
		20	365	27	2	0.55
		100	216	16	1	0.46
	83–05	20	392	29	1	0.26
		100	216	16	0	0.0
	83–21	4	405	34	4	0.99
		20	336	32	2	0.60
		50	396	33	2	0.51

TABLE 2 (continued)

Chemical	Expt.	Concentration (μg/ml)	Total colonies	Cloning efficiency (%)	Total transformed colonies	Transformation frequency (%)
Caprolactam	83-05	60	336	28	1	0.30
		300	372	31	4	1.1
	83-17	100	370	28	2	0.54
		300	365	27	4	1.1
Acrylonitrile	82-29	5	175	23	2	1.1
		30	210	23	4	1.9
	82-31	1	234	26	0	0.0
		5	312	26	0	0.0
		20	241	23	5	2.1
	83-04	2	365	27	7	1.9
		10	392	29	2	0.51
		50	243	18	6	2.5
Diethylhexyl-phthalate	82-30	100	84	11	5	6.0
		300	96	14	5	5.2
	82-31	4	276	23	4	1.5
		20	220	21	4	1.8
		100	120	10	2	1.7
	83-04	0.8	378	28	3	0.79
		4	365	27	5	1.4
		20	338	25	2	0.59
		133	42	4	3	7.1
Benzoin	82-29	1	246	27	2	0.81
		10	216	24	1	0.46
	83-02	1.6	418	31	1	0.24
		8	391	29	0	0.0
		40	337	25	3	0.89
Diethylstil-bestrol	82-29	0.5	246	27	1	0.41
		3	228	25	1	0.44
	83-03	0.2	365	27	0	0.0
		1	391	29	1	0.26
Phenobarbital	82-30	100	186	26	2	1.1
		300	162	23	2	1.2
	83-02	4	405	30	1	0.25
		20	391	29	2	0.51
		100	391	29	2	0.51
	83-17	100	418	31	9	2.1
		300	391	29	8	2.0
Benzo[*a*]pyrene	82-31	0.005	276	23	7	2.5
		0.05	180	15	3	1.7
	83-02	0.005	378	28	5	1.3
		0.05	310	23	7	2.3
	83-03	0.0005	324	24	3	0.93
		0.005	297	22	1	0.34
		0.05	216	16	6	2.8
	83-04	0.005	338	25	4	1.2
		0.05	216	16	6	2.8

assay (see Table 1), it had to have given positive response in at least 2 experiments.

Hexamethylphosphoramide (HMPA)

HMPA has previously been reported by Styles (1981) to be positive in the BHK-21 cell transformation test; Daniel and Dehnel (1981), using the same test, did not obtain any response. In the present study, HMPA gave positive response in 3 of 4 experiments. Concentrations higher than 300 μg/ml were needed in order to give transformation frequencies above 1%.

o-Toluidine (TOL)

TOL has previously been reported to give positive responses in 2 independent experiments using the BHK-21 cell transformation test (Daniel and Dehnel, 1981; Styles, 1981), as well as in mouse embryo cells infected with ACR leukemia virus (Rhim et al., 1974). In the present study, positive results were obtained in 3 of 4 experiments. The highest transformation frequency was obtained at 100 μg/ml, and the frequency decreased at higher concentrations of the compound.

Safrole (SAF)

SAF has been reported to transform SHE cells (Pienta et al., 1977). In the BHK-21 cell transformation test, SAF gave response in one study (Styles, 1981) while it was negative in another (Daniel and Dehnel, 1981). In the present study, SAF gave positive response in 1 experiment, but was negative in 4 other experiments. With SAF there were difficulties with the solubility. Spots of etching could be observed in the exposed dishes, indicating that the substance had not been completely dissolved. In the interpretation of the results for this substance, it was given a ?. Further studies are necessary.

Benzene (BEN)

BEN has been reported by Casto and Hatch (1978) to be negative in the SHE cell-transformation enhancement assay using SA7 viruses. Recently it was reported that benzene gives a low transformation frequency in the SHE cell test (Amacher and Zelljadt, 1983). In the present experiment, we found positive responses with BEN in 2 of 3 experiments. Positive responses were obtained with concentrations of 20 μg/ml BEN. At concentrations higher than 300 μg/ml, the transformation frequency decreased.

Caprolactam (CAP)

CAP has been reported by Pienta et al. (1977) to be negative in the SHE transformation assay. Two experiments were carried out with CAP. In both cases, a transformation frequency of 1.1% was obtained at the highest concentration (300 μg/ml). According to the criteria for result assessment, this should be judged as a positive result. However, because both responses were on the borderline, the overall decision with this compound was to give it a ?. Further studies are necessary.

Acrylonitrile (ACN)

ACN has been reported by Parent and Casto (1979) to give a positive response in the enhancement assay using SHE cells and SA7 viruses. ACN gave a positive response in all 3 experiments carried out. The lowest concentration giving positive response was 2 μg/ml.

Diethylhexylphthalate (DEHP)

DEHP gave positive responses in all 3 experiments carried out. Positive response was obtained at a DEPH concentration of 4 μg/ml. DEHP was the compound which gave the highest transformation frequencies. Thus, in 2 experiments, a transformation frequency of 6% or higher was obtained.

Benzoin (ZOIN)

ZOIN was negative in both experiments performed. The compound was tested in concentrations up to 40 μg/ml.

Diethylstilbestrol (DES)

DES has previously been found to give positive response in Rauscher murine leukemia virus-infected Fischer 344 rat embryo cells, while it gave a transformation frequency on the border line with the SHE cell assay and did not induce transformation in Balb/3T3 cells (Dunkel et al., 1981). Barrett et al. (1981) have in several experiments reported positive responses with DES. In the present test DES was negative in 2 independent experiments. DES was tested at concentrations up to 3 μg/ml.

Phenobarbital (PB)

PB has previously been reported to be negative in the SHE test (Pienta, 1980). In the present assay it was found to give positive response in 2 out of 3 experiments with PB concentrations of 100 μg/ml and higher.

Conclusions

8 of the 10 compounds tested are carcinogens. CAP and ZOIN are considered to be noncarcinogenic. All the carcinogenic compounds except SAF and DES were positive in the present assay. With SAF, further studies are needed before the response can be evaluated. DES was negative in all experiments. Barrett et al. (1981) have previously found DES to induce transformation at a low frequency in the SHE cell assay. One explanation for this discrepancy could be different metabolic capacities of the cells used. The noncarcinogen ZOIN was negative, while the interpretation in the case of CAP was more difficult. The results were on the borderline, and further studies are needed before the response can be evaluated. Thus, the 8 compounds for which an evaluation could be made all carcinogens except DES gave positive responses, and the noncarcinogen ZOIN was negative.

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Assay for the carcinogenicity of chemical agents using enhancement of anchorage-independent survival of retrovirus-infected Fischer rat embryo cells

William A. Suk and Joan E. Humphreys

Laboratory of Cellular and Molecular Oncology, In Vitro Toxicology Program, Northrop Services, Inc., Research Triangle Park, NC 27709 (U.S.A.)

Summary

The ability of carcinogen-treated retrovirus (Rauscher leukemia virus)-infected Fischer rat-embryo (RIFRE) cells to exhibit enhanced anchorage-independent survival is associated with neoplastic transformation. This study provides a preliminary evaluation of the survival responses of selected chemicals of interest to the WHO/IPCS in the Collaborative Study on Short-Term Tests for Genotoxicity and Carcinogenicity. The carcinogens assayed were safrole, *o*-toluidine, hexamethylphosphoramide, diethylhexylphthalate and diethylstilbestrol; the noncarcinogens were caprolactam and benzoin. In general, enhancement of anchorage-independent survival was induced by the known carcinogens, but not by the noncarcinogens. Certain variability of the survival responses was seen with some of the carcinogens. However, the most uniformity was obtained with diethylstilbestrol, which showed induction of anchorage-independent survival in a concentration-dependent manner. All chemicals were tested without the addition of an exogenous metabolic activating system. This study expands the base of information on the potential of this in vitro transformation system.

Fischer rat-embryo cells infected with Rauscher leukemia virus (RLV) were reported susceptible to chemically induced neoplastic transformation (Freeman et al., 1970, 1973a, b, 1975; Price et al., 1972b). The initial research showed that low generation ($D < 60$; D = population doublings) Fischer rat-embryo cell cultures were resistant to spontaneous transformation, chemically induced transformation, or transformation by type C-RNA murine leukemia viruses (Price et al., 1972b; Price and Mishra, 1980). However, cultures chronically infected with leukemia virus had an increased sensitivity to transformation by chemical carcinogens. At high generation levels ($D > 60$), the cells retained their metabolic competence and were readily transformed by carcinogenic chemicals in the absence of added virus (Price et al., 1975, 1978b; Price and Mishra, 1980).

The RLV-infected Fischer rat embryo cell focal assay has been used to determine the in vitro transformation potential of diethylnitrosamine (Freeman et al., 1970); 3-methylcholanthrene (Price et al., 1971); extract of city smog (Freeman et al., 1971); 7,12-dimethylbenzanthracene (Rhim et al., 1971); (−)-*trans*-Δ^9-tetrahydrocannabinol (Price et al., 1972a); more than 30 polycyclic hydrocarbons, azo dyes, aromatic amines and miscellaneous chemicals (Freeman et al., 1973b); antischistosomal drugs (Hetrich and Kos, 1973); *N*-methyl-*N*-nitrosourea and aflatoxin (Auletta and Suk, 1977); food dyes (Price et al., 1978b); industrial chlorinated hydrocarbons (Price et al., 1978a); and antituberculosis drugs (Suk and Price, 1982). Other examples of transforming agents in Fischer rat-embryo cells are the antitumor drugs 1-β-D-arabinofuranosylcytosine (Kouri et al., 1975), and

adriamycin (Price et al., 1975).

In 1981, Traul et al. derived a new RLV-infected Fischer rat-embryo cell line, $2FR_450$, for use in assessing chemical carcinogenicity. The evaluation of this system has been extended to more than 75 compounds covering almost every class or type of chemical carcinogen (Dunkel et al., 1981; Traul et al., 1981a).

The ability of cells to proliferate in an anchorage-independent state in semisolid medium (agar, methyl cellulose) has been considered a confirmation of neoplastic transformation (Barrett et al., 1979; Freedman and Shin, 1974; Kakunaga, 1978; Shin et al., 1975). The ability of transformed cells to aggregate and/or survive in liquid media above a base layer of solid agar is an extension of their anchorage independence and has been used to confirm transformation of a variety of cells exposed to either viruses or chemicals (Steuer and Ting, 1976; Steuer et al., 1977; Cho et al., 1976). Anchorage-independent survival as an indicator of neoplastic transformation has been measured and applied to the RLV-infected Fischer rat-embryo (RIFRE) cells (Traul et al., 1979, 1981b). Recently, a number of modifications have been made in the assay that have increased its sensitivity and reproducibility (Suk et al., 1983, 1984), and have enabled the RIFRE cells to respond to carcinogen treatment in a dose-responsive manner. Moreover, cells exhibiting carcinogen-mediated enhancement of anchorage-independent survival subsequently exhibited changes in their cellular morphology, growth in semisolid agar, and tumors in animals, indicating progression to a transformed phenotype (Suk et al., 1984).

The efficacy of the RIFRE cell anchorage-independent survival assay system is presently being determined by evaluating a number of compounds under code. This research is part of a National Toxicology Program project to evaluate in vitro mammalian cell-transformation systems. In this report, we evaluate a number of agents selected by the World Health Organization's International Program for Chemical Safety (IPCS) for the Collaborative Study on Short-Term Tests for Genotoxicity and Carcinogenicity (CSSTT). Safrole (SAF), *o*-toluidine (TOL), hexamethylphosphoramide (HMPA), diethylhexylphthalate (DEHP), caprolactam (CAP), benzoin (ZOIN), and diethylstilbestrol (DES) were evaluated for their neoplastic transforming potential.

Materials and methods

Cells

The Rauscher leukemia virus (RLV)-infected Fischer rat-embryo (RIFRE) cell cultures ($2FR_450$) used in this assay have been previously described (Traul et al., 1981a). K. Traul, through the American Type Culture Collection, provided frozen ampules of the $2FR_450$ cells. The cells, at population doublings (*D*) 7 and 15, were propagated and cryopreserved by suspending 3×10^6 cells per ml in complete medium containing 7.5% dimethyl sulfoxide (DMSO). Cultures described in this report were used between *D* 17 and 35. The cultures were maintained by seeding at 2.67×10^4 cells per cm^2. They were passaged at 7-day intervals with refeeding at day 3 or 4.

Retrovirus infection of the cell was confirmed by immunofluorescence using antiviral serum (Tennant et al., 1973), reverse transcriptase assay (Grandgenett et al., 1972), and XC plaque-induction (Rowe et al., 1970). Cell cultures were routinely checked and found free of mycoplasma contamination.

The cells were cultivated in Eagle's minimum essential medium (EMEM) with 10% heat-inactivated fetal calf serum (FCS). No antibiotics were used. Cell-culture medium and related reagents were obtained as a powder from Gibco, Grand Island, NY. The FCS was obtained from Sterile Systems, Logan, UT. Trypsin [Enzar-T (Armour)] diluted 1:40 was used routinely for dissociating the cells at subdivision.

Chemicals

The IPCS provided all chemicals used in these experiments, except 7,12-dimethylbenzanthracene (DMBA). The National Toxicology Program repository provided the DMBA. Chemicals were stored in airtight containers at $-20\,^{\circ}C$ and stock solutions were prepared immediately prior to use. Weightings and assays were performed under yellow lights.

Determination of test dosages

Cytotoxicity, determined prior to the assay using

a modification of a previously described procedure (Traul et al., 1981a), was used as an indicator of test dose. RIFRE cells were seeded into 24-well Multiwell Plates (2 cm^2, Falcon Plastics) at 5.2×10^4 cells per well. After 24 h, a range of chemical doses was added in complete medium; all determinations were done in triplicate. The cells were incubated for 72 h, at which time they were washed twice with Earle's Balanced Salt Solution (EBSS), then fixed and stained with methylene blue. The LD_{50} and highest LD_0 doses were identified by macroscopic and microscopic examination. Comparisons with controls were made and a range of desired doses selected.

Anchorage-independent survival assays procedure

The anchorage-independent survival assay has been described (Suk et al., 1984) and is schematically represented in Fig. 1. The RIFRE cells were seeded at $6.66 \times 10^3/cm^2$ and incubated at 37 °C in a humidified 5% CO_2 and 5% air incubator. 1–3 flasks were trypsinized and counted on day 1 to obtain plating efficiency at the time of initiation of chemical treatment. Selected doses of chemical in complete medium were then added to the test flasks, while the control flasks received either complete medium alone or solvent. Following incubation for 72 h, the chemical was removed and the flasks were washed twice with Earle's Balanced Salt Solution (EBSS), and then refed with complete medium without chemical. The cultures were then allowed to incubate and replicate/recover an additional 72 h. Cells were then dissociated with Enzar T, pooled, and resuspended in complete medium. Viable cell counts were made on the basis of trypan blue exclusion. 600 000 viable cells were seeded onto 6-cm dishes (an equal number of dishes for controls and for chemical treatment

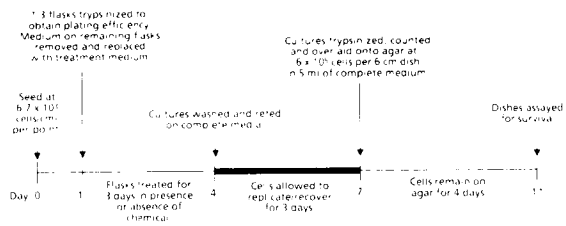


Fig. 1. Schematic representation of the RIFRE cell anchorage-independent survival assay. The details of the procedure are described in "Materials and Methods".

points) in 5 ml of complete media over a solid agar base: 1% agar Noble medium was made by combining 2 × concentrations of complete EMEM and agar at 1 : 1. Dishes were incubated an additional 4 days. Cells from 1–3 dishes were harvested by decanting the suspended cells into a 15-ml conical centrifuge tube, washing the dishes twice with EBSS, and decanting the wash into the tube. The tubes were centrifuged at 600–1200 × *g* at 4 °C for 10 min, supernatant was removed and the cell pellet was resuspended in 2 ml EBSS. Cells for cell counting were taken directly from these tubes. Cells were counted using trypan blue exclusion to determine total viable cell count. When the viable cell counts were made, 3–5 hemacytometer readings were performed for each treatment point and the number averaged. In this way, the amount of variability inherent in the counting procedure was reduced.

Calculating enhanced survival

A positive response was determined as a 2-fold increase in the expression of anchorage-independent surviving viable cells induced by carcinogen treatment relative to the untreated solvent or media control cells. This was computed as:

$$\frac{\text{Treated VCN} - \text{control VCN}}{\text{Control VCN}} \times 100$$

where VCN equals viable cell number (Traul et al., 1981b).

Response criteria for the RIFRE cell anchorage-independent survival assay

The RIFRE cell anchorage-independent survival assay is undergoing development; therefore, the response criteria are not in final form. However, the following is our working guideline for response criteria:

Positive	a 2-fold survival enhancement at 2 or more concentrations; a positive dose–response; and a significant (> 200%) survival enhancement at 2 or more concentrations.
Strong suspect	a significant survival enhancement at 1 concentration.
Weak suspect	a 2-fold survival enhancement at 1 concentration; and approaching a 2-fold survival enhancement at 2 or more concentrations.

Negative	no survival enhancement at any of the concentrations tested while producing cytotoxicity.
No response	no survival enhancement or toxicity produced at the concentrations tested.
Null	an experiment that fails to meet technically acceptable criteria, e.g., no survival enhancement by the positive control.

Results

The RIFRE cell anchorage-independent survival assay uses enhanced cell survival between carcinogen-treated and control cells to indicate neoplastic transformation. Carcinogen-induced anchorage-independent survival precedes the expression of neoplastic transformation (Suk et al., 1984). RIFRE cells exhibiting enhanced cell survival, plated back onto a solid substrate and carried in culture, expressed changes in their cellular morphology, produced macroscopic foci in semisolid agar, and induced tumors in animals.

When RIFRE cells are treated with increasing concentrations of carcinogen, the number of viable anchorage-independent surviving cells is significantly higher when compared to the medium- or solvent-treated controls. For example, a dose–response relationship that was achieved with DMBA is shown in Fig. 2. This agent is routinely used as the positive control in all assay procedures. Other known carcinogens positive in the assay system in a concentration-dependent frequency are benzo [*a*]pyrene [B(a)P]; 3-methylcholanthrene, diethylnitrosamine, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and 4-nitroquinoline-*N*-oxide (Suk et al., 1983, 1984).

