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Phthalate Ester Testing in the National Toxicology Program's Environmental Mutagenesis Test Development Program

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A number of phthalate esters and related chemicals were tested for mutagenicity in *Salmonella typhimurium*. The chemicals were tested blind in three laboratories by a preincubation modification of the Ames Salmonella/mammalian microsome test using S-9 prepared from Aroclor-induced rats and Syrian hamsters. All chemicals tested were judged to be nonmutagenic.

Introduction

The Cellular and Genetic Toxicology Branch of the National Toxicology Program (NTP) is responsible for developing and validating new and established mutagenicity test protocols and methodologies and for testing chemicals of interest to the NTP for mutagenicity (*1*). All chemicals entering the program are tested in *Salmonella typhimurium* for the induction of gene mutations, and selected chemicals are tested further in *Drosophila melanogaster* for the induction of sex-linked recessive lethal mutations and reciprocal translocations, in cultured Chinese hamster ovary cells for the induction of chromosome aberrations and sister chromatid exchanges, or other test systems.

A series of phthalate esters and related chemicals were selected for testing in Salmonella and other systems. This report lists chemicals selected for testing and presents the Salmonella test results from those chemicals for which testing has been completed. All tests reported here were done by three different laboratories under contract to the NTP: EG&G Mason Research Institute, Rockville, Maryland, under the direction of Dr. Stephen Haworth; Case Western Reserve University, Cleveland, Ohio, under the direction of Dr. William Speck; and SRI International, Menlo Park, California, under the direction of Dr. Kristien Mortelmans.

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Methods

Protocol

All testing was done using a preincubation modification of the method of Ames et al. (*2*). Briefly, *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 (obtained from B. N. Ames, Berkeley, Calif.) were used with 9000 *g* liver homogenate preparations (S-9) from Aroclor 1254-induced male Sprague-Dawley rats and Syrian hamsters prepared according to the method of Ames et al. (*2*). A 100 μl portion of an overnight culture of cells was mixed with either 0.5 ml of S-9 mix or 0.1*M* pH 7.4 phosphate buffer, and 50 or 100 μl of test chemical or solvent in a tube. This mixture was incubated at 37°C for 20 min; 2.0 ml of top agar was added and the mixture poured onto a minimal agar plate and incubated at 37°C for 2 days, after which the histidine revertant colonies were machine counted.

Each chemical was tested at five doses separated

Table 1. Phthalates and related chemicals tested in Salmonella.

Name	CAS #	Supplier (purity/grade)	Laboratory[a]	Solvent	Dose range (μg/plate)
Butyl benzyl phthalate	85-68-7	Monsanto (97%)[b]	EGG	DMSO	100-10000
			CWR	"	333-11550
Butyl cyclohexyl phthalate	84-64-0	—[c]			
Diallyl phthalate	131-17-9	Hardwicke (99%)	CWR	DMSO	100-10000
			EGG	DMSO	1-10000
Dibutyl phthalate	84-74-2	Aldrich (99%)	EGG	DMSO	100-10000
Dicyclohexyl phthalate	84-11-7	—			
Di(2-ethylhexyl) adipate	103-23-1	Pfaltz & Bauer (N.S.)[d]	EGG	95% EtOH	100-10000
			SRI	DMSO	100-10000
Di(2-ethylhexyl) phthalate	117-81-7	MC/B (Practical)	EGG	DMSO	100-10000
			CWR	DMSO	100-10000
		WR Grace (99.5%)[b]	SRI	DMSO	100-10000
			EGG	95% EtOH	100-10000
Diethyl phthalate	84-66-2	Chem. Tech. Ind. (99.9%)[b]	EGG	DMSO	10-3333
			CWR	DMSO	100-10000
Di(*n*-hexyl) phthalate	84-75-3	—			
Diisobutyl phthalate	84-69-5	Eastman (Reagent)	EGG	DMSO	100-10000
Diisodecyl phthalate	26761-40-0	Pfaltz & Bauer (NS)	SRI	95% EtOH	100-10000
			EGG	95% EtOH	100-10000
Diisononyl phthalate	28553-12-0	—			
Dimethyl phthalate	131-11-3	Aldrich (99%)	EGG	DMSO	33-6666
Dimethyl terephthalate	120-61-6	Aldrich (99%)	EGG	DMSO	3.3-333
Dioctyl phthalate	117-84-0	Eastman (98%)	SRI	DMSO	100-10000
Ditridecyl phthalate	119-06-2	Polysciences (NS.)	EGG	Acetone	100-10000
Diundecyl phthalate	3648-20-2	—			
2-Ethoxyethanol	110-80-5	Dow Chem. (99%)[b]	EGG	Water	100-10000
2-Ethyl-l-hexanol	104-76-7	Aldrich (99%)	EGG	DMSO	3.3-333
Mono(2-ethylhexyl) adipate	4337-65-9	—			
Mono(2-ethylhexyl) phthalate	4376-20-9	—			
Phthalamide	88-96-0	Sherwin Williams (99+%)[b]	EGG	DMSO	33-1500
Phthalic anhydride	85-44-9	Aldrich (99+%)	EGG	DMSO	1-666
Terephthalic acid	100-21-0	Eastman (98%)	SRI	DMSO	100-10000
Tetrachlorophthalic anhydride	117-08-8	MC/B (Prac.)	CWR	DMSO	33-6666.7
Tris(2-ethylhexyl)phosphate	78-42-2	Chem. Systems Lab (97.6%)[b]	CWR	DMSO	100-10000
Tris(2-ethylhexyl)phosphite	301-13-3	Pfaltz & Bauer (NS)	CWR	DMSO	100-8270

