Mutagenesis vol.2 no.2 pp.111–113, 1987

The bone marrow clastogenicity of eight halogenated benzenes in male NMRI mice

E.Mohtashamipur, R.Triebel, H.Straeter and K.Norpoth

Institute of Hygiene and Occupational Medicine, University Medical Centre, Essen University, Hufelandstrasse 55, D-4300 Essen, FRG

Eight widely used halogenated benzenes, including bromobenzene (BB), chlorobenzene (CB), three isomers of dichlorobenzene (DCB) and three isomers of trichlorobenzene (TCB) were tested for acute toxicity (LD_{50}) and clastogenicity in 8-week-old NMRI mice by intraperitoneal administration. Four doses of each chemical (up to 70% of LD_{50}) were tested for clastogenic activity. Each compound was administered in two equal doses, 24 h apart. Increased formation of micronucleated polychromatic erythrocytes, observed in femoral bone marrow, 30 h after the first injection, was considered to be due to the clastogenic activity of the test compound. All the halogenated benzenes tested were found to be clastogenic ($P < 0.01$). The highest clastogenic activities were induced by *m*-DCB and BB. Among the three isomers of DCB, *m*-DCB significantly ($P < 0.05$) induced more micronuclei than *o*-DCB or *p*-DCB. No significant differences were found between the clastogenic activities of TCB isomers.

Introduction

Halogenated benzenes are widely used in relatively large quantities in various industries. The total production of all chlorinated benzenes in western Europe is estimated to have been more than 208 million kg in 1980, half of which was located in the Federal Republic of Germany (IARC, 1982a). In addition to direct occupational exposure of man, halogenated benzenes are found in most parts of the ecosystem and have been detected in air, soil, food and water (Pellizzari, 1979; Zoeteman *et al.*, 1979; IARC, 1982a; Lai, 1984). Some of these chemicals are not limited to industrial use. For example, dichlorobenzene (*p*-DCB) is used as a fungicide, an air freshener, a moth repellent on clothes, and its crystals can be used to counteract odours in garbage and other refuse (IARC, 1982a; Giavini *et al.*, 1986).

Chlorobenzenes and their metabolites have been detected in human and animal tissues and body fluids indicating the extent of environmental pollution (IARC, 1982a; Jan, 1983). The mean levels of chlorobenzenes in human milk and adipose tissue are found to range from traces to 25 μg/kg in milk and from not detectable to 146 μg/kg in adipose tissue (Jan, 1983). Few biological data relevant to toxicity and genotoxicity of halogenated benzenes are available. Although the hepatotoxic compound, bromobenzene (BB), is found to bind covalently to DNA/RNA to an extent comparable to that of moderately oncogenic substances (Colacci *et al.*, 1985), no data are available on its carcinogenicity. The results of a host-mediated mutagenicity assay on BB were negative (Simmon *et al.*, 1979). No adequate information exists on the carcinogenicity/mutagenicity of chlorinated benzenes (IARC, 1982a). Wester *et al.* (1985) carried out a lifetime carcinogenicity study with a 'mixture' of 11 halogenated hydrocarbons including chlorobenzenes administered to rats in the drinking water, and found no significant carcinogenic effect. The results of some teratogenicity assays with chlorobenzenes were either negative (Hayes *et al.*, 1985), or if positive, they were considered to be the consequence of other factors rather than teratogenic effects (Giavini *et al.*, 1986).

This paper describes an investigation of the effects of a series of halogenated benzenes on the incidence of micronucleus induction within bone marrow polychromatic erythrocytes after intraperitoneal administration to mice.

Materials and methods

Chemicals

Fetal calf serum was obtained from Boehringer Mannheim (Mannheim, FRG). Benzene and halogenated benzenes were purchased from Merck Co. (Hohenbrunn, FRG). All test compounds were of synthetic grade and their purities are given in Table I. *p*-DCB, trichlorobenzene (1,2,3-TCB) and 1,3,5-TCB were supplied as solids which were dissolved in the minimum possible amount of dimethylsulphoxide (Merck, Darmdstadt, FRG).

Animals

Male 8-week-old NMRI mice were obtained from the Zentralinstitut für Versuchstiere (Hannover, FRG). Animals were housed five per cage and were given free access to water and laboratory Altromin chow (Altromin, Lage, FRG).

Micronucleus test

Median lethal doses (LD_{50}) of the test compounds administered by i.p. injection, were conducted according to the standard method (Fowler, 1983) as described elsewhere (Mohtashamipur *et al.*, 1985).