In Tables 1–7, the results of testing a number of the agents of interest to the IPCS/CSSTT in the RIFRE cell anchorage-independent survival assay are shown. In Table 1, the results of SAF as an inducer of cell survival are shown. The first experiment shows SAF to induce anchorage-independent survival at 100 μg/ml. In the second experiment, the same concentration of enhanced anchorage-independent survival was 34% above the control; higher SAF concentrations failed to induce a higher cell survival value, possibly due to increased cytotoxicity. Nevertheless, SAF appears to be a weak suspect carcinogen in this system. Table 2 shows the results of TOL as an inducer of

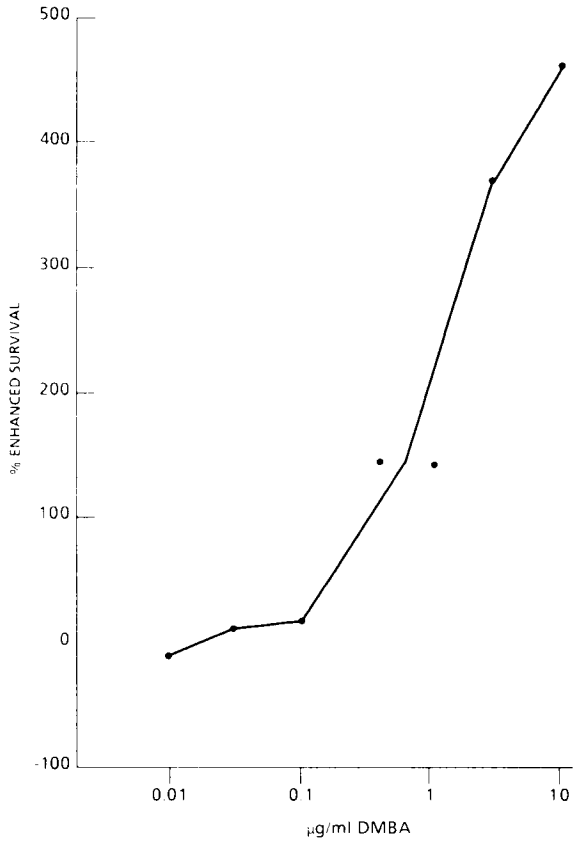


Fig. 2. DMBA-mediated enhancement of anchorage-independent survival as a function of concentration. Monolayer cultures of RIFRE cells were exposed to increasing concentrations of DMBA, and subsequently assayed as described in "Materials and Methods." The data are expressed as the percent enhanced survival of the number of viable cells with respect to DMSO-control obtained with each concentration following 4 days in medium above an agar base. All values were determined in triplicate for duplicate dishes per treatment point.

anchorage-independent survival. The results of Expt. 1 show that TOL approached a 2-fold increase in anchorage-independent survival at 10 and 100 μg/ml. Moreover, in attempts to quantitate the percent enhanced survival value, we use a *t*-test that incorporates a level of significance. When TOL, in Expt. 2, was expressed in this way, there was a significant difference between the 50 μg/ml concentration and the solvent control that was not necessarily reflected in the induced 31% enhanced survival value. Therefore, the results taken together appear to indicate that TOL is a suspect

TABLE 1

RESULTS OF SAFROLE (SAF) IN THE RIFRE CELL ANCHORAGE-INDEPENDENT SURVIVAL ASSAY

Treatment (μg/ml) [a]	Cytotoxicity [(cells/cm^2)]10^3	Mean number of cells $\pm$ SD ($\times 10^3$) [b]	Enhanced survival (%) [c]
Expt. 1			
Medium	88.3	22.0 $\pm$ 1.0	0
DMBA (1)	23.3	135.2 $\pm$ 1.7	514
SAF (100)	48.3	42.0 $\pm$ 2.1	91
SAF (10)	76.7	20.8 $\pm$ 4.0	−5
SAF (1)	67.5	20.8 $\pm$ 9.0	−5
Expt. 2			
Medium	96.6	42.0 $\pm$ 1.5	8
DMSO (0.1%)	65.0	38.8 $\pm$ 6.0	0
DMBA (1)	10.8	142.0 $\pm$ 2.1	266
SAF (500)	11.7	12.0 $\pm$ 7.0	−69
SAF (250)	86.7	46.8 $\pm$ 1.4	21
SAF (100)	72.5	52.0 $\pm$ 2.4	34

[a] RIFRE cells were treated with varying concentrations of known carcinogen for 3 days, washed, and refed for an additional 3 days. They were subsequently trypsinized, adjusted for cytotoxicity, and overlaid at 6×10^5 cells per 6-cm dishes that contained 1% agar Noble medium. Viable cell counts were made following 4 days in suspension above agar.

[b] The numbers represent the mean and standard deviation of the viable number of cells counted.

[c] The percent survival for each treatment point is calculated based on either the solvent control, 0.1% DMSO, or the medium control.

carcinogen in this assay system.

HMPA was tested a number of times, with varying results (Table 3). In Expt. 1, HMPA approached a 2-fold increase in anchorage-independent survival at all 3 concentrations; in Expt. 2, this agent responded positively and induced

TABLE 2

RESULTS OF *o*-TOLUIDINE (TOL) IN THE RIFRE CELL ANCHORAGE-INDEPENDENT SURVIVAL ASSAY

Treatment (μg/ml) [a]	Cytotoxicity [(cells/cm^2)]10^3	Mean number of cells $\pm$ SD ($\times 10^3$) [b]	Enhanced survival (%) [c]
Expt. 1			
Medium	88.3	22.0 $\pm$ 1.0	0
DMBA (1)	23.3	135.0 $\pm$ 1.7	514
TOL (100)	48.3	34.0 $\pm$ 1.2	55
TOL (10)	87.5	39.2 $\pm$ 1.9	78
TOL (1)	75.0	26.0 $\pm$ 1.5	18
Expt. 2			
Medium	96.6	42.0 $\pm$ 1.5	8
DMSO (0.1%)	65.0	38.8 $\pm$ 7.0	0
DMBA (1)	10.8	142.0 $\pm$ 2.1	266
TOL (500)	44.2	43.2 $\pm$ 1.2	11
TOL (50)	68.3	50.8 $\pm$ 1.4	31
TOL (5)	75.0	38.8 $\pm$ 1.1	0

[a] RIFRE cells were treated with varying concentrations of known carcinogen for 3 days, washed, and refed for an additional 3 days. They were subsequently trypsinized, adjusted for cytotoxicity, and overlaid at 6×10^5 cells per 6-cm dishes that contained 1% agar Noble medium. Viable cell counts were made following 4 days in suspension above agar.

[b] The numbers represent the mean and standard deviation of the viable number of cells counted.

[c] The percent survival for each treatment point is calculated based on either the solvent control, 0.1% DMSO, or the medium control.

TABLE 3

RESULTS OF HEXAMETHYLPHOSPHORAMIDE (HMPA) IN THE RIFRE CELL ANCHORAGE-INDEPENDENT SURVIVAL ASSAY

Treatment (μg/ml) [a]	Cytotoxicity [(cells/cm^2)]10^3	Mean number of cells $\pm$ SD ($\times 10^3$) [b]	Enhanced survival (%) [c]
Expt. 1			
Medium	28.7	29.2 $\pm$ 2.0	0
DMBA (1)	28.0	293.2 $\pm$ 7.0	900
HMPA (100)	30.0	50.8 $\pm$ 2.4	73
HMPA (10)	32.7	41.2 $\pm$ 1.3	41
HMPA (1)	44.0	48.8 $\pm$ 1.3	66
Expt. 2			
Medium	68.5	43.0 $\pm$ 3.1	−20
DMSO (0.1%)	76.8	54.3 $\pm$ 4.2	0
DMBA (1)	18.5	227.0 $\pm$ 7.1	320
HMPA (1000)	54.8	192.3 $\pm$ 6.3	256
HMPA (150)	68.5	128.3 $\pm$ 4.5	137
HMPA (10)	75.4	103.0 $\pm$ 3.3	91
Expt. 3			
Medium	135.8	28.7 $\pm$ 2.6	0
DMSO (0.1%)	137.5	33.3 $\pm$ 5.7	16
DMBA (10)	14.2	108.7 $\pm$ 5.0	279
HMPA (1000)	128.3	27.3 $\pm$ 4.1	−5
HMPA (500)	137.5	26.7 $\pm$ 3.0	−7
HMPA (100)	130.0	24.0 $\pm$ 3.8	−17

[a] RIFRE cells were treated with varying concentrations of known carcinogen for 3 days, washed, and refed for an additional 3 days. They wre subsequently trypsinized, adjusted for cytotoxicity, and overlaid at 6×10^5 cells per 6-cm dishes which contained 1% agar Noble medium. Viable cell counts were made following 4 days in suspension above agar.

[b] The numbers represent the mean and standard deviation of the viable number of cells counted.

[c] The percent survival for each treatment point is calculated based on either the solvent control, 0.1% DMSO, or the medium control.

TABLE 4

RESULTS OF DIETHYLHEXYLPHTHALATE (DEHP) ANCHORAGE-INDEPENDENT SURVIVAL ASSAY

Treatment (μg/ml) [a]	Cytotoxicity [(cells/cm^2)]10^3	Mean number of cells $\pm$ SD ($\times 10^3$) [b]	Enhanced survival (%) [c]
Expt. 1			
Medium	78.3	44.0 $\pm$ 3.4	0
DMSO (0.25%)	79.2	48.7 $\pm$ 4.5	10
DMBA (1)	27.5	NT	NT
DEHP (2000)	65.0	41.3 $\pm$ 6.5	−6
DEHP (1000)	66.7	18.7 $\pm$ 2.6	−58
DEHP (200)	80.8	32.0 $\pm$ 4.9	−28
Expt. 2			
Medium	80.8	28.0 $\pm$ 8.0	0
DMSO (0.2%)	83.3	28.3 $\pm$ 3.3	0
DMBA (1)	35.6	116.8 $\pm$ 5.1	317
DEHP (2000)	62.2	73.0 $\pm$ 11.5	161
DEHP (1000)	65.5	45.3 $\pm$ 2.0	61
DEHP (500)	75.9	27.3 $\pm$ 9.5	−4

[a] RIFRE cells were treated with varying concentrations of known carcinogen for 3 days, washed, and refed for an additional 3 days. They were subsequently trypsinized, adjusted for cytotoxicity, and overlaid at 6×10^5 cells per 6-cm dishes that contained 1% agar Noble medium. Viable cell counts were made following 4 days in suspension above agar.

[b] The numbers represent the mean and standard deviation of the viable number of cells counted.

[c] The percent survival for each treatment point is calculated based on either the solvent control, 0.2% DMSO, or the medium control.

TABLE 5

RESULTS OF CAPROLACTAM (CAP) IN THE RIFRE CELL ANCHORAGE-INDEPENDENT SURVIVAL ASSAY

Treatment (μg/ml) [a]	Cytotoxicity [(cells/cm^2)]10^3	Mean number of cells ± SD ($\times 10^3$) [b]	Enhanced survival (%) [c]
Medium	66.7	25.2 ± 1.1	−25
DMSO (0.1%)	63.3	34.0 ± 9.0	0
DMBA (1)	22.5	179.2 ± 3.1	427
CAP (50)	73.3	20.8 ± 7.0	−39
CAP (5)	69.2	20.8 ± 5.0	−39
CAP (0.5)	57.5	33.2 ± 1.8	−2

[a] RIFRE cells were treated with varying concentrations of known noncarcinogen for 3 days, washed, and refed for an additional 3 days. They were subsequently trypsinized, adjusted for cytotoxicity, and overlaid at 6×10^5 cells per 6-cm dishes that contained 1% agar Noble medium. Viable cell counts were made following 4 days in suspension above agar.

[b] The numbers represent the mean and standard deviation of the viable number of cells counted.

[c] The percent survival for each treatment point is calculated based on the solvent control, 0.1% DMSO.

anchorage-independent survival in a concentration-dependent manner; and in Expt. 3, negative results were shown. HMPA was tested twice more, with ambiguous results both times (data not shown). The variability in the response of HMPA was attributed to its volatility: the cells may not have received adequate chemical concentration(s) to elicit a response. Further testing of this agent is indicated, perhaps using a sealed treatment chamber to control the level of exposure better. Nevertheless, based on the results thus far, HMPA appears to be a suspect carcinogen.

The results of DEHP are shown in Table 4. In Expt. 1, this agent did not induce anchorage-independent survival; however, because the positive control was lost due to contamination, this was a null experiment. In Expt. 2, DEHP induced a positive anchorage-independent survival response at the highest concentration tested (2000 μg/ml). The behavior of DEHP-treated cells in the assay, as well as their morphology in suspension above the agar base layer, was similar to the responses seen when the cells are treated with a tumor promoter (unpublished). Therefore, DEHP gave interesting, yet mixed, results, and requires further testing. Nevertheless, our results indicate, thus far, that DEHP is a weak suspect carcinogen.

An important aspect of an in vitro carcinogenesis assay is its ability to discriminate between carcinogens and noncarcinogens. Tables 5 and 6

TABLE 6

RESULTS OF BENZOIN (ZOIN) IN THE RIFRE CELL ANCHORAGE-INDEPENDENT SURVIVAL ASSAY

Treatment (μg/ml)	Cytotoxicity [(cells/cm^2)]10^3	Mean number of cells ± SD ($\times 10^3$) [b]	Enhanced survival (%) [c]
Medium	93.3	14.0 ± 7.0	−52
DMSO (0.1%)	83.3	29.2 ± 1.4	0
DMBA (1)	14.2	149.2 ± 10.3	409
ZOIN (50)	113.3	20.0 ± 2.7	−32
ZOIN (5)	63.3	26.0 ± 3.0	−1
ZOIN (0.5)	100.0	19.2 ± 4.0	−34

[a] RIFRE cells were treated with varying concentrations of known noncarcinogen for 3 days, washed, and refed for an additional 3 days. They were subsequently trypsinized, adjusted for cytotoxicity, and overlaid at 6×10^5 cells per 6-cm dishes which contained 1% agar Noble medium. Viable cell counts were made following 4 days in suspension above agar.

[b] The numbers represent the mean and standard deviation of the viable number of cells counted.

[c] The percent survival for each treatment point is calculated based on the solvent control, 0.1% DMSO.

TABLE 7

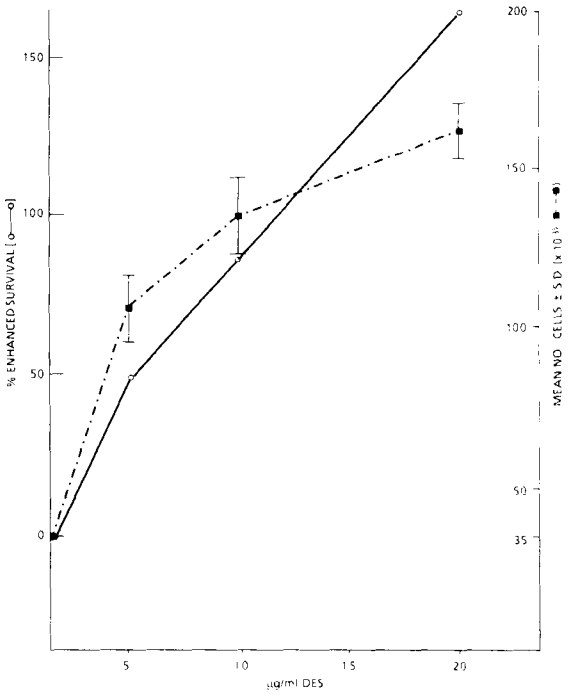
RESULTS OF DIETHYLSTILBESTROL (DES) IN THE RIFRE CELL ANCHORAGE-INDEPENDENT SURVIVAL ASSAY

Treatment (μg/ml) [a]	Cytotoxicity [(cells/cm^2)]10^3	Mean number of cells ± SD ($\times 10^3$) [b]	Enhanced survival (%) [c]
Expt. 1			
Medium	136.0	28.0 ± 1.3	0
DMBA (1)	13.3	188.0 ± 6.6	571
DMBA (0.1)	19.3	48.7 ± 3.7	74
DES (10)	18.7	59.3 ± 3.4	112
DES (5)	28.7	52.7 ± 6.7	88
DES (2.5)	68.7	28.0 ± 3.2	0
DES (1.0)	92.7	14.0 ± 2.3	−50
DES (0.5)	77.3	16.7 ± 1.9	−40
Expt. 2			
Medium	89.3	36.0 ± 2.5	0
DMSO (0.2%)	161.3	44.7 ± 3.8	24
DMBA (1)	13.3	153.3 ± 8.9	326
DMBA (0.1)	15.3	99.3 ± 4.1	176
DES (20)	11.7	66.0 ± 1.4	83
DES (10)	10.0	80.0 ± 5.2	122
DES (5)	18.7	46.7 ± 3.9	30
DES (2.5)	82.7	26.7 ± 3.0	−26
DES (1.0)	58.7	40.0 ± 3.0	11
DES (0.5)	108.0	22.7 ± 1.6	−37

[a] RIFRE cells were treated with varying concentrations of known carcinogen for 3 days, washed, and refed for an additional 3 days. They were subsequently trypsinized, adjusted for cytotoxicity, and overlaid at 6×10^5 cells per 6-cm dishes that contained 1% agar Noble medium. Viable cell counts were made following 4 days in suspension above agar.

[b] The numbers represent the mean and standard deviation of the viable number of cells counted.

[c] The percent survival for each treatment point is calculated based on either the solvent control, 0.2% DMSO, or the medium control.



show the results of testing CAP and ZOIN, respectively, for anchorage-independent survival. The results indicate that these agents are negative and, therefore, noncarcinogens. Similar results were achieved in other experiments (data not shown), consistent with the testing of other known noncarcinogens such as anthracene, pyrene and benzo-[*e*]pyrene (Suk et al., 1983, 1984). Moreover, the results affirm RIFRE cells' capacity to differenti-

Fig. 3. DES-mediated enhancement of anchorage-independent survival as a function of concentration. Monolayer cultures of RIFRE cells were exposed to increasing concentrations of DES and assayed as described in "Materials and Methods." The data are expressed in the percent enhanced survival (○) of the number of viable cells with respect to the solvent control, and as the mean number of viable cells plus or minus their standard deviation (■). All values were determined in triplicate for duplicate dishes per treatment point. The positive controls of 1.0 and 0.1 μg/ml DMBA showed an enhanced survival of 249% and 128%, respectively.

ate between similar structures when determining the carcinogenicity of an agent.

The results in Table 7 and Fig. 3 indicate that DES is a strong inducer of anchorage-independent survival. Expts. 1 and 2 show DES inducing anchorage-independent survival at two concentrations, 5 and 10 μg/ml, and 10 and 20 μg/ml, respectively; 5 μg/ml in Expt. 2 induced a 30% enhanced survival value. The results of Expt. 3 are shown in Fig. 3. The data are expressed as the percent enhanced survival and as the mean number of cells, plus or minus the standard deviation (SD). The results of Fig. 3 indicate that DES induces anchorage-independent survival in a concentration-dependent manner and is, therefore, a carcinogen.

Discussion

A number of investigators have shown that a variety of spontaneous, viral, and/or chemical transformed cells, having the ability to proliferate in semisolid agar and to produce tumors in animals also have increased anchorage-independent survival (Cho et al., 1976; Mason and Takemoto, 1977; Putnam et al., 1977; Steuer and Ting, 1976). There are also a number of studies that have evaluated malignant transformation in vitro and have correlated it with anchorage-independent survival (Eker and Sanner, 1983; Steuer et al., 1977; Traul et al., 1981b; Suk et al., 1984). In this way, cellular transformation is measured by means of survival in an aggregate/anchorage-independent form when the cells are suspended in liquid medium above an agar base. Recent studies have shown that carcinogen-treated retrovirus-infected Fischer rat-embryo cells, which exhibit enhanced anchorage-independent survival, undergo subsequent changes in their cellular morphology, produce macroscopic foci in semisolid agar, and induce tumors in syngenic hosts and immunologically suppressed nude mice (Suk et al., 1984). These observations lend further support to the usefulness of this assay system.

In this study, we present data on the testing of selected chemicals of interest to the IPCS as part of an international collaborative study of short-term assays for chemical carcinogens. This paper provides a comparative evaluation of responses to 6 chemicals as inducers of anchorage-independent survival. In tests performed on SAF and TOL, these agents were determined to be weak suspect carcinogens. The results of experiments performed on HMPA and DEHP, although considered variable, indicate that these chemicals are also weak suspect carcinogens; however, additional investigations are needed to confirm the observed neoplastic transformation by these two chemicals. CAP and ZOIN failed to induce anchorage-independent survival and are therefore noncarcinogenic. DES was shown to be a carcinogen by inducing anchorage-independent survival in a concentration-dependent frequency. All chemicals were tested in the assay without the addition of an exogenous metabolic activation system; the positive responses to these carcinogens that are metabolized to reactive intermediates suggest that the RIFRE cells have the capacity to perform these same functions; however, the lack and/or variability of responses with some of the chemicals underscores the need for the incorporation of a metabolic activating system.

All experiments were performed according to the 3-day treatment schedule discussed in "Materials and Methods." However, it appears that for certain carcinogens, e.g., DMBA and B(a)P, the 3-day treatment is optimal; a 4-h treatment appears optimal for MNNG, and three 24-h repetitive treatments appear necessary for such agents as 2-acetylaminofluorine to be responsive (unpublished). Therefore, a determination of when to change from the standard protocol to either or both of these two other exposure procedures is being reviewed.

Previous studies have shown that nontransforming retroviruses play a specific and significant role in promoting the expression of transformation (Freeman et al., 1973a, b; 1973c, Price et al., 1972b, 1977; Fisher et al., 1979; Fisher and Weinstein, 1980). Recently, we have shown that the presence of retrovirus in low-passage rat cells increases the sensitivity to chemically-induced anchorage-independent survival (Suk et al., 1984), which appears to be an early event in the transformation process. The process of carcinogen-induced immortality/establishment predisposes cells to further progression to malignancy and yields cellular functions beyond those achieved by transforming

oncogenes alone (Land et al., 1983; Newbold and Overel, 1983). Therefore, it is possible that the retrovirus integrates specific oncogenes and that a mutation to an oncogene function may be all that is necessary to achieve complete transformation in these cells.