[a]Laboratory: EGG: EG&G Mason Research Institute; CWR: Case Western Reserve University; SRI: SRI International.
[b]Sample used for carcinogenesis bioassay.
[c]Chemical not yet received.
[d]NS = purity/grade not specified.

clearing of the background/lawn), the preferred high dose was 10 mg/tube. All tests were repeated at least one week following completion of the test. The positive controls, dissolved in DMSO, were without S-9: for TA98, 4-nitro-*o*-phenylenediamine; for TA100 and TA1535, sodium azide; for TA1537, 9-aminoacridine; with S-9 for all strains, 2-aminoanthracene. The solvents used were water, dimethyl sulfoxide or 95% ethanol. Concurrent positive and solvent controls were run with all experiments.

Chemicals

The chemicals selected for testing, supplier and purity or grade are listed in Table 1. Butyl benzyl phthalate, di(2-ethylhexyl) phthalate, diethyl phthalate, 2-ethoxyethanol, phthalamide, and tris(2-ethylhexyl) phosphate were obtained from the NCI Bioassay Repository; the other chemicals were purchased commercially.

Coding

All chemicals were tested as coded aliquots and were sent to the testing laboratories interspersed with unrelated chemicals, including known mutagens and nonmutagens, so that the testing laboratories were unaware of the identity of the chemicals or chemical classes under test. All testing and evaluation of data for any aliquot was completed and a decision made before the chemical was decoded. A positive mutagenic response was defined as a reproducible, dose-related increase in his^+ revertants over the spontaneous level.

Results

Testing has been completed in Salmonella for 20 chemicals, six of which were tested in more than one laboratory (Table 1). No mutagenic responses were seen with any of the phthalates tested in any of the laboratories. The concurrent positive controls produced the expected mutagenic results.

The di(2-ethylhexyl) phthalate tested came from two sources: the first was purchased commercially for this program; the second was received from the NCI Repository and was from the same batch as was used for the NCI Bioassay Program. The data from these tests will be published elsewhere after all testing is completed.

Discussion

A series of 20 phthalates and related chemicals were demonstrated to be nonmutagenic in a preincubation modification of the Salmonella microsome test. Butyl benzyl phthalate, di(2-ethylhexyl) adipate and di(2-ethylhexyl) phthalate were classified as carcinogens in the NCI Bioassay Program; phthalic anhydride was classified as noncarcinogenic.

There is an apparent disagreement between the results reported here and elsewhere in this issue. Two other studies, using different Salmonella protocols, have reported diethyl phthalate to be mutagenic, in disagreement with the results reported here; however, these other reports agree that di(2-ethylhexyl) phthalate is not mutagenic in Salmonella.

Based on the results from these three carcinogenic chemicals, it would appear that the Salmonella microsome test is not responsive to phthalates and would be a poor predictive test for their potential carcinogenicity. However, mutagenicity and carcinogenicity results from a larger number of phthalates are necessary before definitive statements can be made concerning the predictability of the Salmonella microsome test for carcinogenesis in rodents.

A number of these phthalates are currently on test or have been selected for testing in Drosophila for their ability to induce sex-linked recessive lethal mutations and heritable translocations and in cultured Chinese hamster ovary cells for induction of chromosome aberrations and sister chromatid exchanges.

REFERENCES

1. Zeiger, E., and Drake, J. W. An environmental mutagenesis test development programme. In: Molecular and Cellular Aspects of Carcinogen Screening Tests, R. Montesano, H. Bartsch and L. Tomatis, Eds., IARC Scientific Publications, No. 27, Lyon, France, 1980, pp. 303-313.
2. Ames, B. N., McCann, J., and Yamasaki, E. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome test. Mutat. Res. 31: 347-364 (1975).