Since the dosing regime of Schmid (1977) for the micronucleus test implies that the highest dose used should be tolerated by the animals, the doses were chosen in such a way that they did not exceed 70% of the LD_{50}. Each single group of five animals was treated by subacute i.p. injection of half of each chosen dosage of a test chemical 24 h before the other half was administered. The chemicals were given in corn oil (when the doses were too small to be injected alone) or alone. The control group of 10 mice received i.p. injections of corn oil only. Benzene, as the mother compound for halogenated benzenes, was chosen as the positive control. The animals were killed by an overdose of ether 30 h after the first injection of the test chemical; the femora were removed and the marrow was suspended in serum. Two smears per femur were prepared and coded. The smears were studied by two different examiners. One-thousand polychromatic erythrocytes per smear were examined for the presence of micronuclei.

Statistical analysis

LD_{50} doses and their 95% confidence limits were calculated using the method of Cavalli-Sforza and Lorenz (1964); significant testing of the micronucleus results was done using the Student's *t* test (Hill, 1967).

Results

The highest toxicity was found with BB and the least toxicity with 1,3,5-TBC, when the median lethal doses were determined (Table I). In general, trichlorobenzenes were less toxic to the animals than dichlorobenzenes (Table I).

All the halogenated benzenes tested were found to be clastogenic ($P < 0.01$) (Table II). The highest clastogenic activities were observed in animals dosed with *m*-DCB and BB. Among the three isomers of DCB, *m*-DCB induced significantly ($P < 0.05$) more micronuclei than *o*-DCB or *p*-DCB (Table II). No significant differences were found between the clastogenic activities of TCB-isomers (Table II).

Discussion

Although the chromosome-damaging effects of benzene in marrow cells of mammals, including man, and the capacity of

Table I. Median lethal doses (LD_{50}) of benzene and eight halogenated benzenes for 25–30 g male NMRI mice by i.p. administration. Purities of the compounds are those given by the manufacturer

Compound	Purity (%)	LD_{50} mg/kg body weight
Benzene	99.7	1714.24 ± 101.37
Bromobenzene	99.0	817.11 ± 50.71
Chlorobenzene	99.0	1355.24 ± 250.55
m-Dichlorobenzene	99.0	1061.84 ± 38.73
o-Dichlorobenzene	99.0	1228.12 ± 262.99
p-Dichlorobenzene	99.0	2000.42 ± 49.70
1,2,3-Trichlorobenzene	99.0	1390.21 ± 225.98
1,2,4-Trichlorobenzene	98.0	1223.25 ± 320.54
1,3,5-Trichlorobenzene	98.0	2259.83 ± 76.96

Table II. Effects of eight halogenated benzenes on frequency of micronucleated polychromatic erythrocytes of femoral bone marrow of male NMRI mice (significant value for all doses: $P < 0.01$). Values represent means ± SD of five individual experiments per dose

Compound	mg/kg body weight	Micronuclei/ 1000 PCE
Vehicle (corn oil)		1.80 ± 0.748
Benzene	264 (2 × 132)	4.40 ± 0.800
	528 (2 × 264)	8.10 ± 0.943
	1056 (2 × 528)	12.40 ± 1.356
	528 (1 × 528)	10.83 ± 1.343
Bromobenzene	125 (2 × 62.5)	3.70 ± 0.640
	240 (2 × 125)	5.50 ± 0.806
	375 (2 × 187.5)	6.50 ± 1.024
	500 (2 × 250)	8.30 ± 1.100
Chlorobenzene	225 (2 × 112.5)	3.10 ± 0.830
	450 (2 × 225)	3.90 ± 0.830
	675 (2 × 337.5)	4.90 ± 0.943
	900 (2 × 450)	7.20 ± 0.871
m-Dichlorobenzene	175 (2 × 87.5)	3.40 ± 0.663
	350 (2 × 175)	4.40 ± 0.916
	525 (2 × 262.5)	6.30 ± 1.100
	700 (2 × 350)	9.20 ± 1.248
o-Dichlorobenzene	187 (2 × 93.5)	3.90 ± 0.943
	375 (2 × 187.5)	4.50 ± 1.204
	562 (2 × 281)	5.44 ± 1.257
	750 (2 × 375)	5.90 ± 1.135
p-Dichlorobenzene	355 (2 × 177.5)	4.00 ± 0.632
	710 (2 × 355)	4.90 ± 1.135
	1065 (2 × 532.5)	6.00 ± 1.000
	1420 (2 × 710)	6.60 ± 0.916
1,2,3-Trichlorobenzene	250 (2 × 125)	4.10 ± 0.943
	500 (2 × 250)	5.20 ± 1.249
	750 (2 × 375)	6.80 ± 0.979
	1000 (2 × 500)	7.30 ± 1.005
1,2,4-Trichlorobenzene	210 (2 × 105)	3.50 ± 0.806
	420 (2 × 210)	4.50 ± 0.806
	630 (2 × 315)	5.80 ± 0.871
	840 (2 × 420)	7.40 ± 0.916
1,3,5-Trichlorobenzene	425 (2 × 212.5)	3.60 ± 0.800
	850 (2 × 425)	4.40 ± 0.916
	1275 (2 × 637.5)	6.60 ± 0.894
	1700 (2 × 850)	7.20 ± 0.979

benzene to induce myelogenous leukaemia and other blood dyscrasias in rodents and supposedly in man have been well documented (Diaz *et al.*, 1980; Meyne and Legator, 1980; Snyder *et al.*, 1980; Goldstein and Snyder, 1982; Harper *et al.*, 1984; Cronkite *et al.*, 1985; Styles and Richardson, 1984; Erexson *et al.*, 1986; Dean, 1985; Infante and White, 1985), little adequate information exists on the carcinogenic/mutagenic effects of halogenated benzenes.