It must be reiterated that this system, based on anchorage-independent survival as an event that precedes neoplastic transformation, is being evaluated and systematically modified to increase its sensitivity and reproducibility. Therefore, the results presented here should not be construed as definitive. However, we do feel that this assay system is a rapid, sensitive, reproducible, and potentially quantitative bioassay. Moreover, in evaluating the interaction between carcinogens and viruses in this system, we should facilitate our understanding of the multifactorial nature of the carcinogenic process.

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Assays for the induction of cell transformation in Chinese hamster ovary (CHO) cells and in Syrian hamster embryo (SHE) cells

M.Z. Zdzienicka, A.J. de Kok and J.W.I.M. Simons

Department of Radiation Genetics and Chemical Mutagenesis, State University of Leiden (The Netherlands)

Summary

5 compounds were tested for induction of cell transformation in CHO cells by means of a 4-day treatment in the absence and presence of a metabolic activation system. Cocultivation with lethally irradiated Syrian hamster cells was used as a metabolic activation system. One compound (hexamethylphosphoramide) was also tested for morphological transformation of primary Syrian hamster cells; it was found to be positive. In the CHO cells an indication for carcinogenic activity of hexamethylphosphoramide was obtained but further testing is necessary before a definite conclusion can be drawn. The 4 other compounds, toluidine, benzene, safrole and caprolactam) were found to be negative.

Within the framework of the IPCS Collaborative Study on in vitro Short-term Tests the induction of cell transformation was studied for 5 compounds (HMPA, TOL, BEN, SAF and CAP). Two assay systems were employed: (1) morphological transformation in primary Syrian hamster embryo (SHE) cells; (2) clonal variation in Chinese hamster ovary (CHO) cells. In the latter assay system already transformed cells are used. They are able to grow in agar in which they give rise to solid clumps of cells. After treatment with mutagens and carcinogens clonal variants are induced. They are characterized by an invasive growth in agar (INGA transformation). The CHO cells were treated over a period of 4 days in the presence and the absence of a metabolic activation system. Metabolic activation was achieved by cocultivation of the cells with primary Syrian hamster embryo cells.

Materials and methods

Culture

The experiments were carried out with Chinese hamster ovary (CHO) cells. For each experiment one ampoule, containing approximately 10^6 cells, was taken from a stock and stored in liquid nitrogen. The cells were seeded in a petri dish, 145 mm in diameter (P145, Greiner) containing 25 ml of Ham's F10 medium, modified by the omission of hypoxanthine and supplemented with 10% newborn calf serum, penicillin (100 μg/ml) and streptomycin (0.1 mg/ml). The cells were incubated in a humidified CO_2-incubator (5% CO_2) at 37°C. For subcultures a trypsin solution (0.5%) containing EDTA (0.02%) was used. After 3 days cells were subcultured and seeded in P145 dishes at a density of 3.5×10^5 cells per dish. After 3 more days the cells were trypsinized and seeded at a density of 10^6 cells per roller bottle, containing 50 ml of medium.

Treatment

Chemicals were stored at 4°C in a refrigerator until use. The compounds were dissolved or diluted in DMSO (Merck). This stock solution was added to the cells in the roller bottles in the appropriate amount to obtain the desired concentration. The compounds were added 4 h after seeding the cells and the treatment occurred over 4

consecutive days. Metabolic activation of the compounds was achieved by cocultivation with irradiated primary Syrian hamster embryo cells (4000 rad) which were added to the roller bottle at the same time as the chemical, 5×10^6 cells per bottle. After 4 days the cells were suspended and cloned in soft agar.

Determination of cell-transformation frequencies

The transformed phenotype of CHO cells consists out of colonies in agar that show an INvasive Growth in Agar (INGA transformation). Untransformed wild-type cells also grow in agar, but they give rise to small solid clumps of cells without invasive properties. There are indications that the transformed phenotype correlates with an increased tumorigenicity and an increased metastatic potential of the cells.

Immediately after treatment the cells were seeded in Ham's F10 medium containing lactopeptone (0.5 mg/ml), folic acid (8.7 μg/ml), bicarbonate (0.08%), agar (0.32%) and fetal calf serum (0.4%), 4000 cells/P90 with 40 ml of medium, 15 P90 per group. The cultures were kept in a humidified incubator at 37 °C with 5% CO_2 for 3 weeks and subsequently the number of transformed clones was counted with the naked eye and the number of untransformed clones estimated by counting under the microscope the number of untransformed colonies in 3 petri dishes chosen at random from each experimental group. The transformation frequencies were calculated by dividing the number of transformed clones by the total number of clones in the agar.

For the determination of cell-transformation frequencies in primary Syrian hamster embryo cells, the cells were seeded in the presence of the compounds. The assay was performed according to the protocol, published by Pienta et al. (1977).

Results and discussion

As the CHO cells were seeded in agar immediately after the 4-day treatment, and as un-

TABLE 1

CLONING EFFICIENCIES OF CELLS IN AGAR AFTER 4 DAYS CONTINUOUS TREATMENT

Compound		Metabolic activation	Experimental group			
			1	2	3	4
HMPA	Concentration (μl/ml)		0.0	1.0	10.0	
	Cloning efficiency (%)	−	26	40	4	
		+	46	40	24	
TOL	Concentration (μl/min)		0.0	0.10	0.25	0.50
	Cloning efficiency (%)	−	39	29	28	17
		+	35	27	33	50
BEN	Concentration (μl/ml)		0.0	0.5	1.0	
	Cloning efficiency (%)	−	n.d.	n.d.	17	
		+	14	7.1	20	
SAF	Concentration (μl/ml)		0.0	0.5	1.0	
	Cloning efficiency (%)	−	n.d.	36	29	
		+	45	45	53	
CAP	Concentration (mg/ml)		0.0	1.0	2.0	
	Cloning efficiency (%)	−	31	10	23	
		+	36	23	21	
B(a)P	Concentration (μg/ml)		0.0	10.0	50.0	
	Cloning efficiency (%)	−	23	n.d.	34	
		+	22	14	13	
EMS	Concentration (μl/ml)		0.0	0.2	0.6	
	Cloning efficiency (%)	−	31	13	15.6	

TABLE 2

CELL TRANSFORMATION FREQUENCIES IN CHO CELLS AFTER TREATMENT WITH THE COMPOUNDS

Compound		Metabolic activation	Experimental group			
			1	2	3	4
HMPA	concentration (μl/ml)		0.0	1.0	10.0	
	Transf. freq. ($\times 10^{-4}$)	−	0.7	0.9	9.2	
		+	0.8	2.2	30.8	
TOL	concentration (μl/ml)		0.0	0.10	0.25	0.50
	Transf. freq. ($\times 10^{-4}$)	−	1.4	3.6	2.1	n.d
		+	1.5	3.3	1.6	n.d.
		−	n.d.	n.d.	0.0	2.2
		+	n.d.	n.d.	0.0	1.7
BEN	concentration (μl/ml)		0.0	0.5	1.0	
	Transf. freq. ($\times 10^{-4}$)	−	2.7	n.d.	4.2	
		+	n.d.	2.5	4.5	
SAF	concentration (μl/ml)		0.0	0.5	1.0	
	Transf. freq. ($\times 10^{-4}$)	−	n.d.	2.0	5.5	
		+	1.2	2.3	1.0	
CAP	concentration (mg/ml)		0.0	1.0	2.0	
	Transf. freq. ($\times 10^{-4}$)	−	3.4	5.4	4.6	
		+	3.0	5.5	7.6	
B(a)P	concentration (μg/ml)		0.0	10.0	50.0	
	Transf. freq. ($\times 10^{-4}$)	−	4.4	n.d.	3.1	
		+	3.3	22.7	59.5	
EMS	concentration (μl/ml)		0.0	0.2	0.6	
	Transf. freq. ($\times 10^{-4}$)	−	3.4	12.8	22.9	

transformed cells also give rise to clones in agar, the cytotoxicity of the treatment can be inferred from the cloning efficiencies in agar (Table 1).

TABLE 3

CELL TRANSFORMATION FREQUENCIES IN SYRIAN HAMSTER EMBRYO CELL AFTER TREATMENT WITH HMPA

Concentration (μg/ml)	Relative survival		Transformation frequency ($\times 10^{-2}$)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
0.00	100	100	0.0	0.0
0.01	89	-	0.0	-
0.05	96	-	0.8	-
0.10	100	-	0.4	-
0.50	94	81	0.4	0.4
1.00	77	72	2.0	0.4
2.50	-	57	-	1.1
5.00	25	22	3.2	1.4
10.00	0	-	-	-

Hexamethylphosphoramide and caprolactam probably are cytotoxic both in the presence and in the absence of a metabolic activation system; no cytotoxicity was observed for *o*-toluidine and

TABLE 4

CONCLUSIONS ON INDUCTION OF CELL TRANSFORMATION IN CHO CELLS AND IN SYRIAN HAMSTER EMBRYO CELLS AFTER TREATMENT WITH THE COMPOUNDS

Compound	CHO cells		SHE cells
	− Metabolic activity	+ Metabolic activity	
Hexamethylphosphoramide	−	?	+
o-Toluidine	−	−	n.d.
Benzene	−	−	n.d.
Safrole	−	−	n.d.
Caprolactam	−	−	n.d.

safrole. The data for benzene are inconclusive. (For the effect of the compounds on the growth rate during the 4-day treatment, see Zdzienicka and Simons, this volume.) Hexamethylphosphoramide was also toxic for primary Syrian hamster embryo cells (Table 3). The transformation frequencies in CHO cells are given in Table 2 and the conclusions drawn from these experiments are shown in Table 4. No induction of cell transformation was observed for *o*-toluidine, benzene, safrole or caprolactam. For hexamethylphosphoramide a strong indication for induction of cell transformation was obtained in the presence of the metabolic activation system but this compound should be tested further before a definite conclusion can be drawn. Hexamethylphosphoramide certainly transforms primary Syrian hamster embryo cells (Table 3).

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PART III

APPENDICES

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Appendix 1

Source, purification and analytical details of the 10 test chemicals

John Ashby and David Paton

Imperial Chemical Industries PLC, Central Toxicology Laboratory, Alderley Edge, Cheshire (Great Britain)

General comments about the meaning of the phrase 'a pure chemical' are made in the Overview. The present chemicals were 'pure' by currently accepted standards for the bioassay of a chemical, they are each of 99% or greater purity. The analytical criteria were essentially as employed in the previous International Study (de Serres and Ashby, 1981).

The chemicals were all commercially available and material of the highest purity was acquired. In the case of benzoin the commercial material was recrystallized prior to dispatch. Similarly, the sample of *o*-toluidine was re-distilled upon receipt from the supplier. The data shown in Table 1 records the analytical procedures applied to those chemicals in order to confirm a purity of 99% or greater. The ^{1}H-nuclear magnetic (nmr) spectrum and mass spectrum of each chemical is shown following Table 1. Specific assignments of chemical shifts and coupling patterns in the nmr spectra,

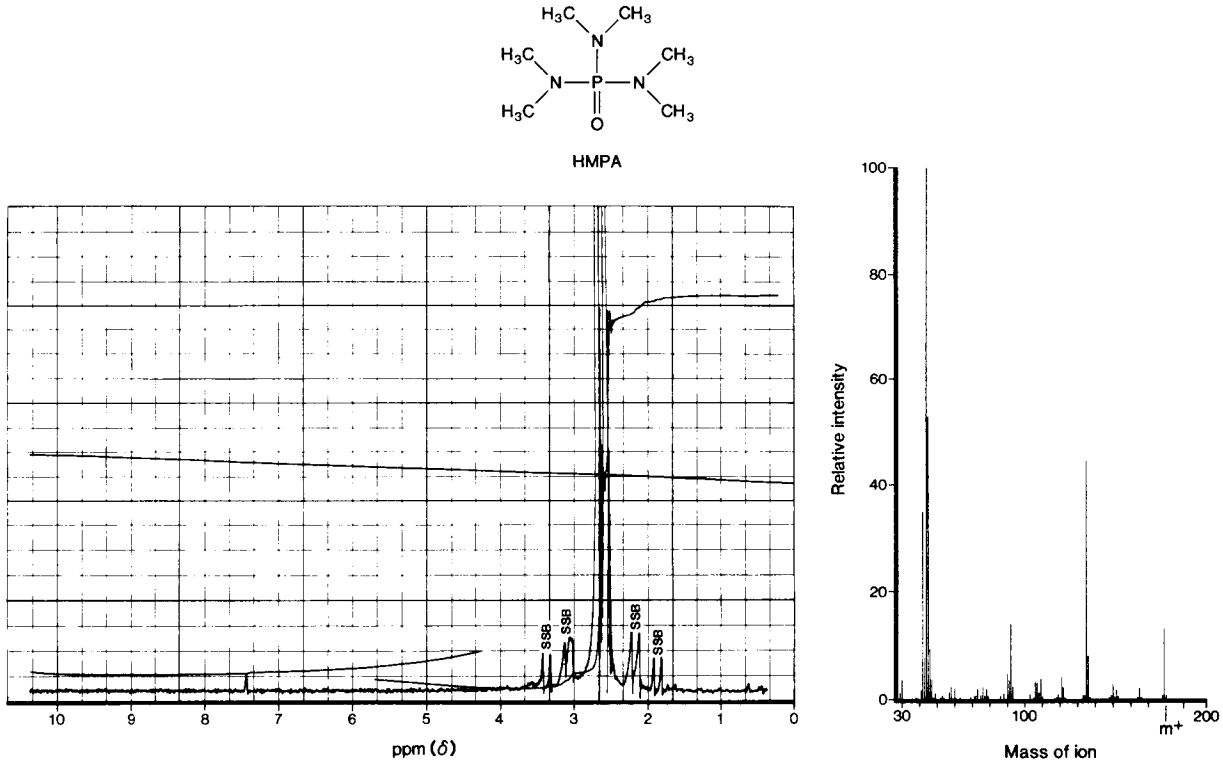


Fig. 1. Hexamethylphosphoramide.

TABLE 1

Compound	Symbol	Original supplier	C, H and N analysis Largest variance from theory (%)	ms nmr Corresponds to structure. No impurities seen unless stated		m.p. (°C)	Literature m.p. (°C)	TLC No impurities seen unless stated	HPLC	Comments
Hexamethyl-phosphoramide	HMPA	A (99%)	−0.12 (CH)	√	√	–	–	–	–	Coleman N 22.7, requires 23.4%
Safrole	SAF	F	+0.4 (CH)	√	Traces at 0.7–1.3 and 1.3–1.8 ppm	–	–	–	*Trace at T_R 4.9 min	*T_R Safrole 4.1 min Conditions: RP18, water/methanol 1:19 1 ml/min, 254 nm
o-Toluidine	TOL	A, redist.	+0.06 (CHN)	√	√	–	–	Trace at R_f 0.7 *	–	* Merck silica gel GF_{254}, $CHCl_3$ R_f *o*-toluidine = 0.5
Benzene	BEN	A (99%)	−0.35 (CH)	√	√	–	–	–	–	
Di(2-ethylhexyl)-phthalate	DEHP	F (99%)	+0.2 (CH)	√	√	–	–	–	–	CH analysis indicates 1/3 H_2O present
Acrylonitrile	ACN	A (99%)	−0.1 (CHN)	√	√	–	–	–	–	
Diethylstilboestrol	DES	LS	−0.26 (CH)	√	√	173 –174	172 [2]	√	–	
Phenobarbital	PB	F (99%)	+0.34 (CHN)	√	√	176.5–177.5	177 [3]	√	–	
Caprolactam	CAP	A (99%)	−0.42 (CHN)	√	√	70 – 70.5	70.5–71.5 [4]	√	–	
Benzoin	ZOIN	LS (recryst)	+0.32 (CH)	√	√	134 –135	133 [5]	√	–	

A dash (–) indicates method not applicable or not undertaken. HPLC analysis was undertaken on safrole only because of the trace impurities seen in the nmr spectrum. A, Aldrich; F, Fluka; LS, Lancaster Synthesis.

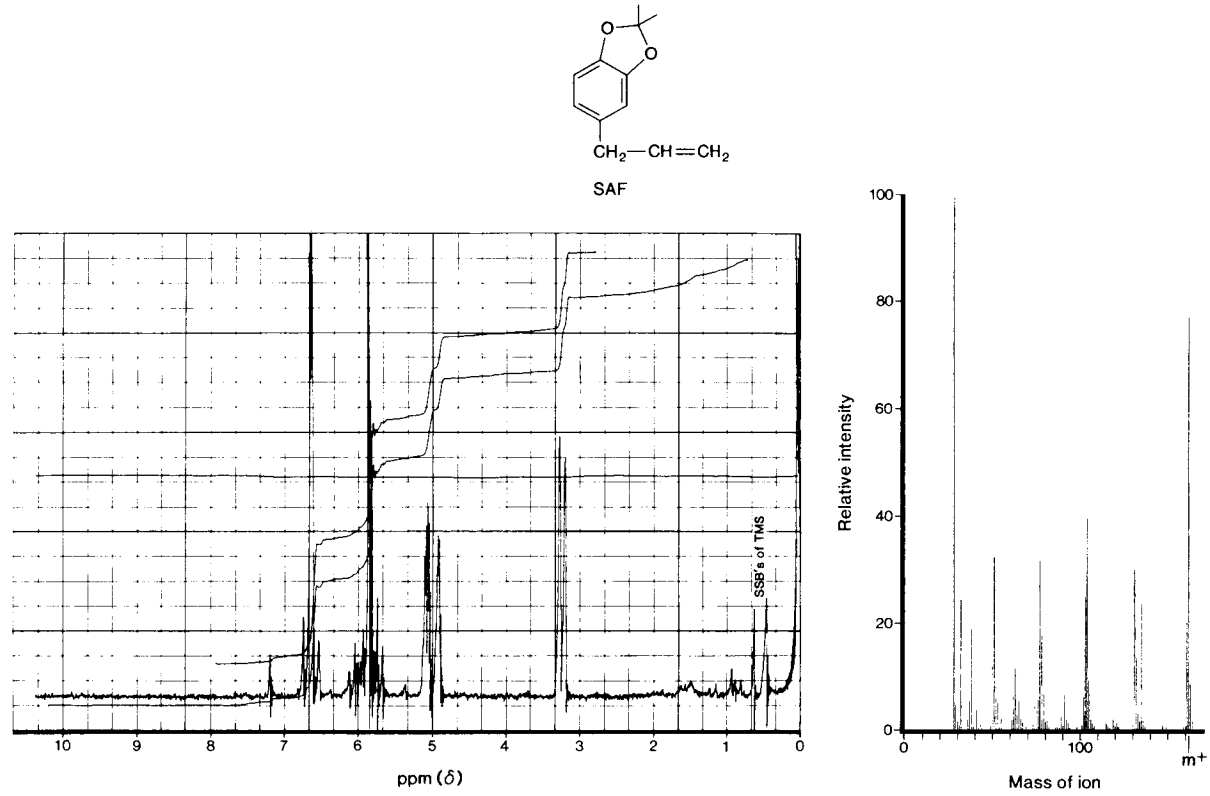


Fig. 2. Safrole.

