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.

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 0.1M 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

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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-amino-acridine; 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, phthalimide, 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.

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             |
| Butyl cyclohexyl phthalate    | 84-64-0  |                                   c            | CWR          | DMSO     | 333-11550             |
| Diallyl phthalate             | 131-17-9 | Hardwicke (99%)                               | CWR          | DMSO     | 100-10000             |
| Dibutyl phthalate             | 84-74-2  | Aldrich (99%)                                  | EGG          | DMSO     | 100-10000             |
| Dicyclocxethyl phthalate      | 84-11-7  |                                   -            | EGG          | DMSO     | 100-10000             |
| Di(2-ethylhexyl) adipate      | 103-23-1 | Pfaltz & Bauer (N.S.) b                        | EGG          | 95% EtOH | 100-10000             |
| Di(2-ethylhexyl) phthalate    | 117-81-7 | MC/B (Practical)                               | EGG          | DMSO     | 100-10000             |
| Di(2-ethylhexyl) adipate      |          | WR Grace (99.5%) b                             | SRI DMSO     |          | 100-10000             |
| Di(2-ethylhexyl) phthalate    | 84-66-2  | Chem. Tech. Ind. (99.9%) b                     | EGG          | DMSO     | 10-3333               |
| Di(n-hexyl) phthalate         | 84-75-9  |                                   -            | CWR DMSO     |          | 100-10000             |
| Diisobutyl phthalate          | 84-69-5  | Eastman (Reagent)                              | SRI DMSO     | 95% EtOH | 100-10000             |
| Diisodecyl phthalate          | 25761-40-0 | Pfaltz & Bauer (NS) d                        | EGG          | 95% EtOH | 100-10000             |
| Diisononyl phthalate          | 28553-12-0 |                                   -            | EGG          | 95% EtOH | 100-10000             |
| Dimethyl phthalate            | 131-11-3 | Aldrich (99%)                                  | EGG          | DMSO     | 33-6666               |
| Dimethyl terephthalate        | 120-61-6 | Aldrich (99%)                                  | EGG          | DMSO     | 3.3-3333              |
| 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 |                                   -            | EG          |          | 3.3-3333              |
| 2-Ethoxyethanol               | 110-80-5 | Dow Chem. (99%) b                             | EGG          | Water    | 100-10000             |
| 2-Ethyl-1-hexanol             | 104-76-7 | Aldrich (99%)                                  | EGG          | DMSO     | 3.3-3333              |
| Mono(2-ethylhexyl) adipate    | 4337-65-9 |                                   -            | EGG          |          | 100-10000             |
| Mono(2-ethylhexyl) phthalate  | 4376-20-9 |                                   -            | EGG          |          | 100-10000             |
| Phthalimide                   | 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) phosphate  | 301-13-3 | Pfaltz & Bauer (NS)                            | CWR DMSO     |          | 100-8270              |

aLaboratory: EGG: EG&G Mason Research Institute; CWR: Case Western Reserve University; SRI: SRI International.
bSample used for carcinogenesis bioassay.
cChemical not yet received.
dNS = purity/grade not specified.
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.

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