Our data demonstrate the clastogenic activity of these compounds (Table II) and indicate the desirability of further testing of benzene derivatives in order to assess the mutagenic hazard and carcinogenic potential associated with the use of this class of compounds.

The number of human cases of leukaemia associated with DCB-exposure is too small to assess a causal relationship (IARC, 1982b). This observation and the possibility of weak mutagenic activity of chlorinated benzenes (IARC, 1982a) suggest, however, that they may possess the ability to bind to DNA and cause genetic alterations in cells. BB has been found to bind covalently to DNA/RNA in mouse and rat liver cells at rates comparable to those of some moderate oncogenic compounds (Colacci *et al.*, 1985). In this connection, the negative results of Wester *et al.*, 1985) following lifetime carcinogenicity testing of chlorinated benzenes on rat may not be a very reliable assessment of the carcinogenicity of chlorobenzene (CB) isomers. This view is based on a fact that a mixture of 11 halogenated hydrocarbons (including six CB isomers) was tested together rather than testing each compound separately. Although Lai (1984), in a review article, cites a study conducted by the US National Toxicology Program implying that monochlorobenzene is a rat carcinogen, the results of this study do not appear to have been published.

Further studies are in progress to identify the metabolite(s) and the mechanism(s) involved in the induction of clastogenic effects in bone marrow cells of mice by halogenated benzenes.

Acknowledgements

Our thanks are due to Ms. I.Bäcker-Bödecker from the Institute of Informatic and Biomathematics of University Medical Center of Essen for her assistance in computer programming and analysis of data. We are also grateful to Karin Wöhrmeyer for her valuable technical assistance, and to Iris Funke-Kaiser and Doris Münz for expert typing of the manuscript.

References

Cavalli-Sforza,L. and Lorenz,R.J. (1964) Grundbegriffe der Biometrie insbesondere der statistischen Methoden bei der Wertbemessung biologisch wirksamer Substanzen. Gustav Fischer Verlag, Suttgart, pp. 171–177.

Colacci,A., Arfellini,G., Mazzullo,M., Prodi,G. and Grilli,S. (1985) The covalent binding of bromobenzene with nucleic acids. *Toxicol. Pathol.*, **13**, 276–282.

Cronkite,E.P., Drew,R.T., Inoue,T. and Bullis,J.E. (1985) Benzene hematotoxicity and leukemogenesis. *Am. J. Ind. Med.*, **7**, 447–456.

Dean,B.J. (1985) Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. *Mutat. Res.*, **154**, 153–181.

Diaz,M., Reiser,A., Bruier,L. and Diez,J. (1980) Studies on benzene mutagenesis. I. The micronucleus test. *Experientia*, **36**, 297–298.

Erexson,G.L., Wilmer,J.L., Steinhagen,W.H. and Klingerman,A.D. (1986) Induction of cytogenetic damage in rodents after short-term inhalation of benzene. *Environ. Mutagenesis*, **8**, 29–40.

Fowler,I.S.L. (1983) Short-term testing in drug development. In Balls,M., Riddell,R.J. and Worden,A.N. (eds), *Animals and Alternatives in Toxicity Testing*. Academic Press, London, pp.16–23.

Giavini,E., Broccia,M.L., Prati,M. and Vismara,C. (1986) Teratologic evaluation of *p*-dichlorobenzene in the rat. *Bull. Environ. Contam. Toxicol.*, **37**, 164–168.