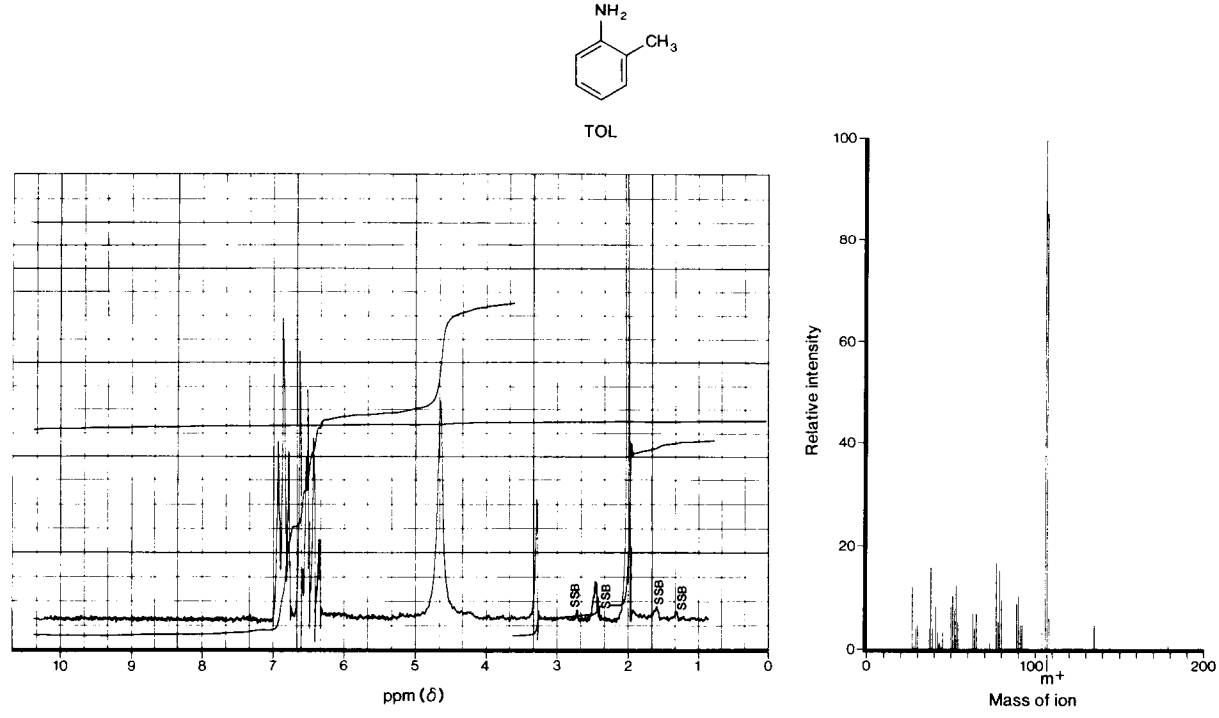


Fig. 3. *o*-Toluidine.

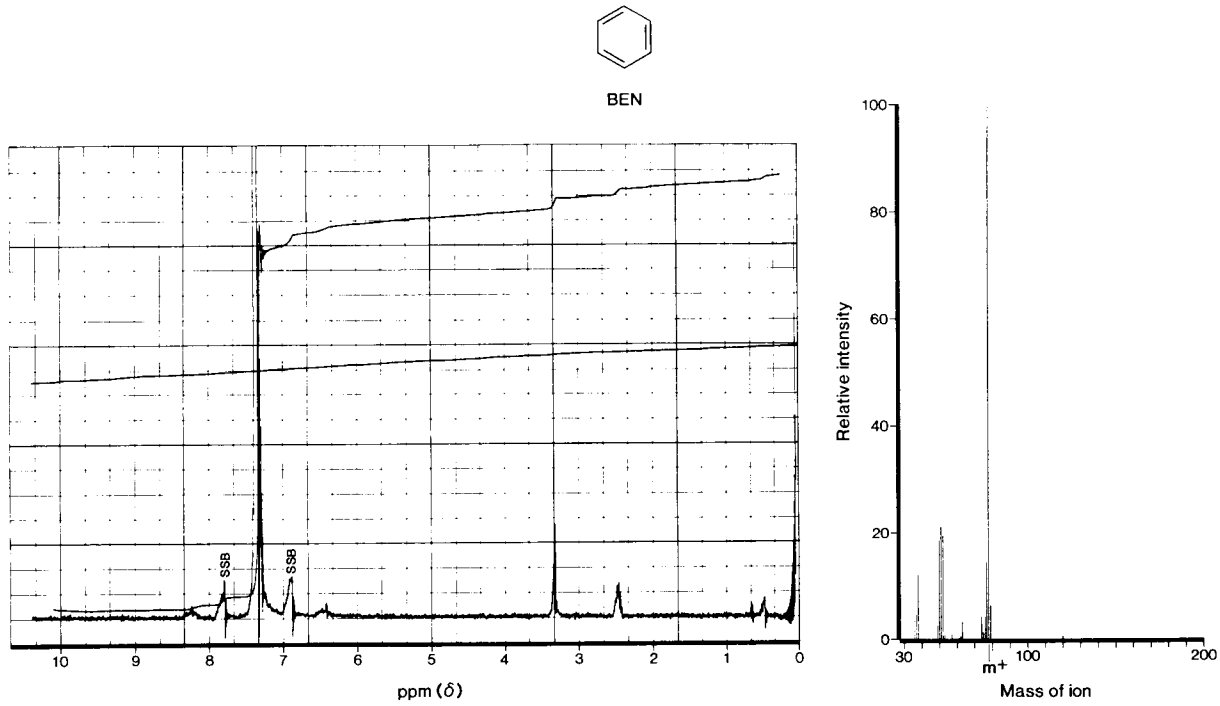


Fig. 4. Benzene.

or fragmentation patterns in the mass spectra have not been indicated, but these spectra are consistent with the structures shown.

The analysis of the chemicals was undertaken in these laboratories and their labelling and dispatch was undertaken by Lancaster Synthesis Ltd., Morecambe, U.K. Rigorous attention was paid to labelling, only one chemical being processed at any given time. In order to double check the validity of the labelling method a set was subse-

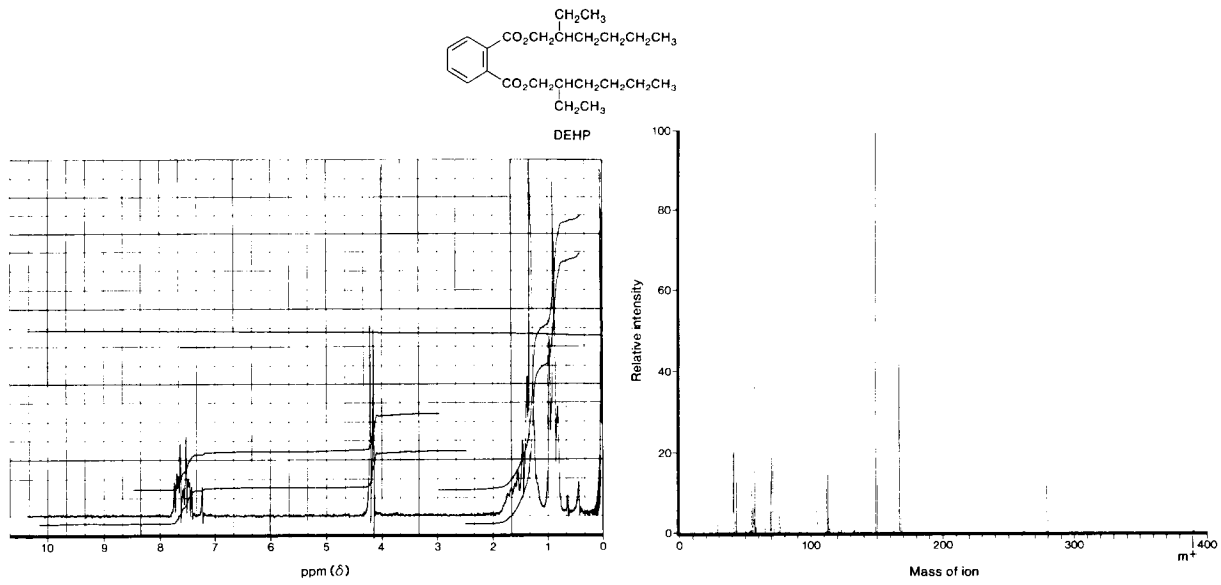


Fig. 5. Di(2-ethylhexyl)phthalate.

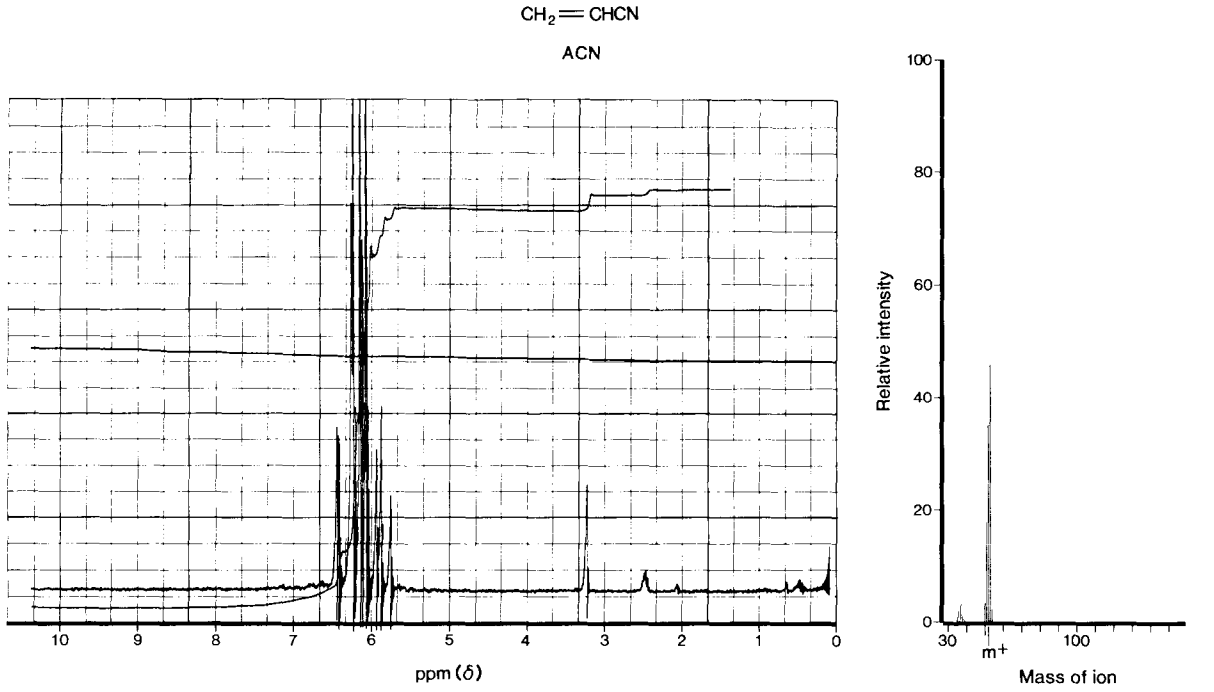


Fig. 6. Acrylonitrile.

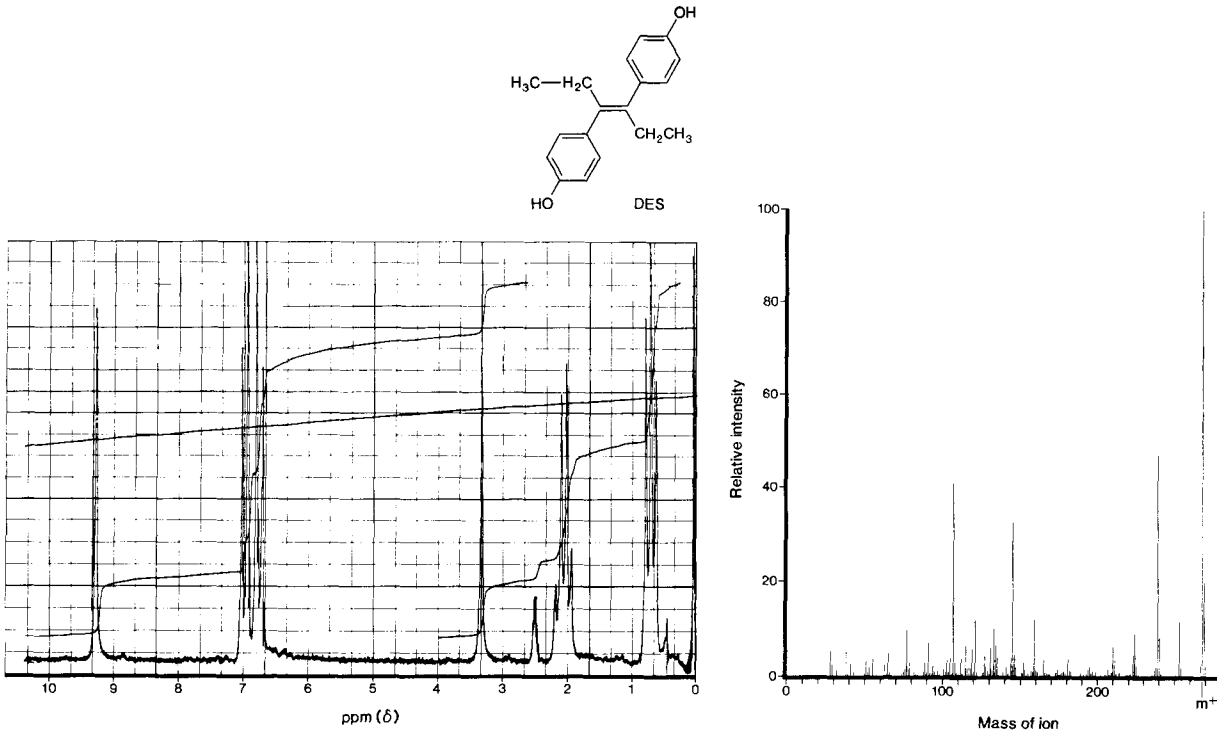


Fig. 7. Diethylstilboestrol.

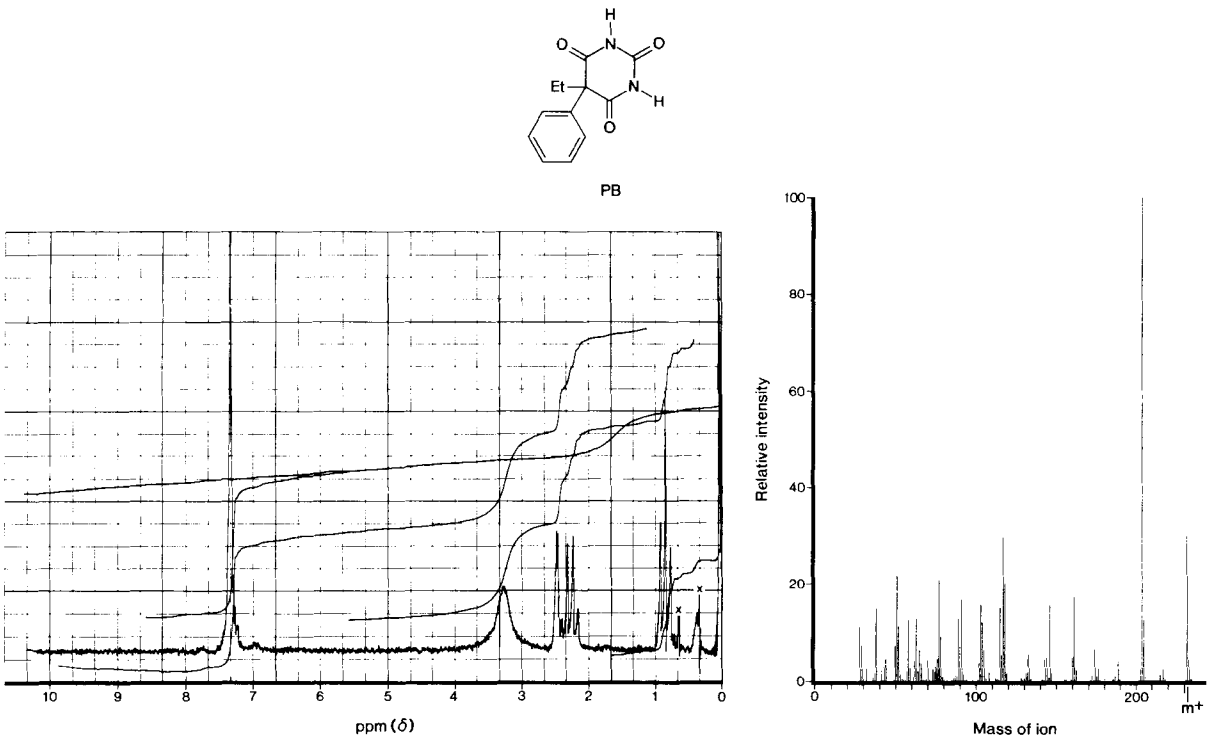
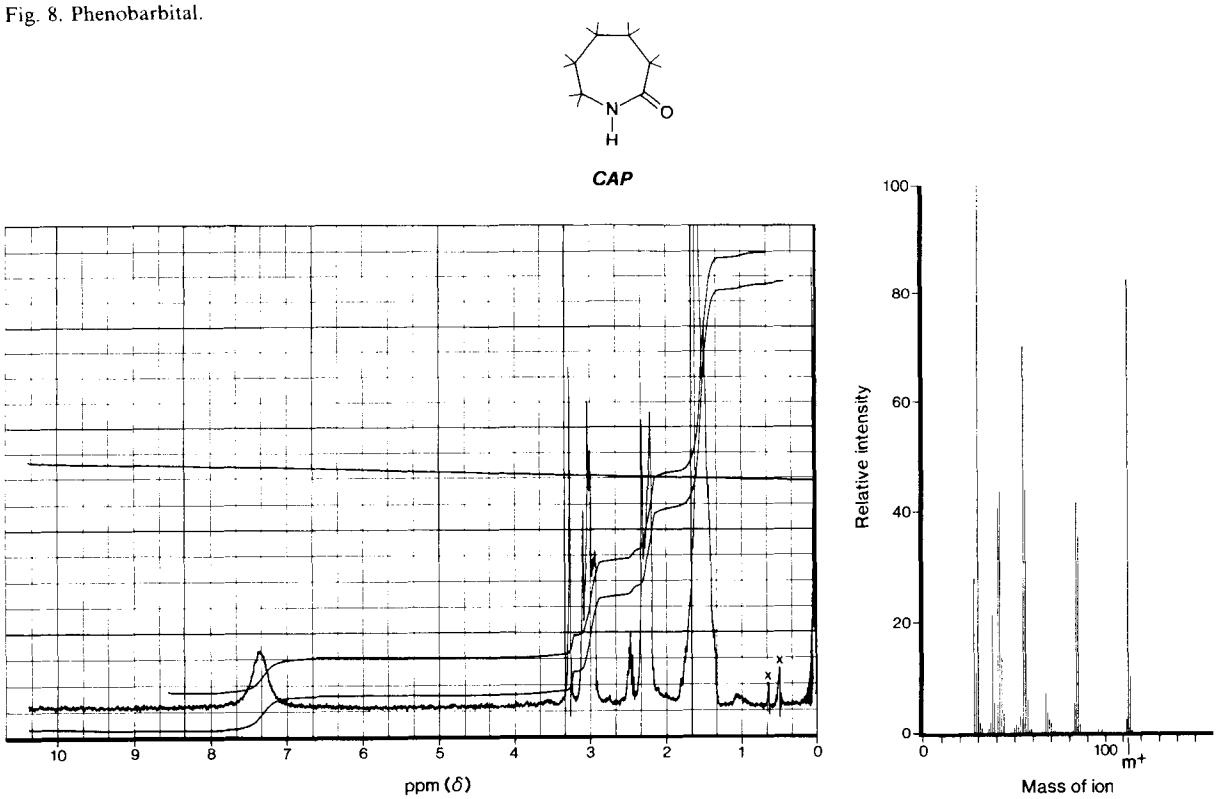


Fig. 8. Phenobarbital.