Goldstein,B.D. and Snyder,C.A. (1982) Benzene leukemogenesis. *Environ. Sci.*

Res., 25, 2778–2789.
Harper,B.L., Sadagopa Ramanujam,V.C., Gad-El-Karim,M.M. and Legator,M.S. (1984) The influence of simple aromatics on benzene clastogenicity. *Mutat. Res.*, 128, 105–114.
Hayes,W.C., Hanley,T.R., Gushow,T.S., Johnson,K.A. and John,J.A. (1985) Teratogenic potential of inhaled dichlorobenzenes in rats and rabbits. *Fund. Appl. Toxicol.*, 5, 190–202.
Hill,A.B. (1967) The 't' test for small numbers. In Hill,A.B. (ed.), *Principles of Medical Statistics*. The Lancet Ltd., London, pp. 146–151.
IARC (1982a) Monographs on the evaluation of the carcinogenic risks of chemicals to humans. *Some Industrial Chemicals and Dyestuffs*. Vol. 29, pp. 213–238.
IARC (1982b) Monographs on the evaluation of the carcinogenic risk of chemicals to humans. *Chemicals, Industrial Processes and Industries Associated with Cancer in Human*. Vol. 29 (Suppl. 4), pp. 108–109.
Infante,P.F. and White,M.C. (1985) Projections of leukemia risk associated with occupational exposure to benzene. *Am. J. Ind. Med.*, 7, 403–413.
Jan,J. (1983) Chlorobenzene residues in human fat and milk. *Bull. Environ. Contam. Toxicol.*, 30, 595–599.
Lai,D.Y. (1984) Halogenated benzenes, naphthalenes, biphenyls and terphenyls in the environment: their carcinogenic, mutagenic and teratogenic potential and toxic effects. *J. Environ. Sci. Health*, 2, 135–184.
Meyne,J. and Legator,M.S. (1980) Sex-related differences in cytogenetic effects of benzene in the bone marrow of Swiss mice. *Environ. Mutagenesis*, 2, 43–50.
Mohtashamipur,E., Norpoth,K., Woelke,U. and Huber,P. (1985) Effects of ethylbenzene, toluene, and xylene on the induction of micronuclei in bone marrow polychromatic erythrocytes of mice. *Arch. Toxicol.*, 58, 106–109.
Pellizzari,E.D. (1979) Quantification of chlorinated hydrocarbons in previously collected air samples. *Gov. Rep. Announce. Index (US)*, 79(8), 68.
Schmid,W. (1975) The micronucleus test. *Mutat. Res.*, 31, 9–15.
Simmon,V.F., Rosenkranz,H.S., Zeiger,E. and Poirier,A. (1979) Mutagenic activity of chemical carcinogens and related compounds in the intraperitoneal host-mediated assay. *J. Natl. Cancer Inst.*, 62, 911–918.
Snyder,C.A., Goldstein,B.D., Sellakumar,A.R., Bromberg,I., Laskin,S. and Albert,R.E. (1980) The inhalation toxicology of benzene: incidence of hematopoietic neoplasms and hematotoxicity in AKR/J and C57BL/6J mice. *Toxicol. Appl. Pharmacol.*, 54, 323–331.
Styles,J.A. and Richardson,C.R. (1984) Cytogenetic effects of benzene: dosimetric studies on rats exposed to benzene vapour. *Mutat. Res.*, 135, 203–209.
Wester,P.W., van der Heijden,C.A., Bisschop,A., van Esch,G.J., Wegman,R.C. and de Vries,T. (1985) Carcinogenicity study in rats with a mixture of eleven volatile halogenated hydrocarbon drinking water contaminants. *Sci. Total Environ.*, 47, 427–432.
Zoeteman,B.C.J., Piet,G.J., Kussmaul,H. and Muehlhausen,D. (1979) Behavior of organic water components during drinking water preparation, especially in groundwater enrichment. *Reinhalt. Wasser*, 1, 89–106.

Received on December 17, 1986; accepted on January 29, 1987

Environmental Mutagenesis Supplement 1:3–142 (1983)

Salmonella Mutagenicity Test Results for 250 Chemicals

Steve Haworth, Timothy Lawlor, Kristien Mortelmans, William Speck, and Errol Zeiger

Genetic Toxicology Department, Microbiological Associates, Bethesda, Maryland (S.H., T.L.), Microbial Genetics Department, SRI International, Menlo Park, California (K.M.), Department of Pediatrics, Case Western Reserve University, Cleveland (W.S.), and Cellular and Genetic Toxicology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina (E.Z.)

Key words: Salmonella, mutagenicity, Ames, testing

INTRODUCTION

The Salmonella/mammalian microsome test developed by Ames and his associates [Ames et al, 1975] has become widely accepted as an initial test for the identification of chemicals with mutagenic activity. Substances that produce a positive mutagenic effect are considered to be potential animal mutagens and carcinogens and, by extension, potential human mutagens and carcinogens. This is because of the reported high correlations between mutagenicity in Salmonella, and genetic and carcinogenic effects in mammalian systems [McCann et al, 1975; Sugimura et al, 1976; Purchase et al, 1978; Rinkus and Legator, 1979; Bartsch et al, 1980]. However, chemicals that are not mutagenic in Salmonella cannot be considered benign. It has been well documented [McCann et al, 1975; Rinkus and Legator, 1979] that certain chemical classes, such as chlorinated hydrocarbons, contain a large number of carcinogens that are not mutagenic to Salmonella. Other chemicals are negative in the standard plate or preincubation protocol but are positive in protocols modified to achieve improved metabolism of the chemical to a mutagen or optimal exposure of the cells to the mutagen. Examples of these are the requirement for flavin mononucleotide (FMN) and reducing conditions or riboflavin for benzidine-based or other azo dyes [Prival and Mitchell, 1982; Hartman et al, 1978; Sugimura et al, 1977; Robertson et al, 1982], or the need for testing volatile liquids in a sealed chamber [Simmon et al, 1977].