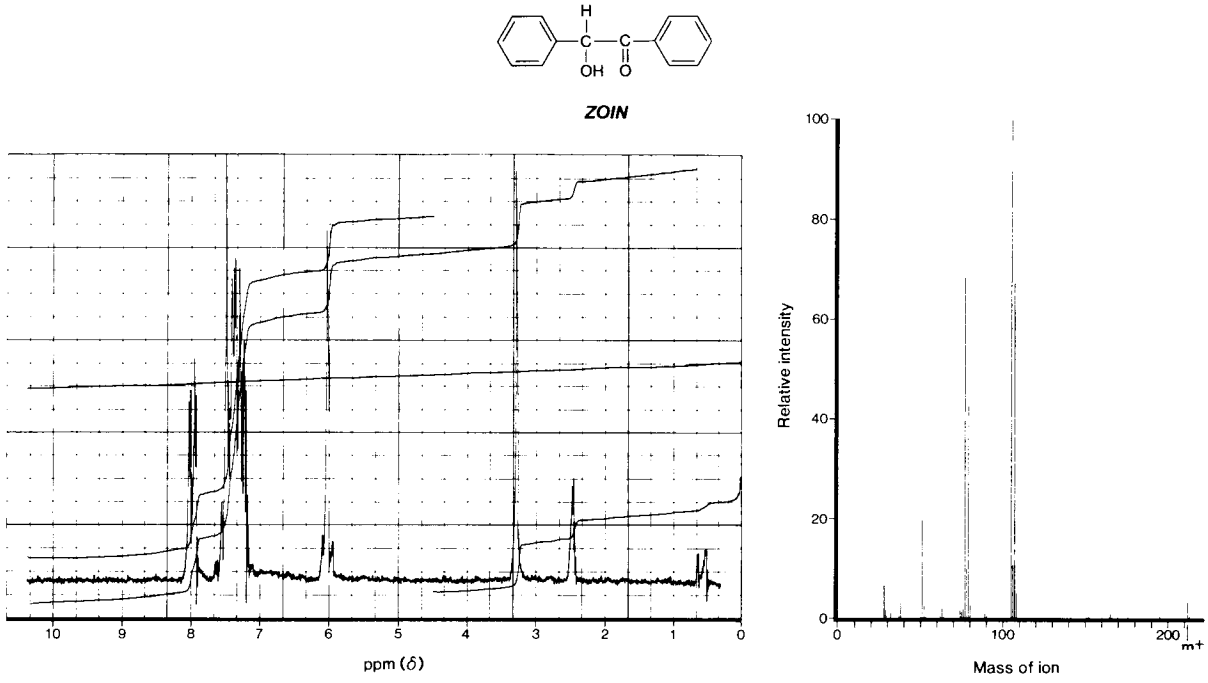


Fig. 10. Benzoin.

quently opened by Lancaster Synthesis and a further two in our chemistry laboratory. The contents were as stated on the label in each case. The chance for labelling errors to occur in this study were substantially reduced by the physical appearance of the test agents.

The nuclear magnetic spectra (^{1}H) were determined in DMSO (d_6) using a varian EM-390 spectrometer (SSB in the figures represents spinning side bands). The mass spectra were measured using an LKB 2091-051 mass spectrometer (M + in the figures represents the mass ion). The infrared (IR) spectrum of each compound was measured as a neat liquid or as a suspension in nujol using a Perkin Elmer 157 spectrometer. These spectra were each consistent with the presumed structure, but as such data does not contribute to the definition of chemical purity beyond the 95% pure point, they are not shown here. The HPLC data on safrole were recorded using a Perkin Elmer series 3B solvent delivery system, and LC-75 spectrophotometric detector under reverse phase conditions (Lichrosorb 10 μm RP-18) (see Table 1).

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Appendix 2

A tabular review of the published mutagenicity literature for IPCS study compounds

Elizabeth S. Von Halle

Environmental Mutagen Information Center, Oak Ridge National Laboratory, Oak Ridge, TN (U.S.A.)

Introduction

The published mutagenesis literature for the compounds selected for the IPCS study is summarized in this Appendix. There are 9 tables, one for each compound. No publications were found on the mutagenicity testing of benzoin. There is also a summary table on the carcinogenicity of all 10 compounds as evaluated by the International Agency for Research on Cancer (IARC) and the National Cancer Institute/National Toxicology Program (NCI/NTP).

For the mutagenicity studies on the 9 chemicals, three sections were compiled. Section 1 was reserved for Salmonella studies using plate incorporation, any of the 5 standard strains (TA98, TA100, TA1535, TA1537, TA1538), and uninduced, Aroclor- or phenobarbital-induced rat-liver S9 mix (Ames et al., 1975). Any departures from these parameters (e.g., strain TA1530, hamster- or mouse-liver S9 mix, pre-incubation or desiccator incubation) are found in Section 2.

Section 2 includes, in addition to the 'non-standard' Salmonella studies, all other in vitro studies from prokaryotes through human. Non-mammalian in vivo studies (e.g., Drosophila) are also in this section.

Section 3 is reserved for mammalian in vivo mutagenicity test results.

Four of the compounds selected for the present study, diethylstilbestrol, hexamethylphosphoramide, safrole, and *o*-toluidine, were also used in the first international collaborative study (de Serres and Ashby, 1981). Results from that exercise are not included in these tables. These 4 compounds had conflicting results, which is one of the major reasons they were selected for further testing.

It should be noted that benzoin, evaluated as a noncarcinogen, is used in consumer products. Benzoin is a flavor ingredient and is found in ice cream, ices, candy, baked goods, gelatins, puddings, and nonalcoholic beverages, and is a diluent in inks for marking fruits and vegetables (Furia, 1980; Furia and Bellanca, 1975).

Benzoin methyl ether is used as a photosensitizer in the preparation of dentures (Jpn. Patent No. 81138106 10/28/81), and benzoin preparations can be found in oral health care products as oral mucosal protectants (Hecht, 1982).

Patents have been granted for using benzoin in medicinal preparations for the treatment of acne (Bonnet, 1979) and for the treatment of hemorrhoid symptoms (Haimowitz and Arrington, 1979).

Caprolactam, also evaluated as a noncarcinogen, is considered an indirect food additive (USFDA, 1983) in that it is used in the preparation of polyamide film food packaging.

Readers who would like a complete listing of the references reporting on the evaluation of the compounds reviewed in this Appendix may obtain it by request from the Environmental Mutagen Information Center, Oak Ridge National Laboratory, P.O. Box Y, Bldg. 9224, Oak Ridge, TN 37831 (U.S.A.)

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Abbreviations

Assay

AG	8-Azaguanine resistance
arg+	Arginine+
BFB/R	Body fluid, bile/rat
BFU/H	Body fluid, urine/human
BFU/M	Body fluid, urine/mouse
BFU/R	Body fluid, urine/rat
CT	Cell transformation
chem/CT	Chemically induced cell transformation
desicc	Desiccator
DLT	Dominant lethal test
fluct	Fluctuation test
gal	Galactose
grad pl	Gradient plate
HMA	Host-mediated assay
Mtp-R	5-Methyltryptophan resistance
oua-R	Ouabain resistance
plate inc	Plate incorporation
pre-inc	Pre-incubation
SA7/CT	Simian adenovirus 7-mediated cell transformation
SCE	Sister-chromatid exchange
SHA	Sperm-head abnormality
SLRL	Sex-linked recessive lethal
susp	Suspension
TG	6-Thioguanine resistance
UDS	Unscheduled DNA synthesis
?	Not indicated

Inducer

A	Aroclor or PCB
BF	Benzoflavone
3-MC	3-Methylcholanthrene
P	Phenobarbital
P, A	Phenobarbital and Aroclor
?	Not indicated

Source of microsomes

HMA/M	Host-mediated assay/mouse
M	Mouse
R	Rat
RSV	Ram seminal vesicles

Route

inh	Inhalation
inj	Injection
ip	Intraperitoneal
iv	Intravenous
sc	Subcutaneous

Result (Author's interpretation)

+	Positive
–	Negative
(+)	Weak positive
?	Indeterminate

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
S. typhi-murium	8 TA strains	–	grad pl	A	R	No data	McMahon et al., 1979	31237
	TA98	–	plate	A	hamster	No data	Lijinsky and Andrews, 1980	41109
		–	spot	A	R	No data	Florin et al., 1980	37207
		(+)	desicc	A	R		de Meester et al., 1978	30046
		–	?	?	?	No data	Kawachi et al., 1980	40875
	TA100	–	plate	A	hamster	No data	Lijinsky and Andrews, 1980	41109
		–	spot	A	R	No data	Florin et al., 1980	37207
		(+)	desicc	A	R	Different inducers positive	de Meester et al., 1978	30046
		+	pre-inc	A	R	No data	Ishidate et al., 1981	44406
		?	?	?	?	No data	Kawachi et al., 1980	40875
	TA1530	+	fluct	A	R		de Meester et al., 1978	30046
		+	pre-inc	P	M		Duverger-van Bogaert et al., 1982	45524
		+	pre-inc	none	none		Duverger-van Bogaert et al., 1981	37867
		+	pre-inc	A	R			
		+	pre-inc	P	R			

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
S. typhi-murium (continued)	TA1530 (continued)	–	desicc	none	R		de Meester et al., 1978	30046
		–	desicc	none	none			
		+	desicc	A	R			
		–	desicc	none	none			
		+	desicc	P	R	Different inducers positive	de Meester et al., 1979	31997
		+	desicc	P	M			
		+	desicc	A	R			
		+	desicc	A	M			
		+	desicc	none	R			
		+	desicc	styrene	R		Roberfroid et al., 1978	31476
		+	BFU/R	none			Lambotte-Vandepaer et al., 1980	38184
		–	BFU/R	P				
		+	BFU/M	none				
		–	BFU/M	P				
		+	BFU/R	none			Lambotte-Vandepaer et al., 1981	37868
	TA1535	–	spot	A	R	No data	Florin et al., 1980	37207
		+	desicc	A	R		de Meester et al., 1978	30046
		–	BFB/R	none			Connor et al., 1979	34279
		+	plate	A	hamster		Lijinsky and Andrews, 1980	41109
		–	plate	none	none			
		+	desicc	A	M		Milvy and Wolff, 1977	24887
		?	pre-inc	A	M			
	TA1537	–	spot	A	R	No data	Florin et al., 1980	37207
		–	plate	A	hamster	No data	Lijinsky and Andrews, 1980	41109
	TA1538	+	desicc	A	M		Milvy and Wolff, 1977	24887
		–	plate	A	hamster	No data	Lijinsky and Andrews, 1980	41109
	TA1950	+	desicc	A	R		de Meester et al., 1978	30046
	TA1978	(+)	desicc	A	R			
		–	pre-inc	A	M		Milvy and Wolff, 1977	24887
E. coli	WP21exA	–	fluct	A	R		Venitt et al., 1977	27172
		–	plate	A	R			
	WP2uvrA	–	grad pl	A	R	No data	McMahon et al., 1979	31237
		+	plate	A	R		Venitt et al., 1977	27172
	WP2uvrA polA	+	fluct	A	R			
		(+)	plate	A	R			
	WP2 (PKM101)	+	plate	A	R			
	WP2	+	fluct	A	R			
		+	plate	A	R			
		–	grad pl	A	R	No data	McMahon et al., 1979	31237

ACRYLONITRILE (107-13-1) (continued) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
B. subtilis		+	rec assay	none	none	No data	Kawachi et al., 1980	40875
B. mori		–	egg color			No data	Kawachi et al., 1980	40875
D. melano-gaster		–	SLRL				Benes and Sram, 1969	6618
C. griseus	Don-6 cells	+	SCE	none	none		Sasaki et al., 1980	43143
	CHO	+	SCE	co-cultured	rat hepatocytes		Ved Brat and Williams, 1982	48814
		–	SCE	none	none			
	lung cells	+	chromosome aberrations	none	none	No data	Kawachi et al., 1980	40875
	CHL cells	+	chromosome aberrations	A	R		Ishidate et al., 1981	44406
M. auratus	embryo cells	+	SA7/CT				Parent and Casto, 1979	31458
		+	DNA breaks					
		(+)	chem/CT					
Calf thymus	DNA	+	DNA binding	P	R		Guengerich et al., 1981	43166
		+	DNA binding	BF	R			

ACRYLONITRILE (107-13-1) *SECTION 3*

Test object	Cells observed	Result	Protocol or assay	Sex	Route	Comment	Reference	EMIC No.
Rat	Bone marrow	+	chromosome aberrations	?	?	No data	Kawachi et al., 1980	40875
Mouse	Bone marrow	–	chromosome aberrations	M	gavage		Rabello-Gay and Ahmed, 1980	37509
		–	chromosome aberrations	M	ip			
		–	chromosome aberrations	M	ip		Leonard et al., 1981	37869
		–	micronuclei	M	ip			
	Germ cells	–	DLT	M	ip			
Human	Lymphocytes	–	chromosome aberrations	M?	inh		Thiess and Fleig, 1978	31169

BENZENE (71-43-2) *SECTION 1*

TA strain	Result	Inducer	Source of microsomes	Comment	Reference	EMIC No.
98	–	A	R	No data	Ho et al., 1981	41662
	–	A	R	No data	Hermann, 1981	45038

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of microsomes	Comment	Reference	EMIC No.
S. typhimurium	TA98	–	pre-inc	A	R		Shimizu et al., 1983	48947
		–	plate	none	M		Shahin and Fournier, 1978	30004
		–	desicc	A	R		Bartsch et al., 1980	37078
		–	spot	A	R	No data	Florin et al., 1980	37209
		–	spot	none	none			
	TA100	–	pre-inc	A	R		Shimizu et al., 1983	48947
		–	plate	none	M		Shahin and Fournier, 1978	30004
		–	plate	none	none			
		–	desicc	A	R		Bartsch et al., 1980	37078
		–	spot	A	R	No data	Florin et al., 1980	37209
		–	spot	none	none			
	TA1530	–	desicc	A	R		Bartsch et al., 1980	37078
	TA1535	–	pre-inc	A	R		Shimizu et al., 1983	48947
		–	plate	none	M		Shahin and Fournier, 1978	30004
		–	desicc	A	R		Bartsch et al., 1980	37078
		–	spot	A	R	No data	Florin et al., 1980	37209
		–	spot	none	none			
	TA1537	–	pre-inc	P, A	R		Seixas et al., 1982	48129
		–	pre-inc	A	R		Shimizu et al., 1983	48947
		–	plate	none	M		Shahin and Fournier, 1978	30004
		–	spot	A	R	No data	Florin et al., 1980	37209
		–	spot	none	none			
	TA1538	–	pre-inc	A	R		Shimizu et al., 1983	48947
		–	plate	none	M		Shahin and Fournier, 1978	30004
		–	desicc	A	R		Bartsch et al., 1980	37078
	TM677	–	AG	P, A	R	No data	Seixas et al., 1982	48129
		–	AG	P, A	R		Kaden et al., 1979	31969
	hisG46	–	desicc	A	R		Bartsch et al., 1980	37078
	8 TA strains	–	grad pl	A	R		Probst et al., 1981	37980
E. coli	lambda	–	phage induction	A	R		Ho and Ho, 1981	43495
	WP2	–	grad pl	A	R		Probst et al., 1981	37980
	WP2/WP100	?	susp	none	none		McCarroll et al., 1981a	41597
	WP2uvrA	–	grad pl	A	R		Probst et al., 1981	37980
B. subtilis	H17/M45	–	rec assay	A	R	No data	McCarroll et al., 1981b	41826
	TKJ5211	–	his rev	A	R		Tanooka, 1977	24162
	HJ15	–	differential killing	A	R			
S. cerevisiae		–	petite	none	none		Egilsson et al., 1979	33568
Grasshopper	embryos	+	mitotic effects				Liang et al., 1983	50962
D. melanogaster	germ cells	–	SLRL				Kale and Baum, 1983	50725
		–	translocations					

BENZENE (71-43-2) (continued) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of microsomes	Comment	Reference	EMIC No.
D. melanogaster (continued)	spermatogonia	+	recombination				Kale and Baum, 1983	50725
	eye anlage	–	zeste mosaic				Nylander et al., 1978	27482
C. griseus	V79 cells	–	DNA breaks	Ames	75 R		Swenberg et al., 1976	23583
M. auratus	SHE cells	+	CT	feeder cells			Amacher and Zelljadt, 1983	49916
Rat	hepatocytes	–	UDS				Probst et al., 1981	37980
		–	DNA breaks				Sina et al., 1983	50841
Human	lymphocytes	+	chromosome aberrations	none	none		Morimoto, 1976	29359
		–	chromosome aberrations	none	none		Gerner-Smidt and Friedrich, 1978	31107
		+	chromosome aberrations	none	none		Mnatsakonov and Pogosyan, 1973	10254
	leukocytes	+	chromosome aberrations	none	none		Koizumi et al., 1974	19405
	lymphocytes	+	chromosome aberrations	none	none		Morimoto, 1975	21381
		–	SCE	none	none		Gerner-Smidt and Friedrich, 1978	31107
		+	SCE	Litton	S9R		Morimoto, 1983	49576
		–	SCE	none	none		Morimoto and Wolff, 1980	37345
		+	SCE	Litton	S9R		Morimoto et al., 1983	48971
		–	DNA repair	P	R		Perocco et al., 1983	48986
		+	DNA synthesis	none	none		Dobashi, 1974	20657
	HeLa cells	+	DNA synthesis	none	none		Koizumi et al., 1974	19405
		+	DNA synthesis	none	none		Dobashi, 1974	20657
		–	DNA synthesis	A	R		Painter and Howard, 1982	45209

BENZENE (71-43-2) *SECTION 3*

Test object	Cells observed	Result	Protocol or assay	Sex	Route	Reference	EMIC No.
Rabbit	bone marrow	+	chromosome aberrations	M, F	sc	Kissling and Speck, 1971	12912
		+	chromosome aberrations	?	sc	Kissling and Speck, 1972	27571

Test object	Cells observed	Result	Protocol or assay	Sex	Route	Reference	EMIC No.
Rat	bone marrow	+	chromosome aberrations	M, F	sc	Philip and Krogh Jensen, 1970	7871
		+	chromosome aberrations	?	sc	Lyapkalo, 1973	18215
		+	chromosome aberrations	M	inh	Dobrokhotov and Enikeev, 1977	25832
		+	chromosome aberrations	M	inh	Anderson and Richardson, 1981	41697
		+	chromosome aberrations	M, F	sc	Dean, 1969	6584
	lymphocytes	+	chromosome aberrations	M, F	sc		
	bone marrow	–	DNA synthesis	M	sc	Irons et al., 1979	35812
	adrenal cortex	–	mitotic index	?	?	Amlacher and Ziebarth, 1979	34316
	liver	+	DNA binding	M	inh	Lutz and Schlatter, 1977	25232
Mouse	bone marrow	+	micronuclei	M, F	gavage	Hite et al., 1980	34357
		+	micronuclei	M	sc	Diaz et al., 1980	34503
		+	micronuclei	M, F	gavage	Meyne and Legator, 1980	34811
		–	micronuclei	F	ip		
		+	micronuclei	M	ip		
		+	micronuclei	M, F	gavage	Siou et al., 1981	41698
		+	micronuclei	M	sc	Tunek et al., 1982	45310
		+	micronuclei	M	inh	Toft et al., 1982	50123
		+	micronuclei	M	gavage	Siou et al., 1980	37627
		+	micronuclei	M, F	gavage	Siou et al., 1979	36485
		+	micronuclei	M	gavage	Siou et al., 1977	36486
		–	chromosome aberrations	M, F	inh	Tice et al., 1980	39661
		+	chromosome aberrations	M, F	gavage	Siou et al., 1981	41698
		+	chromosome aberrations	M	gavage, ip	Meyne and Legator, 1980	34811
		+	chromosome aberrations	F	gavage, ip		
		+	SCE	M, F	inh	Tice et al., 1980	39661
	sperm	+	SHA	M	ip	Topham, 1980	37395
	kidney	+	DNA synthesis	?	?	Amlacher and Rudolph, 1972	32461
C. griseus	bone marrow	–	chromosome aberrations	M, F	gavage	Siou et al., 1981	41698
		–	micronuclei	M, F	gavage		

CAPROLACTAM (105-60-2) *SECTION 1*

TA strain	Result	Inducer	Source of microsomes	Comment	Reference	EMIC No.
98	–	none	none		Greene et al., 1979	34467
100	–	none	none			
1535	–	none	none			
1537	–	none	none			
1538	–	none	none			
98	–	A	R			
100	–	A	R			
1535	–	A	R			
1537	–	A	R			
1538	–	A	R			

DIETHYLHEXYL PHTHALATE (DEHP) (117-81-7) *SECTION 1*

TA strain	Result	Inducer	Source of microsomes	Comment	Reference	EMIC No.
98	–	?	?	No data	Kawachi et al., 1980	40875
100	–	?	?			
98	–	none	none		Robertson et al., 1983	48687
100	–	none	none			
98	–	A	R		Kirby et al., 1983	50951
	–	none	none			
100	–	A	R			
	–	none	none			
1535	–	A	R			
	–	none	none			
1537	–	A	R			
	–	none	none			
1538	–	A	R			
	–	none	none			

DIETHYLHEXYL PHTHALATE (DEHP) (117-81-7) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
S. typhi-murium	TA98	–	pre-inc	none	RSV		Robertson et al., 1983	48687
		–	pre-inc	A	R			
		–	pre-inc	A	R		Yoshikawa et al., 1983	50114
		–	pre-inc	none	none			
	TA100	–	pre-inc	none	RSV		Robertson et al., 1983	48687
		–	pre-inc	A	R			
		–	pre-inc	A	R		Yoshikawa et al., 1983	50114
		–	pre-inc	none	none			
E. coli	WP2uvrA	–	pre-inc	A	R		Yoshikawa et al., 1983	50114
		–	pre-inc	none	none			

DIETHYLHEXYL PHTHALATE (DEHP) (117-81-7) (continued) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
B. subtilis	H17/M45	–	rec assay	none	none	No data	Kawachi et al., 1980	40875
B. mori	germ cells	–	egg color	none	none			
C. griseus	CHL cells	–	SCE	none	none		Kawachi et al., 1980	40875
		–	chromatid gaps	none	none		Ishidate and Odashima, 1977	24895
	Don cells	–	chromosome aberrations	none	none		Abe and Sasaki, 1977	25199
		+	SCE	none	none	No dose response		
	CHO cells	–	chromosome aberrations	none	none		Phillips et al., 1982	48138
Mouse	L5178Y cells	–	TK test	A	R		Kirby et al., 1983	50951
		–	TK test	none	none			
Human	lymphocytes	–	chromosome aberrations	none	none		Stenchever et al., 1976	24479
		–	chromosome aberrations	none	none		Tsuchiya and Hattori, 1976	9992
		–	chromosome aberrations	none	none		Turner et al., 1974	20167
		+	mitotic index	none	none			

DIETHYLHEXYL PHTHALATE (DEHP) (117-81-7) *SECTION 3*

Test object	Cells observed	Result	Protocol or assay	Sex	Route	Comment	Reference	EMIC No.
Rat	bone marrow	–	chromosome aberrations	none	none	No data	Kawachi et al., 1980	40875
	bone marrow	–	chromosome aberrations	M	gavage		Putman et al., 1983	50726
Mouse	post-meiotic sperm	+	DLT	M	ip		Singh et al., 1974	17788
	pre-meiotic sperm	–	DLT	M	ip			
	sperm	–	DLT	M	ip		Hamano et al., 1979	39576

DIETHYLSTILBESTROL (DES) (56-53-1) *SECTION 1*

TA strain	Result	Inducer	Source of microsomes	Comment	Reference	EMIC No.
98	–	A	R	No data	Dunkel, 1979	31963
	–	A	R		McCann et al., 1975	21338
	–	A	R	No data	Anderson and Styles, 1978	29183
	–	A	R	Also negative with other activation systems	Glatt et al., 1979	31660

DIETHYLSTILBESTROL (DES) (56-53-1) (continued) *SECTION 1*

TA strain	Result	Inducer	Source of microsomes	Comment	Reference	EMIC No.
98	–	A	R	No data	Lang and Redmann, 1979	31781
	–	A	R		Ruediger et al., 1979	35072
	–	Ames 75	R		Ingerowski et al., 1981	37853
	–	?	?	No data	Kawachi et al., 1980	40875
	–	A	R		Affolter et al., 1983	48685
100	–	A	R		McCann et al., 1975	21338
	–	A	R	No data	Anderson and Styles, 1978	29183
	–	A	R		Glatt et al., 1979	31660
	–	A	R	No data	Lang and Redmann, 1979	31781
	–	A	R	No data	Dunkel, 1979	31963
	–	A	R		Ruediger et al., 1979	35072
	–	Ames 75	R		Ingerowski et al., 1981	37853
	–	?	?	No data	Kawachi et al., 1980	40875
	–	A	R		Affolter et al., 1983	48685
1535	–	A	R		McCann et al., 1975	21338
	–	A	R	No data	Anderson and Styles, 1978	29183
	–	A	R	No data	Lang and Redmann, 1979	31781
	–	A	R		Dunkel, 1979	31963
	–	A	R		Ruediger et al., 1979	35072
	–	Ames 75	R		Ingerowski et al., 1981	37853
1537	–	A	R		Glatt et al., 1979	31660
	–	A	R		McCann et al., 1975	21338
	–	A	R	No data	Lang and Redmann, 1979	31781
	–	A	R	No data	Dunkel, 1979	31963
	–	A	R		Ruediger et al., 1979	35072
	–	Ames 75	R		Ingerowski et al., 1981	37853
1538	–	A	R	No data	Anderson and Styles, 1978	29183
	–	A	R		Glatt et al., 1979	31660
	–	A	R	No data	Lang and Redmann, 1979	31781
	–	A	R	No data	Dunkel, 1979	31963
	–	Ames 75	R		Ingerowski et al., 1981	37853

DIETHYLSTILBESTROL (DES) (56-53-1) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
S. typhi-murium	TA98	–	pre-inc	Ames 75		No data	Ishidate et al., 1981	44405
		–	plate inc	A or P	M	No data	Bartsch et al., 1980	37078
		–	plate inc	A or P				
	TA100	–	plate inc	A or P	M	No data	Bartsch et al., 1980	37078
		–	plate inc	A or P	hamster			
		–	pre-inc	Ames 75		No data	Ishidate et al., 1981	44405

DIETHYLSTILBESTROL (DES) (56-53-1) (continued) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
S. typhimurium (continued)	8 TA strains	–	grad pl	A	R	No data	Cline and McMahon, 1977	24620
	8 TA strains	–	grad pl	A	R	No data	Probst et al., 1981	37981
	TA1535	–	BFU/H	P	R	No data	Minnich et al., 1976	23572
	TA1537	–	BFU/H	P	R	No data	Minnich et al., 1976	23572
		–	pre-inc	Ames 75		No data	Ishidate et al., 1981	44405
	TA1538	–	BFU/H	P	R		Minnich et al., 1976	23572
		–	plate inc	3-MC	R or M	No data	Allaben et al., 1979	35089
		–	plate inc	none	none			
		–	plate inc	3-MC	R	No data		
		–	plate inc	none	R or M			
		–	BFB/R	none	none	No data	Connor et al., 1979	34279
		–	BFU/R	none	none			
	5 TA strains	–	plate inc	A	M	No data	Dunkel, 1979	31963
		–	plate inc	none	M			
		–	plate inc	A	hamster			
		–	plate inc	none	hamster			
	4 TA strains	–	plate inc	A	hamster	No data	Glatt et al., 1979	31660
		–	plate inc	none	hamster			
	5 TA strains	–	plate inc	none	M	No data	Lang and Redmann, 1979	31781
E. coli	WP2/WP100	–	rec assay	A	R		Mamber et al., 1983	48782
	WP2uvrA	–	grad pl	A	R	No data	Cline and McMahon, 1977	24620
		–	grad pl	A	R	No data	Probst et al., 1981	37981
	P3110/P3478	–	pol test	none	none		Fluck et al., 1976	24127
B. subtilis	H17/M45	+	rec assay	?	?	No data	Kawachi et al., 1980	40875
	rec⁻	–	rec assay	A	R	No data	Suter and Jaeger, 1982	45031
S. cerevisiae	D6	+	aneuploidy	none	none		Parry et al., 1981	42704
		–	mitotic recombi-nation	none	none			
	XV185-14C	(+)	reversion	none	none		Mehta and von Borstel, 1982	45177
B. mori	germ cells	–	egg color	none	none	No data	Kawachi et al., 1980	40875
C. griseus	CHL cells	–	SCE	none	none		Kawachi et al., 1980	40875
		+	polyploidy	A	R		Ishidate et al., 1981	44405
	V79 cells	–	SCE	none	none		Kinsella, 1982	45695
	Don-6 cells	–	SCE	none	none		Abe and Sasaki, 1982	46525
	Don cells	–	SCE	none	none		Abe and Sasaki, 1977	25199
	CHL cells	+	chromosome aberrations	none	none		Ishidate and Odashima, 1977	24896
	Don cells	–	chromosome aberrations	none	none		Abe and Sasaki, 1977	25199
	thymus cells	+	ploidy	none	none		Sawada and Ishidate, 1978	27484

DIETHYLSTILBESTROL (DES) (56-53-1) (continued) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
C. griseus (continued)	V79 cells	–	TG-R	none	none		Barrett et al., 1983	48772
		–	TG-R	none	SHE feeder cells			
		–	oua-R	none	rat liver feeder cells		Drevon et al., 1981	41074
		–	AG-R	none	rat liver feeder cells			
		–	oua-R	A	rat liver feeder cells			
		–	AG-R	A	rat liver feeder cells			
M. auratus	SHE cells	–	TG	none	none		Barrett et al., 1981	39297
		–	oua-R	none	none			
		+	CT	none	none			
Rat	hepatocytes	–	UDS	none	none		Althaus et al., 1982	48510
		–	UDS	none	none		Williams et al., 1982	45999
		+	DNA breaks	none	none		Sina et al., 1983	50841
	tracheal cells	–	UDS	none	none		Ide et al., 1981	44329
	hepatocytes	–	UDS	A	R		Probst et al., 1981	37981
	AH66-B cells	+	SCE	none	none		Abe and Sasaki, 1982	45265
	R1 cells	+	SCE	none	none			
	liver cells	+	ploidy	none	none		Sawada and Ishidate, 1978	27484
	leukemia cells	+	ploidy	none	none			
Mouse	host-mediated ascites cells	+	chromosome aberrations				Bishun et al., 1980	37641
	S49.1 lymphoma cells	+	dexa-methasone resistance	?	R		Friedrich and Nass, 1983	50794
	L5178Y cells	+	TK test	A	R		Clive et al., 1979	31144
	C3H/10T1/2 cells	–	CT	none	none		Lillehaug and Djurhuus, 1982	46797
Human	lymphocytes	+	chromosome aberrations	none	none		Dzhangulashvilli, 1982	48373
	lymphocytes (pregnant)	+	SCE	none	none		Hill and Wolff, 1982, 1983	45225 50904
	lymphocytes (pre-menopausal)	+	SCE	none	none		Hill and Wolff, 1982	45225
	lymphocytes (post-menopausal)	–	SCE	none	none			
	lymphocytes (male)	–	SCE	none	none		Hill and Wolff, 1982, 1983	45225 50904
	lymphocytes	+	DNA repair inhibition	none	none		Gaudin et al., 1971	11780
	fibroblasts	+	abnormal mitosis	none	none		Parry et al., 1982a	48166
		+	abnormal mitosis	none	none		Danford and Parry, 1982	45115
		+	abnormal mitosis	none	none		Parry et al., 1982b	45530

DIETHYLSTILBESTROL (DES) (56-53-1) (continued) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
Human (continued)	skin fibroblasts	+	SCE	A	R		Ruediger et al., 1979	35072
	HeLa S3 cells	+	ploidy	none	none		Sawada and Ishidate, 1978	27484
		+	UDS	P	R		Martin et al., 1978	29113

DIETHYLSTILBESTROL (DES) (56-53-1) *SECTION 3*

Test object	Cells observed	Result	Protocol or assay	Sex	Route	Comment	Reference	EMIC No.
M. auratus	liver, kidney	(+)	DNA binding	M	sc		Lutz et al., 1982	48354
Rat	liver	(+)	DNA binding	F	oral		Lutz et al., 1982	48354
	bone marrow	−	chromosome aberrations	?	?		Kawachi et al., 1980	40875
Mouse	bone marrow	−	micronuclei	M, F	ip		Chrisman and Baumgartner, 1979	31664
		+	chromosome aberrations	F	iv		Ivett and Tice, 1981	41598
		−	SCE	F	iv			
	testis	+	DNA synthesis	M	implant	Positive in BALB/c mice; negative in (C3H×A)F1 mice	Uchikawa et al., 1970	9273
	sperm	+	SHA	M	ip		Topham, 1980	34345

HEXAMETHYLPHOSPHORAMIDE (HMPA) (680-31-9) *SECTION 1*

TA strain	Result	Inducer	Source of microsomes	Comment	Reference	EMIC No.
98	−	A	R	No data	Ashby et al., 1977	28451
100	+/−	A	R			
1535	+/−	A	R			
1538	−	A	R			
98	−	A	R	No data	Anderson and Styles, 1978	29184
100	+	A	R			
1535	+	A	R			
1538	−	A	R	No data		

HEXAMETHYLPHOSPHORAMIDE (HMPA) (680-31-9) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
Lambda DNA		+	breaks	none	none		Kappen and Goldberg, 1979	38439
E. coli	K39(lambda)	−	phage induction	none	none		Zetterberg, 1971	11209

HEXAMETHYLPHOSPHORAMIDE (HMPA) (680-31-9) (continued) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
V. faba	root tips	–	chromosome aberrations	none	none		Sturelid, 1971	11210
D. melano-gaster		+	trans-locations			No data	Vogel et al., 1982	45179
		+	trans-locations				Sram, 1972	12349
		+	SLRL				Benes and Sram, 1969	6618
		+	SLRL				Sram, 1972	12349
C. griseus	lung cells	–	chromosome aberrations	none	none		Sturelid, 1971	11210
M. auratus	BHK-21-C13 cells	+	CT	A	R		Ashby et al., 1977	28451
Human	leukocytes	–	chromosome aberrations	none	none		Chang and Klassen, 1968	6084

HEXAMETHYLPHOSPHORAMIDE (HMPA) (680-31-9) *SECTION 3*

Test object	Cells observed	Result	Protocol or assay	Sex	Route	Reference	EMIC No.
Rabbit	germ cells	+	sperm count	M	oral	Jackson and Craig, 1966	8193
Rat	bone marrow	–	micronuclei	M	ip	Trzos et al., 1978	30009
	germ cells	+	spermato-genesis	M	oral	Kimbrough and Gaines, 1966	36396
		–	oogenesis	F	oral		
		+	spermato-genesis	M	oral	Jackson et al., 1969	6741
Mouse	bone marrow	–	chromosome aberrations	?	ip	Mana and Das, 1973	15932
	sperm	–	DLT	M	ip	Scheufler, 1980	39453
	spermatozoa	+	SHA	M	ip	Topham, 1980	37395
	germ cells	+	spermato-genesis	M	ip	Scheufler, 1980	39453
		+	DLT	M	ip	Sram et al., 1970	7907
		–	DLT	M	ip	Epstein et al., 1972	12985
	bone marrow	+	micronuclei	M	ip	Styles et al., 1983	52071
	erythrocytes (C57Bl/6J)	+	micronuclei	M	ip		
	erythrocytes (hybrid strains)	–	micronuclei	M	ip		

PHENOBARBITAL (50-06-6) AND SODIUM PHENOBARBITAL (57-30-7) *SECTION 1*

TA strain	Result	Inducer	Source of microsomes	Comment	Reference	EMIC No.
98	–	?	R	No data	Rao et al., 1977	26470
100	–	?	R			
1535	–	?	R			
1537	–	?	R			
1538	–	?	R			
98	–	A	R		Anderson and Styles, 1978	29184
100	–	A	R			
1535	–	A	R			
1538	–	A	R			
98	–	A	R		McCann et al., 1975	21339
100	–	A	R			
1535	–	A	R			
1537	–	A	R			

PHENOBARBITAL (50-06-6) OR SODIUM PHENOBARBITAL (57-30-7) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
S. typhi-murium	hisG46	–	HMA	P	none		Propping and Buselmaier, 1971	12961
	TA98	–	BFU/R	A	R		Reddy et al., 1980	37566
	TA100	–	pre-inc	Ames 75		No data	Ishidate et al., 1981	44405
	TA1530	–	BFU/R	P	R		Lambotte-Vandepaer et al., 1980	38184
		–	BFU/M	P	R			
	TA1535	–	BFU/H	?	R	No data	Minnich et al., 1976	23572
		–	BFU/H	none	none			
	TA1536	–	BFU/H	?	R			
		–	BFU/H	none	none			
	TA1537	–	BFU/H	?	R			
		–	BFU/H	none	none			
	TA1538	–	BFU/H	?	R			
		–	BFU/H	none	none			
		–	pre-inc	Ames 75		No data	Ishidate et al., 1981	44405
E. coli	343/113	–	Mtp-R	A	R	No data	King et al., 1979	30876
		–	gal	A	R			
		–	arg +	A	R			
	CM611/WP2	+	differen-tial killing	none	none	6 other strains negative	McCarroll et al., 1981a	41597
D. melano-gaster	germ cells	+	SLRL			C1B method	Filippova et al., 1975	20795
	sperm	–	SLRL	P			Donner et al., 1979	31783
	Berlin-K sperm	–	SLRL			No data	King et al., 1979	30876

PHENOBARBITAL (50-06-6) OR SODIUM PHENOBARBITAL (57-30-7) (continued) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
C. griseus	lung fibroblasts	+	chromosome aberrations	none	none		Ishidate et al., 1978	34497
	CHL cells	+	polyploidy	A	R	No data	Ishidate et al., 1981	44405
		–	chromosome aberrations		R			
	V79 cells	–	DNA breaks	?	R		Swenberg et al., 1976	23583
Rat	tracheal cells	–	UDS	none	none		Ide et al., 1981	44329
	hepatocytes	–	DNA breaks	none	none		Sina et al., 1983	50841
	hepatocyte nuclei	–	UDS	none	none		Althaus et al., 1982	45810
Mouse	L5178Y cells	–	TK test	none	none		Amacher et al., 1980	37387
	S49.1 lymphoma cells	+	dexa-methasone resistance	none	none		Friedrich and Nass, 1983	50794
Human	fetal fibroblasts	–	chromosome aberrations	none	none		Stenchever and Jarvis, 1970	18157
	lymphocytes	+	chromosome aberrations				Nandan and Rao, 1982b	48677
	lymphocytes	–	chromosome aberrations	none	none		Foerst, 1972	9591
	lymphocytes	+	C-mitosis	none	none		Caratzali and Roman, 1969	5854

PHENOBARBITAL (50-06-6) OR SODIUM PHENOBARBITAL (57-30-7) *SECTION 3*

Test object	Cells observed	Result	Protocol assay	Sex	Route	Reference	EMIC No.
M. auratus	embryo	–	chromosome aberrations		transplacental	Nishi et al., 1978	28696
Rat	liver	–	DNA breaks	F	oral	Stout and Becker, 1982	45813
Mouse	bone marrow	–	micronuclei	M, F	ip	King et al., 1979	30876
		–	chromosome aberrations	?	oral	Subramanyam and Murthy, 1978	33020
		–	chromosome aberrations	M, F	inj	Tice et al., 1980	38661
		–	SCE	M, F	inj		
	liver nuclei	–	DNA breaks	M, F	oral	Schwarz et al., 1979	31479
	spermatocytes	+	chromosome aberrations	M	gavage	Nandan and Rao, 1982a	48193
	spermatozoa	–	SHA	M	ip	Topham, 1980	37395
	germ cells	+	DLT	M	gavage	Nandan and Rao, 1983	50777

SAFROLE (94-59-7) *SECTION 1*

TA strain	Result	Inducer	Source of microsomes	Comment	Reference	EMIC No.
98	–	A	R		McCann et al., 1975	21338
	–	none	none	No data	Dorange et al., 1977	27999
	+	A	R		Anderson and Styles, 1979	29184
	–	A	R	No data	Simmon, 1979a	31451
	–	none	none			

SAFROLE (94-59-7) (continued)