Received June 29, 1983; revised and accepted September 20, 1983.

Address correspondence to Dr. Errol Zeiger, Cellular and Genetic Toxicology Branch, N.I.E.H.S., P.O. Box 12233, Research Triangle Park, NC 27709.

Single copies of this Supplement will be available for purchase directly from the publisher: Alan R. Liss, Inc., Subscription Department, 150 Fifth Avenue, New York, NY 10011. Please do *not* address reprint requests to the authors.

© 1983 Alan R. Liss, Inc.

The testing of large numbers of chemicals for mutagenicity in Salmonella and other test systems is being performed within the National Toxicology Program (NTP) [Zeiger and Drake, 1980]. All chemicals selected for genetic toxicology screening are initially tested in Salmonella using a preincubation procedure [Yahagi et al, 1975], which is a modification of the Salmonella/mammalian microsome test of Ames et al [1975]. Additional tests—Drosophila melanogaster sex-linked recessive lethal and heritable translocation tests, induction of chromosome aberrations and sister chromatid exchanges (SCEs) in Chinese Hamster ovary (CHO) cells, and induction of mutation in L5178Y mouse lymphoma cells—are performed on selected chemicals based upon the needs of the NTP and results obtained from other mutagenicity test systems and carcinogenesis tests. The results obtained in these short-term mutagenicity tests will be used by the NTP in the prioritization of chemicals for subchronic or chronic toxicity testing, to assist in the evaluation of rodent test data, and to alert the cognizant government agencies and industries about potentially hazardous chemicals. A number of the chemicals were already reported by other workers as having been tested in Salmonella. Some appeared in the literature after the NTP program had begun; others were tested to develop a NTP data base for these chemicals or because they were members of a larger class of chemicals of interest.

The Salmonella tests were performed by three laboratories: Case Western Reserve University (CWR), Dr. William Speck; Microbiological Associates (formerly EG&G Mason Research Institute [EGG]), Dr. Steve Haworth; and SRI International (SRI), Dr. Kristien Mortelmans. Salmonella strains TA98, TA100, TA1535, and TA1537 were used in a modification of the preincubation test of Yahagi et al [1975]. The preincubation procedure was selected because of reports that it was no less sensitive than the plate test, and was more effective than the plate test for various chemicals such as aliphatic nitrosamines [Prival et al, 1979; Yahagi et al, 1975], pyrrolizidine alkaloids [Yamanaka, et al, 1979], and volatile chemicals [Rosenkranz et al, 1980]. Liver S-9 was prepared from male Sprague-Dawley rats (RLI) and Syrian hamsters (HLI) that were induced with Aroclor 1254 [Ames et al, 1975]. Hamster liver was used because of indications from a prior study (unpublished) and reports that the use of hamster S-9 would detect a number of chemicals undetected with rat S-9 [Prival and Mitchell, 1981; Bartsch et al, 1975; Sugimura, personal communication]. The protocol was standardized among the three laboratories, as discussed below. Each chemical was coded and tested as an unknown; the primary purpose of each test was to determine whether or not the chemical was mutagenic. This is why, in the case of a positive result, only the strains and activation systems that gave the positive results were repeated, not the entire series. The protocol was designed to allow the individual investigators the flexibility to change doses based on their interpretations of the results of the initial experiment. To monitor the performance of each laboratory, a set of positive and negative control chemicals was chosen. These chemicals were included, on a random basis, in batches of coded test chemicals sent to the testing laboratories. In addition to these controls, a small number of test chemicals selected, at random, were resubmitted to the same laboratory or sent to a second laboratory to determine interlaboratory reproducibility.

This publication is a presentation of Salmonella testing results on 250 coded chemicals, encompassing 370 tests (see Table I). The majority of these results were previously summarized in issues of the National Toxicology Program Technical Bulletin [1980a,b, 1981a,b, 1983]. However, some interpretations were changed since

publication in the NTP Bulletin, based upon a reevaluation of the data. The presentation here is designed both to summarize the results in the text and to present the data (Appendix 2) so that the reader has the opportunity of performing an independent evaluation of the data. Results from additional chemicals will be presented in future publications.

MATERIALS AND METHODS

Chemicals

The chemicals tested, their source, and purity (where known) are listed in Table I and their structures are given in Appendix 1. They were supplied to the testing laboratories by a chemical repository (Radian Corporation, Austin, TX), which was responsible for purchase and inventory of the chemicals, collection of physical, toxicological and safety data for each chemical, coding each test sample, shipment to the testing laboratories, and chemical analyses (when requested). Each sample sent out by the repository carried a unique, six-digit code number (Aliquot number) so that it could be tested under code as an unknown. The laboratories were also supplied with available information on the volatility, density, solubility, flammability, and stability of each chemical; Radian only performed solubility tests. Also sent, but in a sealed envelope coded with the Aliquot number, was the chemical name(s) along with the available information on its toxicological effects and decontamination procedures. The laboratories were instructed to open this envelope only in the event of a spill or exposure to the chemical and to treat all coded chemicals as potential mutagens and carcinogens. After completion of the testing, the unopened envelopes were returned to the Radian Corporation.