TA strain	Result	Inducer	Source of microsomes	Comment	Reference	EMIC No.
98	–	A	R	No data	Gocke et al., 1981	41641
	–	none	none		Sekizawa and Shibamoto, 1982	45340
	–	A	R		To et al., 1982	48289
	–	none	none			
100	–	A	R		McCann et al., 1975	21338
	–	A	R		Wislocki et al., 1977	24870
	–	none	none	No data	Dorange et al., 1977	27999
	+	A	R	No data	Anderson and Styles, 1979	29184
	–	A	R	No data	Simmon, 1979a	31451
	–	none	none			
	–	A	R(S13)		Swanson et al., 1979	31466
	–	none	none			
	–	A	R		Cheh et al., 1980	41583
	–	A	R	No data	Gocke et al., 1981	41641
	–	A	R		To et al., 1982	48289
	–	none	none			
	–	none	none		Sekizawa and Shibamoto, 1982	45340
1535	–	A	R		McCann et al., 1975	21338
	–	A	R	No data	Wislocki et al., 1977	24870
	–	none	none	No data	Dorange et al., 1977	27999
	+	A	R	No data	Anderson and Styles, 1979	29184
	–	none	R		Rosenkranz and Poirier, 1979	31449
	–	A	R	No data	Simmon, 1979a	31451
	–	none	none			
	–	A	R	No data	Gocke et al., 1981	41641
	–	none	none		Sekizawa and Shibamoto, 1982	45340
	–	A	R			
	–	none	none		To et al., 1982	48289
	–	A	R			
1537	–	A	R	No data	McCann et al., 1975	21338
	–	none	none	No data	Dorange et al., 1977	27999
	–	A	R	No data	Simmon, 1979a	31451
	–	A	R	No data	Gocke et al., 1981	41641
	–	none	none		Sekizawa and Shibamoto, 1982	45340
	–	none	none		To et al., 1982	48289
	–	A	R			
1538	–	none	none	No data	Dorange et al., 1979	27999
	–	A	R	No data	Anderson and Styles, 1979	29184
	–	none	R	No data	Rosenkranz and Poirier, 1979	31449
	–	A	R	No data	Simmon, 1979a	31451
	–	none	none			
	–	none	none	No data	Gocke et al., 1981	41641
	–	A	R		Sekizawa and Shibamoto, 1982	45340
	–	none	none		To et al., 1982	48289
	–	A	R			

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
S. typhimurium	TA98	–	pre-inc	A	R		Sekizawa and Shibamoto, 1982	45340
		–	grad pl	A	R	No data	Probst et al., 1981	37980
		–	grad pl	none	none			
	TA100	–	pre-inc	A	R	No data	Sekizawa and Shibamoto, 1982	45340
		–	grad pl	A	R	No data	Probst et al., 1981	37980
		–	grad pl	none	none			
		–	susp	Ames 75	Ames 75	No data	Eder et al., 1982	44082
		–	susp	A	R	No data	Eder et al., 1980	36385
		–	plate inc	A	frog liver		Cheh et al., 1980	41583
	TA1530	+	Ames 71	?	M(S21)		Green and Savage, 1978	27476
	TA1531	–	Ames 71	?	M(S21)			
	TA1532	+	Ames 71	?	M(S21)			
	TA1535	–	pre-inc	A	R	No data	Sekizawa and Shibamoto, 1982	45340
		–	grad pl	A	R	No data	Probst et al., 1981	37980
		–	grad pl	none	none			
	TA1536	–	plate inc	A	R	No data	Simmon, 1979a	31541
		–	plate inc	none	none			
	TA1537	–	pre-inc	A	R	no data	Sekizawa and Shibamoto, 1982	45340
		–	grad pl	A	R	No data	Probst et al., 1981	37980
		–	grad pl	none	none			
	TA1538	–	pre-inc	A	R	No data	Sekizawa and Shibamoto, 1982	45340
		–	grad pl	A	R	No data	Probst et al., 1981	37980
		–	grad pl	none	none			
	TA1950	+	Ames test	none	HMA/M		Green and Savage, 1978	27476
	TA1951	–	Ames 71	?	HMA/M			
	TA1952	+	Ames 71	?	HMA/M			
	TA1964	–	Ames 71	?	HMA/M			
		–	Ames 71	?	M(S21)			
E. coli	P3110/P3478	–	pol test	none	none		Fluck et al., 1976	24127
	WP2/WP100	–	rec assay	A	R		Mamber et al., 1983	48782
	repair defi-cient strains	–	rec assay	none	none		Suter and Jaeger, 1982	45031
	(lambda)	–	phage induction	none	none		Speck et al., 1978	29153
	polA	+	pol test	none	none		Rosenkranz and Poirier, 1979	31449
	WP2uvrA	–	trp rev	none	none		Sekizawa and Shibamoto, 1982	45340
		–	trp rev	A	R			
B. sub-tilis	M45/H17	+	rec assay	none	none		Sekizawa and Shibamoto, 1982	45340
	M45/H17	–	rec assay	none	none		Suter and Jaeger, 1982	45031
S. cerevisiae	D3	+	mitotic recombi-nation	A	R		Simmon, 1979	31453
		+	mitotic recombi-nation	none	none			

SAFROLE (94-59-7) (continued) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of micro-somes	Comment	Reference	EMIC No.
D. melano-gaster		–	SLRL				Gocke et al., 1981	41641
	Canton-S	–	SLRL				Valencia, 1977	31499
C. griseus	V79 cells	–	DNA breaks	Ames 75	R		Swenberg et al., 1976	23583
Rat	hepatocytes	–	UDS	none	none		Mirsalis et al., 1982	48204
		–	UDS	none	none		Probst et al., 1981	37980
		+	DNA breaks	none	none		Sina et al., 1983	50841
	hepatocyte nuclei	+	UDS	none	none		Althaus et al., 1982	45810
Human	skin fibro-blasts	–	UDS	none	none		San and Stich, 1975	21394
		+	DNA repair	none	none		Francis et al., 1981	41556
	XP skin fibroblasts	+	DNA repair	none	none			
	HeLa S3 cells	–	UDS	P	R		Martin et al., 1978	29113

SAFROLE (94-59-7) *SECTION 3*

Test object	Cells observed	Result	Protocol or assay	Sex	Route	Comment	Reference	EMIC No.
Mouse	germ cells	–	DLT	M	ip	No data	Epstein et al., 1972	12985
	testis	+	inhibition of DNA synthesis	M	ip		Friedman and Staub, 1976	23169
	spermatozoa	–	SHA	M	ip		Topham, 1980	37395
	bone marrow	–	micronuclei	?	ip		Gocke et al., 1981	41641

o-TOLUIDINE (95-53-4) *SECTION 1*

TA strain	Result	Inducer	Source of microsomes	Comment	Reference	EMIC No.
98	–	A	R		McCann et al., 1975	21337
	–	A	R	No data	Simmon, 1979a	31451
	–	A	R	No data	Zimmer et al., 1980	34657
	–	A	R	No data	Florin et al., 1980	37208
	–	A	R		Tanaka et al., 1980	37372
	–	none	none			
100	–	A	R		McCann et al., 1975	21337
	–	A	R	No data	Simmon, 1979a	31451
	–	A	R	No data	Hecht et al., 1979	31731
	–	none	none			
	–	A	R	No data	Zimmer et al., 1980	34657
	–	A	R	No data	Florin et al., 1980	37208
	–	A	R		Tanaka et al., 1980	37372
	–	none	none			

o-TOLUIDINE (95-53-4) (continued) *SECTION 1*

TA strain	Result	Inducer	Source of microsomes	Comment	Reference	EMIC No.
1535	–	A	R		McCann et al., 1975	21337
	–	A	R	No data	Simmon, 1979a	31451
	–	none	none		Rosenkranz and Poirier, 1979	31449
	–	A	R	No data	Hecht et al., 1979	31731
	–	none	none			
1537	–	A	R		McCann et al., 1975	21337
	–	A	R	No data	Simmon, 1979a	31451
	–	A	R	No data	Zimmer et al., 1980	34657
1538	–	P	R		Garner and Nutman, 1977	25154
	–	none	none			
	–	A	R	No data	Ferretti et al., 1977	25098
	–	A	R	No data	Simmon, 1979a	31451
	–	none	R		Rosenkranz and Poirier, 1979	31449
	–	A	R	No data	Hecht et al., 1979	31731
	–	none	none			

o-TOLUIDINE (95-53-4) *SECTION 2*

Test object	Strain or cells observed	Result	Protocol or assay	Inducer	Source of microsomes	Comment	Reference	EMIC No.
S. typhimurium	TA98	–	pre-inc	A	R	Positive with norharman	Nagao et al., 1977	28014
		–	BFU/R	none	none		Tanaka et al., 1980	37372
		+	BFU/R	A	R			
	TA100	–	BFU/R	none	none			
		–	BFU/R	A	R			
E. coli	WP2uvrA	–	pol test	none	none		Rosenkranz and Poirier, 1979	31449
S. cerevisiae	D3	–	mitotic recombination	A	R		Simmon, 1979b	31453
C. griseus	CHL cells	–	DNA breaks	A	R	No data	Zimmer et al., 1980	34657

o-TOLUIDINE (95-53-4) *SECTION 3*

Test object	Cells observed	Result	Protocol or assay	Sex	Route	Comment	Reference	EMIC No.
Mouse	testis	+	DNA synthesis	M	oral		Seiler, 1977	25108
	kidney	–	inhibition of DNA synthesis	?	ip ?	No data	Amlacher and Ziebarth, 1979	34316
	spermatozoa	–	SHA	M	ip		Topham, 1980	37395
C. griseus	bone marrow	+	SCE	F	ip or oral		Neal and Probst, 1983	48665

CARCINOGENICITY SUMMARIES

Test chemical	IARC evaluation for carcinogenicity		NCI/NTP evaluation for carcinogenicity	Reference
	Animal	Human	Animal (mouse and rat)	
Acrylonitrile (107-13-1)	S [1]	L [2]		IARC Monographs, 19, 73–133, 1979; Suppl. 4 (Appendix 2), 267–270, 1982
Benzene (71-43-2)	L	S		IARC Monographs, 29, 93–148, 1982; Suppl. 4, 56–57, 1982
Benzoin (119-53-9)	* [3]	*	Negative	NIH-80-1760, 1980
Caprolactam (105-60-2)	*	*	Negative	NIH-81-1770, 1981
Diethylhexyl phthalate (DEHP) (117-81-7)	S	S	Positive	IARC Monographs, 29, 257–294, 1982; NIH-82-1773, 1982
Diethylstilbestrol (DES) (56-53-1)	S	I [4]		IARC Monographs, 21, 173–231, 1979; Suppl. 4, 184–185, 1982
Hexamethylphosphoramide (HMPA) (680-31-9)	S	*		IARC Monographs, 15, 211–222, 1977; Suppl. 4 (Appendix 2), 267–270, 1982
Phenobarbitone (50-06-6) and sodium phenobarbital (57-30-7)	L	I		IARC Monographs, 13, 157–183, 1977; Suppl. 4, 208–211, 1982
Safrole (94-59-7)	S	*		IARC Monographs, 10, 231–244, 1976; Suppl. 4 (Appendix 2), 267–270, 1982
o-Toluidine (95-53-4) and *o*-toluidine hydrochloride (636-21-5)	S	I	Positive	IARC Monographs, 27, 155–175, 1982; Suppl. 4, 245, 1982

[1] Sufficient evidence.
[2] Limited evidence.
[3] No data available for evaluation.
[4] Inadequate evidence.

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Appendix 3

A review of the current regulatory requirements for mutagenicity testing

D.J. Berry and M.H. Litchfield

Imperial Chemical Industries PLC, Central Toxicology Laboratory, Alderley Edge, Cheshire (Great Britain)

There are numerous guidelines from international organisations and national regulatory authorities dealing with the toxicological testing requirements in support of the development of new chemicals or for the toxicological evaluation of existing materials. The extent of the testing is governed by the nature and uses of the chemical concerned. Guidance is given on the design of the toxicological tests involved and in many cases there is general acceptance of the principles and details of studies used e.g. a 90-day rat study.

The requirements for mutagenicity testing are among the most recent in the history of toxicological testing requirements. They appear to be the most confusing because of the subject of mutagenicity testing itself which is a comparatively young science and is in a rapidly developing phase. There are many tests, involving in vitro and in vivo techniques and covering several end-points and their validation has been a problem in itself. The regulatory requirements for mutagenicity testing have grown up in this climate of change and this is reflected by the large and varied range of tests suggested by different authorities.

This review has been undertaken to demonstrate the present position with the mutagenicity testing requirements of regulatory authorities. All the major authority requirements and as many as possible of the others have been reviewed but the survey is not to be regarded as exhaustive. The results of the survey are shown in Table 1, commencing with the OECD guidelines. After the initial column the table is split into 4 columns the first of these detailing the general requirements for each authority together with relevant comments. The other 3 columns show the tests suggested grouped under the different genetic end-points.

The major outcome of the review shows that many authorities require a minimum base set of tests and that additional tests are required only if firmly positive results have been obtained from the base set. The selection of these tests is left mainly with the individual scientists undertaking the evaluation.

Abbreviations

S. typh	*Salmonella typhimurium*
E. coli	*Escherichia coli*
B. sub	*Bacillus subtilis*
S. cere	*Saccharomyces cerevisiae*
S. pomb	*Schizosaccharomyces pombe*
N. cras	*Neurospora crassa*
A. nid	*Aspergillus nidulans*
D. melan	*Drosophila melanogaster*
mam	mammalian
cytogen	cytogenetics
micronuc	micronucleus
seg	segregation
recomb	recombination
aneup	aneuploidy
UDS	unscheduled DNA synthesis
SCE	sister-chromatid exchange
RL	recessive lethal
CT	cell transformation
HT	heritable translocation
SLRL	sex-linked recessive lethal
DL	dominant lethal

The superscripts [1] and [2] against the name of a country indicates, respectively a member of the EEC or OECD.

Superscript [3] is a reference to 'Worldwide Genetic Activity (Mutagenicity) Requirements', Warner Lambert Company, Jan. 83 for drug testing.

TABLE 1

A REVIEW OF THE CURRENT REGULATORY REQUIREMENTS FOR MUTAGENICITY TESTING

Regulatory autority or organisation	Comments	Suggested tests for the detection of anomalies		
		Gene mutation	Chromosome damage	Other genotoxic aberrations e.g. DNA damage and repair, numeric abnormalities
ORGANISATION FOR CO-OPERATION AND DEVELOPMENT (OECD Guidelines for Testing of Chemicals, 1981, revised May 1983)	Number and sequence of tests unspecified	*S. typh* (4 strains) *E. coli* (3 strains)	in vitro mam. cytogen micronuc, mouse recommended	
COMMISSION OF THE EUROPEAN COMMUNITIES (Official Journal of the European Communities, 6th Amendment to Directive 67/548/EEC, Annex VII, 15.10.79)	The substance should be examined in two tests, one of which should be bacterial and one non-bacterial. This is the 'Base set' requirement. Tests not specified. Annex VIII says further studies may be required dependent upon the 'Base-set' results. (verification studies)			
(Annex V, EEC Directive 79-831, part B, Toxicological Methods of Annex VIII, Draft July 83).	This Annex lists the following tests, presumably as reference to acceptable verification studies. (See above)	*A. nid* *S. cere* *S. pomb* Mouse lymphoma Chinese hamster D. melan, SLRL CT Mouse spot test Mouse specific locus	DL (rodent) in vivo cytogen Mouse HT	*A. nid* somatic seg. *S. cere* mitotic recomb *S. cere* mitotic aneup UDS-mam cells SCE-in vitro human lymphocytes or Chinese hamster ovary cells
(Notes for Guidance on the Testing of Medicinal (Products for their Mutagenic Potential, Draft, June 80)	Minimum of 4 tests suggested.	Bacterial genome (e.g. Ames) and in vitro mam *OR* *D. melan*	in vitro mam. somatic cell e.g. human lymphocytes or metaphase analysis and in vivo chromosome analysis (e.g. metaphase analysis of rodent micronuc)	

AUSTRIA [2]			
Federal Government Institute for Experimental Pharmacological and Balneological Studies Jun. 80, Draft	Requirement for in vitro and in vivo studies. Reference is made to WHO Technical Report Series Nos. 482 and 546 'Evaluation on Testing of Drugs for Mutagenicity' and 'Assessment of the Carcinogenicity and Mutagenicity of CHemicals'; and also to the U.S.A. Federal Register, Aug. 78, part II Assessment Guidelines, Subdivision F, Nov. 82). Tests not specified		
Drugs [3]:	Required for beta-blockers and beta-agonists. Tests not specified.		
Federal Institute for Plant Protection (Draft guidelines issued in 1982)	Requirement for in vivo and in vitro studies. Tests not specified.		
AUSTRALIA [2]			
Interim Notification Scheme for New Chemicals in Australia, Australia Environmental Council, Oct. 1981	Mutagenicity data required. It is suggested that the OECD guidelines are followed.		
Drug [3]:	Mutagenicity data may be required. Reference is made to WHO Technical Report Services 482/1971.	Beta-blockers	*S. typh*
BELGIUM [1,2]			
Le Moniteur Belge, Jul. 82	A Belgium Royal Decree (24.5.82) was issued giving effect to notification requirements of the EEC's 6th Amendment.		
Conseil Supérieur d'Hygiène (Pesticides et produits phytopharmaceutiques, Dec. 78)	Quick tests to evaluate mutagenesis in vivo and in vitro. Tests not specified.		

TABLE 1 (continued)

Regulatory autority or organisation	Comments	Suggested tests for the detection of anomalies		
		Gene mutation	Chromosome damage	Other genotoxic aberrations e.g. DNA damage and repair, numeric abnormalities
BRAZIL Pesticide Registration Application form.			DL	
CANADA [2] Health Protection Branch, Pesticides Divn. (Guidelines for Pesticide Toxicity Data Requirements, OCt. 81, Draft)	Number or sequence of tests not specified.	in vitro point mutation in microbial and mam. systems. Positive results in point mutation may indicate need for in vivo studies.	Chromosomal aberrations	DNA repair
Health Protection Branch Bureau of Human Prescription Drugs [3] (Preclinical Toxicologic Guidelines, June 81)	May be required. Battery of tests suggested.	Mouse specific locus *OR* Point mutation in eukaryotic microorgs., cultured mam. cells and RL mutations in insects.	HT or sex chromosome loss in mice.	
CZECHOSLOVAKIA Pesticides:	Require mutagenicity test on bacteria, unspecified			
DENMARK [1,2] Ministry of the Environment, National Agency of Environmental Protection (Statutory Order No. 410, Sep. 80; 1984 interpretation of law)	The substance shall be tested in a series of two experiments, one bacterial and one non-bacterial. Tests not specified.			
FRANCE [1,2] Decree of 15.1.79 relating to the technical file to be	A minimum of two short-term tests, one bacterial (with or without metabolic			

supplied prior to manufacture for commercial purposes of a new chemical substance.	activation). Tests not specified.			
Law No. 82-905. Journal Officiel de la République Française Oct. 82.	This law modifies law No. 77-771 of 12.7.77 bringing it into line with the requirements of the EEC 6th Amendment. Tests required not specified.			
Drugs::[3]:				
FINLAND[2] Pesticide Regulation in Finland, Makkula and Blomqvist, Agriculture Research Centre Pesticide Regulation Unit, 1981.	Requirement for investigations on mutagenicity. Tests not specified.			
National Board of Health (Application for Registration of Pharmaceutical Specialties Guidelines[3] May 78)	Battery of tests suggested to cover different types of mutation events, both in vivo and in vitro		e.g. micronuc	e.g. sperm morphology
INDIA Registration Committee of the Central Insecticides Board Report submitted by Pesticides Formulaters Association of India. (MAI, 1978)	Battery of tests required covering all three categories. Number or sequence of tests unspecified. The following tests are suggested.	*S. typ* (4 strains) Somatic cells, mouse or Chinese hamster.	DL in mouse, rat or Chinese hamster. Somatic cells, mouse or Chinese hamster. Nucleus anomally or micronuc, bone marrow. Chromosome studies, bone marrow.	Not specified.
INDONESIA Ministry of Health (Criteria for the Registration of Finished Drugs[3], 1980)	Required. No tests specified.			
IRAN Drugs[3]:	Required 'if appropriate'. No tests specified.			

TABLE 1 (continued)

Regulatory autority or organisation	Comments	Suggested tests for the detection of anomalies		
		Gene mutation	Chromosome damage	Other genotoxic aberrations e.g. DNA damage and repair, numeric abnormalities
IRELAND				
Statutory Instrument 258, Sep. 82	These regulations from the Ministry of Labour, incorporate the EEC VI Amendment Directive. (See EEC).			
Drugs [3]:	Tests not required for drugs given in a single dose regime. Otherwise, required for a number of conditions. Two or more standard tests required, to include in vitro and in vivo tests:	Ames	in vitro mam. metaphase analysis DL rodent hamster micronuc	
ISRAEL				
(Information pertaining to the Application to the Ministry of Health for the registration of a foreign produced medicant intended for human use.) [3] April 82	Required for all new chemicals. Tests not specified.			
ITALY				
Proposed amendment to the 'Regulations Concerning Control of Production, Marketing and Sale of Plant Health Products for the Protection of Stored Foodstuff's Presidential Decree, 1978	Battery of 5 tests is required: two tests for genetic or point mutations (*S. typh* plus one other); two tests for structural and numeric chromosomal mutations (preferably a non-dissociation test + one other); one test for DNA lesions or DNA repair.	*S. typ* (4 strains) or similar on other prokaryotes. eukaryotes. *D. melan* e.g. RL. in vitro somatic cells. in vivo mam. punctate mutations (e.g. mouse specific	Non-dissociation in eukaryotes (yeasts, Aspergillus). Non-dissociation in mam. male germinal cells in vivo. mam. in vivo in bone marrow. mam. in vivo in germinal cells.	Mitotic crossing over in yeasts of *A. nid.* DNA repair. Evaluation of DNA damage by elution technique. Stimulation of DNA synthesis in mam somatic cells. in vitro in vitro SCE

	A list of acceptable tests is given.	locus or somatic mutation).	mam. in vivo in first stage of embryogenesis. Micronuc test. *D. melan* heritable and non-heritable effects mam. in vivo DL F_1 mam translocation.	
Circular of Ministry of Health No. 76, Dec. 82	This circular implements regulations so that they conform to the EEC directive, VI amendment.		Non-disjunction in *D. melan.*	
JAPAN [2] National Institute of Hygienic Sciences (Draft guidelines, 1982)	Initially, in vitro tests should be performed with gene mutation and chromosomal aberration as indices.	*S. typh* (several strains) and *E. coli* (few strains).	in vitro mam	
	When mutagenicity is suspected from either tests, in vivo micronucleus should be performed.		Rodent in vivo micronu	
	Where these tests suggested are not applicable for technical or scientific reasons, they can be substituted with an appropriate test.	in vitro mam *D. melan* Mouse spot test	Rodent bone marrow Rodent micronuc. Rodent generative cells Rodent DL Reciprocal translocation test in mice. Plant cells	Phage induction with bacteria. DNA repair with bacteria in vitro mam UDS in vitro mam SCE Rodent in vivo SCE Mitotic recomb and gene conversion with yeast. Sperm abnormality mouse
Systems of Conducting the Toxicity Investigations of Chemical Substances, Safety and Health law, 1979.	Microbial test will be used (unspecified).			
Labour Safety Law, Jun. 79	Requirement for all new chemicals imported or introduced to Japan after 20.6.79.	*B. sub* (rec. assay) *E. coli* (one strain) *S. typ* (5 strains)		

TABLE 1 (continued)

Regulatory autority or organisation	Comments	Suggested tests for the detection of anomalies		
		Gene mutation	Chromosome damage	Other genotoxic aberrations e.g. DNA damage and repair, numeric abnormalities
Ministry of Agriculture, Fisheries and Forestries, Guidelines for Preparation of Pesticide Toxicological Data (Draft) Dec. 83.	Tests from all 3 categories are required: When it is necessary to carry out further investigations from the data on these assays, any test suitable for detection of genetic toxicity of DL, mouse specific locus, micronuc, SLRL, in vivo cytogen etc. should be carried out.	Microbial reverse mutation test e.g. *S. typh* (5 strains) and *E. coli*	in vitro mam cytogen (e.g. Chinese hamster cells or human lymphocytes)	DNA damage and repair in *B. sub*
LUXEMBOURG [1,2] Ministerial decree (No. 22) of Mar. 79.	Decree implements the EEC directive.			
MEXICO Drugs [3]:	'When necessary'. No tests specified.			
NEW ZEALAND Drigs [3]:	Tests required if compound or metabolites similar to known carcinogen or mutagen. No details of tests required.			
NORWAY [2] Health Hazard Labelling Regulations, Nov. 82.	This Royal Decree, pursuant to the Worker Protection and Working Environment Acta and the Product Control Act is, in principle, based upon the EEC 6th Amendment.			
National Centre for Medical Products Control (Registration of Pharmaceutical Specialities; Guidelines for Applications, 1982). [3]	At least two studies to detect DNA defects; chromosomal deficiencies. Tests not specified.			

Regulations Relating to Pesticides: law of April '63 and subsequent amendments.	Results from bacterial and non-bacterial study are required.		
PHILIPPINES			
Drugs: Philippine FDA [3]	No specific requirement but likes to receive data.		
POLAND			
Pesticides:	List of studies given but no indication of sequence of number.	DL in vitro studies and chromosome studies	Host-mediated
PORTUGAL			
Couissao de Toxicologia des Pesicidas, CTP (D) 17/82, July	Mutagenicity tests required but not specified.		
SOUTH AFRICA			
Drugs [3]:	Required 'if necessary'. No details of tests.		
Pesticides:	Not normally required, depends on situation in U.S.A.		
SWEDEN [2]			
National Environmental Board, Products Control Board General Directives for Applicatioan for Registration of Pesticides etc. in Sweden) PCB/1983-04-08	Studies should be carried out as described in OECD guide-lines. Studies should be designed as a test battery with a sufficient number of methods of which at least one should be microbial and at least one non-microbial. Methods chosen should provide information on mut. effects in vivo and in vitro as well as mutagenic effects on different levels (gene, chromosome, genome) and on sexual and somatic cells. Tests not specified.		
National Board of Health and Welfare (Registration of Pharma-ceutical specialities. Instructions for submission of Applicants 1981) [3]	At least two tests required to detect DNA or chromosomal changes. Tests not specified.		

TABLE 1 (continued)

Regulatory autority or organisation	Comments	Suggested tests for the detection of anomalies		
		Gene mutation	Chromosome damage	Other genotoxic aberrations e.g. DNA damage and repair, numeric abnormalities
SWITZERLAND [2]				
Drugs [3]:	Required under certain conditions. No details of test required.			
THE NETHERLANDS [1,2]				
Application for the Regulation of a Pesticide, Pesticides Bureau of the Commission for the Registration of Pesticides, March 81.	Battery of at least two tests, one bacterial and one non-bacterial. A list of tests for consideration is given.	*S. typh* *D. melan* RL mam. in vitro e.g. mouse lymphoma or Chinese hamster cells	in vitro studies	DNA repair *E. coli* host-mediated assay. in vitro SCE
Report on the Mutagenicity of Chemical Substances, Health Council, ref. B. BRA Bulletin March 82.	Recommends a two-stage approach, stage 1 consisting of a bacterial point mutation assay and two tests in eukaryotic systems (either two point mutation assays or one point mutation and one test for the detection of chromosome abnormalities in vitro). It is recommended that consideration be given to make this stage legally binding.	*S. typh* (4 strains) *OR* *E. coli*	in vitro study (e.g. *D. melan* SLRL)	
	If positive results are obtained, further testing would be required in vivo on mammals.		No tests specified	
UNITED KINGDOM [1,2]				
Health and Safety Commission (Methods for Determination of Toxicity, Notification Regulations, 1982)	Tests recommended for Schedule 1 testing	*E. coli* (3 strains) *OR* *S. typh*	in vitro mam cytogen *OR* in vivo mam (micronuc or rodent cytogen)	
	Further screening may be required as amount of compound			

	produced or the number of people exposed increases. Tests not specified but reference is made to 'Guideline for the Testing of Chemicals for Mutagenicity' HMSO, Apr. 81. (See below).			
Department of Health and Social Security (Consultative Document on Guidelines on Toxicity Testing, July 1981)	A battery of tests (unspecified) is recommended. Reference is made to 'Guidelines for the Testing of Chemicals for Mutagenicity', HMSO, Apr. 81, which recommends the following test strategy for a 'basic package'.	*S. typh OR* *E. coli OR* *B. sub* in vitro mam cells *OR D. melan* RL	in vitro mam in vivo micronuc *OR* in vivo cytogen *OR* DL in rat or mouse	
	If the chemical is not intended for human ingestion and it is expected that its use will be limited and the degree of human exposure small or containable, a case could be made for only doing a bacterial point mutation and in vitro mam chromosome damage tests.			
	Supplementary tests are suggested when unexpected or equivocal results are obtained in the basic package. The number and sequence of tests is unspecified.	Mam cell culture mutations Mouse spot mouse	in vivo micronuc DL in mouse or rat	Prokaryotic tests in repair deficient strains UDS-mam cell Host-mediated assay SCE
		Tests in sub-mam eukaryotic systems such as yeasts, fungi and *D. melan* (tests unspecified)		
	Other studies may be required where a known or suspected mutagen is to be proposed for a full risk benefit evaluation.	Mouse specific locus	Mouse HT Cytogen translocation Studies on spermatocytes and oocytes	Non-disjunction tests in mice
	Other studies ('as yet not validated') which could be of use:			Sperm morphology Chromosomal analysis of sperm used for in vitro fert. of hamster eggs. Non-disjunction tests.

TABLE 1 (continued)

Regulatory autority or organisation	Comments	Suggested tests for the detection of anomalies		
		Gene mutation	Chromosome damage	Other genotoxic aberrations e.g. DNA damage and repair, numeric abnormalities
Department of Health and Social Security (Notes on applications for Clinical Trials Certificates, Medicines for Human Use, MAL 4, May 77 MAIL 32, DHSS Nov. 81 MLX 130, DHSS Jan. 80 [3]	Required under a number of conditions. Attention is also drawn to DHSS 'Guidelines for Testing of Chemicals for Mutagenicity, Apr. 81'.	*S. typh* OR *E. coli*		
Pesticides Safety Precautions Scheme (revised 1982)	Sequential set of tests is recommended (with reference to the DHSS document) Tests include:	Point mutation assay (*S. typh*)	in vitro and in vivo tests. DL in rat or mouse.	
UNITED STATES OF AMERICA [2]				
EPA-FIFRA (Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation, Human and Domestic Animals, Nov. 82)	A battery of tests is required appropriate to address each category: a representative selection of tests is given:	*S. typh* (Ames strains) *E. coli* (2 strains) *B. sub* (2 strains) Forward and reverse mutation in *S. cere* *S. pomb* *N. cras* *A. nid* *D. melan* RL Specific loci forward and reverse mutation in Chinese hamster lung and ovary. Mouse lymphoma Mouse specific locus	*A. nid* *N cras* *D. melan* in vitro mam SCE in vitro mam cytogen Mam micronuc in vivo mam SCE in vivo mam cytogen DL in rat or mouse Mouse H.T.	Differential toxicity in *E. coli* *B. sub* *S. cere*-mitotic recomb *A. nid* mitotic recomb in vitro mam UDS Mouse UDS in vitro DNA alkaline elution in vitro SCE in vivo SCE Eukaryotic mitotic seg in vitro, mitotic interfer. Mam., mitotic interfer in vitro, micronuc Mam., micronuc in vitro, Mam-CT Sperm morphology DNA synthesis inhibition DNA alkylation

EPA-TSCA Premanufacturing Notification (Rule 40 CFR Part 720) Apr. 83.	Mutagenicity required. The guidelines published in the Federal Register (27.1.81) recommend that the OECD guidelines are used as a starting point in setting up a testing program for new chemicals.			
EPA-TSCA (Health Effects Test Guidelines, Aug. 82)	No guidance is given as to when these tests are required.	*S. typh* (at least 4 strains) *E. coli* (3 strains) *A. nid* *N. cras* *D. melan* SLRL Mam. in vitro e.g. mouse lymphoma and Chinese hamster cells. Mouse specific locus.	in vitro cytogen Micronuc (mouse recommended) in vivo cytogen mam. *D. melan* HT DL (rat or mouse) MouseHT	DNA damage/repair tests in *E. coli* or *B. sub* in vitro mam. UDS *S. cere* mitotic gene conversion in vitro SCE
Food and Drug Administration (Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives used in Food, 1982).	A battery of tests is recommended. The mam. CT and *D. melan* SLRL mutation test are cited as being quite useful but lack sufficient commercial availability to be recommended routinely. Other tests than those recommended may be used but must be validated.	Bacterial (e.g. Ames) Mam. (e.g. mouse lymphoma)		Generalised test for DNA damage (e.g. UDS in rat hepatocytes)
WEST GERMANY [1,2] Chemikaliengesetz law, Jan 82.	This law adopts the requirements of the EEC's VI amendment.			
Drugs:	Two in vitro and one in vivo study required, not specified.			

TABLE 1 (continued)

Regulatory autority or organisation	Comments	Suggested tests for the detection of anomalies		
		Gene mutation	Chromosome damage	Other genotoxic aberrations e.g. DNA damage and repair, numeric abnormalities
BBA Leaflet No. 33, Sep. 75	Mutagenicity studies are required, no tests specified.			
YUGOSLAVIA Pesticides:	At least two tests required, one of which must be in mammals. The confirming test of the cancerogenicity is to be done in vivo.	Unspecified	DL Chromosome aberration tests micronuc	DNA damage

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Appendix 4

Phenobarbital: its mutagenicity and toxicity in the Ames' Salmonella test

D.B. McGregor and R.D. Prentice

Inveresk Research International, Ltd., Musselburgh (Scotland)

An unexpected result from the WHO–IPCS study was the small but reproducible mutagenic effect of phenobarbital in *Salmonella typhimurium* TA1535 and TA100. The objective of the short supplementary study reported here was to investigate the involvement of toxicity in the dose–response curve using *S. typhimurium* TA100. Substantial toxicity at high dose levels could be a contributory factor in keeping the observed mutagenic effect low. In pursuit of this objective, two methods of toxicity measurement were used: the filler cell method (Waleh et al., 1982) and the microcolony method (McGregor et al., 1984). Toxicity is measured on his^+ cells in the filler cell method and on his^- cells in the microcolony method.

Materials and methods

Test chemical

Phenobarbital was received from J. Ashby, Central Toxicology Laboratory, ICI Ltd., Macclesfield (Great Britain).

Bacteria

The bacterial strain, *Salmonella typhimurium* TA100, was obtained from B.N. Ames, Department of Biochemistry, University of California, Berkeley, CA. The non-reverting strain, *S. typhimurium* NS1100, was a gift from K. Mortelmans, SRI International, Menlo Park, CA. The derivation of NS1100 was described by Waleh et al. (1982). The strain is similar to TA100, but has, in addition to *hisG46*, a second histidine mutation, *hisD8557*::Tn10. It was used in conjunction with TA100 as the filler cell for the filler cell experiment. His^+ bacteria used in the filler cell experiment were randomly selected at this laboratory from control plates and cloned on minimal medium.

Culture conditions

Bacteria were routinely stored in liquid nitrogen. When required, a scraping from the surface of a frozen culture was transferred to 25 ml nutrient broth in a 125-ml erlenmeyer flask and incubated at 37°C for 16 h in a gyratory incubator at 60 rpm.

Mutagenicity tests

The plate-incorporation assay, with and without S9 mix, was performed as described by Ames et al. (1975). His^+ colonies were counted after 2 days incubation at 37°C using a Biotran III automated colony counter (New Brunswick Scientific Corp., New Brunswick, NJ) fitted with a 50-mm lens. This gave a $2\times$ magnification of the scanning area.

Toxicity measurements

Microcolony method. A normal Salmonella/activation test, as described above, was performed, except that no more than approximately 1.5×10^7 cells were plated. Following the normal 2-day incubation period and the counting of his^+ colonies, microcolonies formed by surviving his^- cells were counted without correction by a Biotran III counter linked to an auxillary Hitachi video camera mounted upon a Leitz Dialux 20 microscope. A $10\times$ objective was used, giving a depth of field of approximately 25 μm. After ocusing on a randomly selected area of the lawn, microcolonies could be counted, provided that the initial plated cell population did not exceed 1.5×10^7 per 90

mm plate. This concentration was achieved by diluting back the 16 h culture. At higher population densities, the microcolonies were smaller and closer together, thereby prohibiting discrimination of individual microcolonies by the counter. Careful adjustment of the microscope was necessary to ensure optimal lighting conditions and contrast. 5 fields per plate were counted and the mean for a particular plate recorded. Since relative microcolony numbers were required, it was not necessary to apply any factors to the data to convert mean microscope field counts to total plate counts. The ratio of the average numbers of microcolonies on the dosed plate and the control plate is defined as the measure of toxicity.

Filler cell method. The method used has been described in detail by Waleh et al. (1982). Briefly, a mutation test was performed as described above. A parallel set of plates was similarly treated, these plates containing approximately 10^8 nonrevertable "filler" cells and approximately 10^2 his^+ cells which could grow up to form colonies visible to the unaided eye, provided that they were not killed by the treatment. Hence, the nonrevertable his^- cells mimicked nonrevertant cells on a normal Ames' Salmonella/activation test plate, while the his^+ cells allowed estimates of survival to be made. Toxicity is defined in a manner similar to that used in the microcolony method.

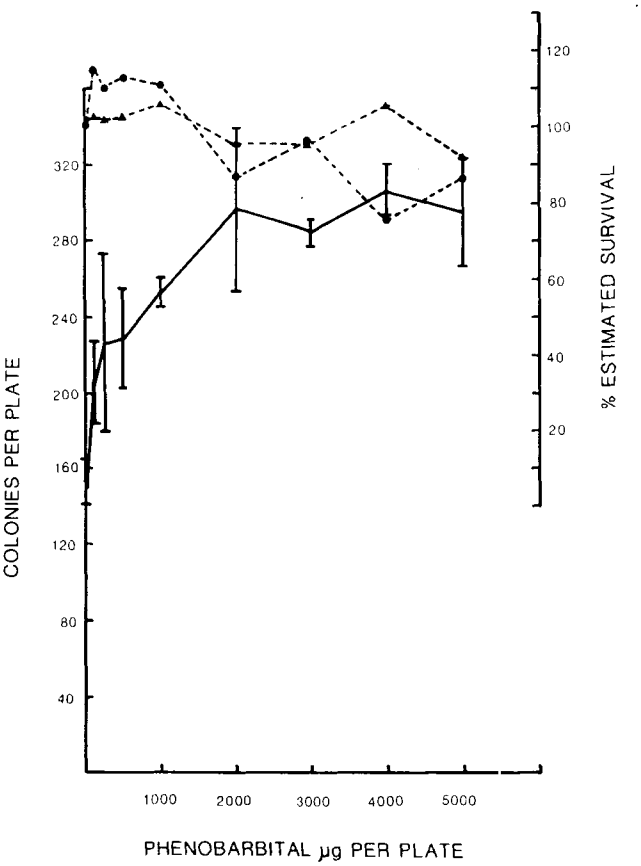

Fig. 1. Mutagenicity and toxicity testing of phenobarbital in *S. typhimurium* TA100. ●, survival, microcolony method; ▲, survival, filler cell method; ————, mutation with ± standard deviation bars.

TABLE 1

PHENOBARBITAL: MUTAGENICITY AND TOXICITY ESTIMATIONS IN THE ABSENCE OF SUPPLEMENTARY METABOLIC CONDITIONS, USING *S. typhimurium* TA100

Quantity per plate	Mutagenicity Colonies per plate [a] ± S.D.	Toxicity	
		Microcolony method Colonies per plate [a] ± S.D.(%)	Filler cell method Colonies per plate [a] ± S.D.(%)
0	153 ± 11.9	996 ± 148 (100	132 ± 18 (100)
125 µg	206 ± 21.7	1149 ± 130 (115)	134 ± 9 (102)
250 µg	227 ± 47.0	1089 ± 135 (109)	133 ± 8 (101) [b]
500 µg	229 ± 25.5	1120 ± 226 (112)	135 ± 15 (102)
1000 µg	253 ± 8.2	1096 ± 109 (110)	139 ± 8 (105)
2000 µg	296 ± 43.1	856 ± 94 (86)	125 ± 21 (95)
3000 µg	284 ± 6.6	953 ± 140 (96)	125 ± 10 (95)
4000 µg	306 ± 12.7	750 ± 132 (75)	138 ± 23 (105)
5000 µg	295 ± 28.9	857 ± 67 (86)	122 ± 11 (92)

[a] Mean of plates in each case, except for [b], where mean of 2.

Results and discussion

The results of the experiments are shown in Table 1 and Fig. 1.

There was a small, dose-related increase in numbers of mutant colonies per plate which reached a plateau at 2000 μg per plate. Further dose increases up to 5000 μg per plate had no effect upon colony numbers.

Over the whole dose range there was no significant change in survival, as estimated by the filler cell method and only a very small reduction in survival according to the microcolony technique. Since the size of the exposed cell population is hardly changed and there is apparently no specific toxicity of phenobarbital for the his^+ filler cells, it is concluded that toxicity does not contribute towards the plateau in mutagenic response.

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