All chemicals were stored at the testing laboratories as recommended by the chemical repository. Each chemical was dissolved and diluted immediately prior to testing. The solvent of choice was distilled water; dimethyl sulfoxide (DMSO) was used if the chemical was insoluble or not sufficiently soluble in water. Ethanol (95%) or acetone was used if the chemical was not soluble or stable in DMSO.

As a rule, if a chemical was mutagenic or gave a questionable response, it was analyzed by Radian for identity and purity. Analyses had been performed previously by Midwest Research Institute (MRI) on selected other chemicals and on chemicals that had been tested in the National Cancer Institute's Carcinogenesis Bioassay Program. The results of these analyses are in Table I.

Bacterial Strains

Salmonella typhimurium strains TA1535, TA1537, TA98, and TA100 were obtained by the individual laboratories from Dr. Bruce Ames, University of California, Berkeley. Cultures of each tester strain were prepared for storage essentially as described in the Supplement to the Methods Paper [Ames et al, 1975] supplied with the tester strains by Dr. Ames. Frozen cultures were stored in liquid nitrogen (EGG) or in a −70°C freezer (CWR, SRI) in 0.2-ml aliquots (EGG), or in 1-ml aliquots (SRI) in sterile, screw-cap vials. To inoculate overnight cultures, CWR transferred a loopful of cells that were maintained on Columbia agar slants kept at 4°C into Columbia broth. EGG transferred a loopful of the thawed cultures into Oxoid Nutrient Broth #2 (CM 67) and discarded the unused portion of the thawed culture. SRI used all of the thawed 1-ml culture to inoculate minimal glucose medium [Vogel and

TABLE I. Sources, Purities, and Mutagenicities of 250 Chemicals in Salmonella (Continued)

	Chemical name	CAS number	Chemical source	Lot number
194	Pentachloroethane	76-01-7	Aldrich	CE 072487
195	Pentachloronitrobenzene	82-68-8	Aldrich	AC 071177
196	Pentachlorophenol	87-86-5	Aldrich	CC 022487
197	Phenol	108-95-2	Fluka; Textile Chem.	187130280; 79380
198	o-Phenyl phenol	90-43-7	Dow Chemical	MM09157
199	Phosphamidon	13171-21-6	Chevron	—
200	β-Picoline	108-99-6	MC/B	IIJ28
201	Picric acid	88-89-1	MC/B	8H09A
202	Piperazine	110-85-0	Eastman	A7C
203	Piperonal	120-57-0	Eastman	A8X
204	Piperonyl butoxide	51-03-6	FNC	—
205	Piperonyl sulfoxide	120-62-7	CPC International	291-N100-12
206	Prednisone	53-03-2	Upjohn	52836
207	β-Propiolactone	57-57-8	Tridom	196050 1178
208	1,2-Propylene glycol	57-55-6	MC/B	C6J13
209	Pyridine	110-86-1	Fisher	732113
210	Pyrimethamine	58-14-0	Burroughs-Wellcome	51416
211	Quinoline	91-22-5	Eastman	D6H
212	Resorcinol (1,3-benzenediol)	108-46-3	MC/B	C3J23
213	Ricinoleic acid, Na salt	5323-95-5	MC/B	D6F04
214	Semicarbazide HCl	563-41-7	Aldrich	EC 050287
215	Sodium fluoride	7681-49-4	Aldrich	DC 030887
216	Sodium phosphate, dibasic	7558-79-4	Fisher	783607
217	cis-Stilbene	645-49-8	Pfaltz & Bauer	—
218	trans-Stilbene	103-30-0	Aldrich	JC 072087
219	Streptomycin sulphate (2:3)	3810-74-0	Sigma	107C-0168
220	Sulfallate	95-06-7	Monsanto	H5215
221	1,2,3,4-Tetrachlorobenzene	634-66-2	Pfaltz & Bauer	—
222	1,2,3,5-Tetrachlorobenzene	634-90-2	Aldrich	101777
223	1,2,4,5-Tetrachlorobenzene	95-94-3	Pfaltz & Bauer	—
224	1,1,1,2-Tetrachloroethane	630-20-6	Aldrich	KE 7912KE
225	1,1,2,2,-Tetrachloroethane	79-34-5	Aldrich	060207
226	Tetrachloroethylene	127-18-4	Fisher	772783
227	1,2,3,4-Tetrachloronaphthalene	20020-02-4	Aldrich	DC 052347
228	2,3,4,5-Tetrachloronitrobenzene	879-39-0	Aldrich	090777
229	2,3,5,6-Tetrachloronitrobenzene	117-18-0	Aldrich	BB 120557
230	Tetramethyl lead	75-74-1	Ethyl Corporation	—
231	Thiocarbanilide	102-08-9	MC/B	11E22
232	Titanocene dichloride	1271-19-8	Pfaltz & Bauer	—
233	Toluene	108-88-3	Fisher	782825
234	Tributoxyethyl phosphate	78-51-3	Pfaltz & Bauer	—
235	Tributyl borate	688-74-4	MC/B	5738
236	1,2,3-Trichlorobenzene	87-61-6	Aldrich	HC 060787
237	1,2,4-Trichlorobenzene	120-82-1	MC/B	B3J08
238	1,3,5-Trichlorobenzene	108-70-3	Aldrich	KC 081087
239	1,1,1-Trichloroethane (chlorothene)	71-55-6	Fluka	31086 680
240	2,4,5-Trichlorophenol	95-95-4	Aldrich	JD 02197
241	2,4,6-Trichlorophenol	88-06-2	Eastman	B7X
242	1,2,3-Trichloropropane	96-18-4	Shell Chemical	JG 32449

TABLE I. Sources, Purities, and Mutagenicities of 250 Chemicals in Salmonella (Continued)

	Chemical name	CAS number	Chemical source	Lot number
243	Tricresyl phosphate (tritolyl phosphate)	1330-78-5	MC/B	C3J16
244	Tri-m-cresyl phosphate (TMCP)	563-04-2	Pfaltz & Bauer	T20605
245	Tris(2-chloroethyl)phosphate	115-96-8	Stauffer	0101F-1-3
246	Tris(2-chloroethyl)phosphite	140-08-9	Aldrich	KC 073187
247	m-Xylene	108-38-3	Eastman	B7B
248	o-Xylene	95-47-6	Aldrich	HD 061297
249	p-Xylene	106-42-3	Aldrich	CD 030197
250	Ziram	137-30-4	Uniroyal	—

[a]CWR, Case Western Reserve University; EGG, EG&G Mason Research Institute; SRI, SRI International.
[b]+, Positive, −, negative; ?, equivocal.
[c]None available.
[d]Purity based on Radian analysis.
[e]Purity based on MRI analysis.
[f]Two different lots tested.

Bonner, 1956] supplemented with biotin and an excess of histidine. All overnight cultures (late log phase) were obtained by incubation at 37°C on a shaker for 12–15 hr and were routinely checked for genetic integrity as recommended by Ames et al [1975].

Preparation of S-9 Fraction

Liver S-9 fractions were routinely prepared from male Sprague-Dawley rats and male Syrian hamsters that were injected, ip, with Aroclor 1254 (200 mg/ml in corn oil) at 500 mg/kg. Five days after injection, the animals were sacrificed by decapitation (EGG, SRI) or cervical dislocation (CWR) and the livers were removed aseptically. The animals were fasted for 12–24 hr immediately preceding sacrifice.

Liver homogenates were prepared aseptically at 0–4°C. Excised livers were rinsed with 0.15 M KCl, then minced and homogenized (3 ml of 0.15 M KCl/g wet tissue) in a Potter-Elvehjem apparatus with a teflon pestle (EGG, SRI) or in a Waring blender (CWR). The homogenate was centrifuged for 10 min at 9,000g at 4°C. The supernatant (S-9) was decanted and distributed into freezing ampules and stored at −70°C.

The microsomal enzyme reaction mix (S-9 mix) was prepared immediately prior to each assay. Unused S-9 mix was discarded and not refrozen. One milliliter of S-9 mix has the following composition: S-9, 0.10 ml; 0.04 M $MgCl_2$, 0.02 ml; 1.65 M KCl, 0.02 ml; 0.04 M β-nicotinamide adenine dinucleotide phosphate (NADP), 0.10 ml; 0.05 M glucose-6-phosphate, 0.10 ml; 1.0 M NaH_2PO_4 (pH 7.4), 0.10 ml; and distilled water, 0.56 ml.

Preincubation Methodology

All chemicals were tested using the preincubation procedure of the Salmonella assay [Ames et al, 1975] as described by Yahagi et al [1975]. Briefly, 0.5 ml of S-9

Label purity	Analyzed purity	Testing lab	Test result	
Technical		CWR,SRI	−,−	243
—		SRI	−	244
99.5%	98.2%[c]	SRI	−	245
—	87.8%[d]	CWR,SRI	+,+	246
Practical		EGG	−	247
97%		EGG	−	248
99%		EGG	−	249
—	86.2%[c]	SRI	+	250

mix or 0.1 M PO_4 buffer was dispensed into an appropriate number of 13 × 100 mm culture tubes maintained at 37°C in a dry-bath. Then, 0.05 ml of cells and 0.05 ml of solvent or chemical dilution were added to each tube. The mixture was vortexed and allowed to incubate with shaking in the early tests (CWR, EGG), or standing (SRI) for 20 min at 37°C. The protocol was later changed to eliminate the shaking procedure, because the commercial shakers available would not fit in the Class II, Type B hoods and, for the purposes of laboratory safety, it was inadvisable to incubate the chemicals at 37°C in the open laboratory. Following the preincubation period, 2.5 ml (EGG) or 2.0 ml (CWR, SRI) of molten top agar (45°C) supplemented with 0.5 mM L-histidine and 0.5 mM d-biotin was pipetted into the tubes, which were immediately vortexed, and their contents poured onto 25 ml of minimal glucose bottom agar [Vogel and Bonner, 1956] in a 15 × 100-mm plastic petri dish (Falcon Muta-Assay, 1028 [EGG, SRI] or Fisher Scientific petri dishes [CWR]). After the overlay solidified, the plates were inverted and incubated at 37°C for 48 h.

At least five doses of test chemical, in addition to the concurrent solvent and positive controls, were tested on each strain in the presence of S-9 mix or buffer. Three plates were used, and the experiment was repeated no less than 1 week after completion of the initial test.

Dose-Setting Experiment

To select the dose range for the mutagenesis assay, the test chemicals were checked for toxicity to TA100 up to a concentration of 10 mg/plate or the limit of solubility, both in the presence and absence of S-9 mix. One or more parameters were used as an indication of toxicity: viability on complete medium (EGG) and reduced numbers of revertant colonies per plate and/or thinning or absence of the bacterial lawn (CWR, EGG, SRI). If toxicity was not apparent in the preliminary toxicity determination, the highest dose tested was 10 mg/plate; otherwise the upper limit of solubility was used. If toxicity was observed, the doses of test chemical were chosen so that the high dose exhibited some degree of toxicity. Occasionally, in the earlier tests, the high dose was greater than 10 mg/plate.

Positive Controls

The positive control chemicals were tested concurrently with each test chemical. 2-Aminoanthracene (2-AA) was tested on all strains in the presence of rat and hamster S-9. 4-Nitro-o-phenylenediamine (NOPD) was tested on TA98 without S-9. Also without S-9, sodium azide (SA) was tested on TA100 and TA1535, and 9-aminoacridine (9-AAD) was tested on TA1537. The actual concentration for each positive control chemical used for each strain and activation condition was selected by the individual laboratory based on dose-response curves generated at the beginning of the testing program. The doses of the positive controls used by each laboratory are given in Table II.

Data Evaluation

Although procedures for the statistical analysis of Salmonella plate test data have been developed [Margolin et al, 1981], they were not incorporated into the initial data evaluations. The data were evaluated in an ad hoc manner by each testing laboratory and by NTP personnel. Prior to statistical analysis no formal rules were used; however, a positive response was indicated by a reproducible, dose-related increase, whether it be twofold over background or not. The matrix of test strains and activation systems used allowed the investigators to detect trends or patterns that might not be as evident if only one strain and activation system were examined. In addition to the standard "positive" and "negative" categories, there is also "questionable" (or "inconclusive"). This applied to low-level responses that were not reproducible within the laboratory or to results that showed a definite trend but with which the investigator did not feel comfortable in making a "+" or "−" decision. It also included tests in which an elevated revertant colony yield occurred at only a single dose level. After a decision on the mutagenicity of a sample was made, a request to decode the sample was sent to the repository, and the code was broken. The data were subsequently evaluated using an analysis based on the models presented by Margolin et al [1981]; this analysis will be described elsewhere [Risko et al, manuscript in preparation]. As a result of these statistical analyses, a number of calls were changed from the original "negative" to "equivocal." The statistical analysis did not result in any "positive" or "equivocal" calls being called "negative."

Because the criteria for "positive" or "questionable" decisions have evolved during the course of this study and because of the recent use of statistical analysis,

TABLE II. Concentrations of Positive Control Chemicals (μg/plate)

	TA98		TA100		TA1535		TA1537	
	−S-9 (NOPD)[a]	+S-9 (2-AA)	−S-9 (SA)	+S-9 (2-AA)	−S-9 (SA)	+S-9 (2-AA)	−S-9 (9AAD)	+S-9 (2-AA)
CWR	3.3	1.0	3.2	1.0	3.3	2.0	33.0	2.0
EGG	12.0	RLI 1.5[b] HLI 0.75	2.5	RLI 1.5 HLI 0.75	2.5	RLI 1.5 HLI 0.75	80.0	RLI 1.5 HLI 0.75
SRI	5.0	1.0	1.0	1.0	1.0	2.5	50.0	2.5

[a]NOPD, 4-nitro-o-phenylenediamine; 2-AA, 2-aminoanthracene; SA, sodium azide; 9AAD, 9-aminoacridine.
[b]Different concentrations for each S-9 source.