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IV. MUTAGENESIS

A. Introduction

Short-term tests possibly predictive of "genotoxic carcinogens" are widely used. They consist primarily of assays for DNA damage, gene mutations and chromosomal aberrations using bacterial and mammalian cells, and, in some cases, whole animals, primarily rodents. Additionally, cell transformation assays have been developed which are predictive of both "genotoxic" as well as "non- genotoxic" carcinogens. The predictivity of most of the assays for carcinogenesis has been characterized with respect to their performance with known carcinogens and non-carcinogens. The most authoritative data base available for performing this task is that assembled under the aegis of the U.S. Environmental Protection Agency's Gene-Tox Program (Waters 1979; Waters and Auletta 1981; Palajda and Rosenkranz 1985).

Other, as yet not so well characterized, assays are being developed to identify "non-genotoxic" carcinogens and enhancers of carcinogenesis (e.g. tumor promoters). These include assays for the inhibition of metabolic cooperation (Murray and Fitzgerald 1979; Williams 1980; Yotti et al. 1979) as well as the enhancement of hepatocellular hyperplastic foci (e.g. Ward et al 1983). In addition, assays for aneuploidy have been suggested as being predictive of tumor enhancers (Parry et al 1981).

DEHP has been extensively tested in short-term assays, chiefly as the results of its reported carcinogenicity and because it has been included as one of the ten test chemicals in the Collaborative Study of

the International Program on Chemical Safety (Ashby et al 1985). The DEHP metabolites MEHP and 2-ethylhexanol, although not as extensively tested as DEHP, have also been studied sufficiently to permit a determination of their genotoxicity.

B. Data Collection

Reprints and reports submitted to the CPSC were made available to the CHAP, as were computer searches. These were supplemented by searches conducted by CHAP members. The completeness of the bibliography was checked further by comparison with several review articles on DEHP (e.g., Butterworth 1984; IARC 1982; Northup et al 1982; Hopkins 1983; Kluwe 1982; Kluwe et al 1983; Rodricks 1984). Some citations that were unavailable but were referred to in the review articles are listed in the data base, but with a subscript indicating that the original data were not available (e.g. Tables IV-1 to IV-3).

Papers containing redundant information, either previously published data or a reconfirmation of previous results by the same investigators, were not entered separately, rather they were listed as co-entries (see Tables IV-1 to VI-3). On the other hand, if the same laboratory participated in an International Collaborative Study which used centrally supplied test chemicals and the study was clearly a different one from that published previously by the same group, then the results were listed separately.

Except for those entries indicated as not providing data (see Tables IV-1 to IV-3), all the experimental results were verified by the CHAP prior to entry. Conflicting results, or results which were challenged by Cooperative Evaluation Groups were identified (see Table IV-1).

C. Di(2-ethylhexyl)phthalate

1. Data Evaluation

The results of the short-term assays for DEHP are detailed in Table IV-1 and summarized in Table IV-4. DEHP has been tested extensively for genotoxicity and even a cursory examination reveals that it is remarkably lacking in mutagenicity and DNA-damaging activity in bacterial and mammalian systems, hence one might classify it as a non-genotoxicant, bearing in mind the difficulty of classifying a chemical by exclusion (Upton et al 1984).

The test results are, however, consistent with a non-genotoxic or tumor enhancing mechanism, i.e. ability of DEHP to transform Syrian hamster embryo cells (a property common to both genotoxic and non-genotoxic carcinogens), and ability to induce aneuploidy, which has been suggested as being characteristic of tumor enhancers. (The induction of aberrant mitotic spindles is consistent with and may provide a mechanism for aneuploidy.) Finally, DEHP demonstrated the ability to induce anchorage independence and hyperplastic foci in mice. It should, however, be mentioned that, unlike the short-term genotoxicity assays, the above mentioned tests (with the possible exception of the cell transformations) have not been as fully validated.

2. Formal Analysis of the Test Results

The CHAP used three independent assessments of the genotoxicity of DEHP:

a. Criteria of the International Agency for Research on Cancer

The International Agency for Research on Cancer developed criteria (IARC, 1984) for assessing the genetic activity of chemicals. The tests are grouped according to endpoints, i.e.;

Tests of DNA damage. These include tests for covalent binding to DNA, induction of DNA breakage or repair, induction of prophage in bacteria and differential survival of DNA repair-proficient/-deficient strains of bacteria.

Tests of mutation (measurement of heritable alterations in phenotype and/or genotype). These include tests for detection of the loss or alteration of a gene product, and change of function through forward or reverse mutation, recombination and gene conversion; they may involve the nuclear genome, the mitochondrial genome and resident viral or plasmid genomes.

Tests of chromosomal effects. These include tests for detection of changes in chromosome number (aneuploidy), structural chromosomal aberrations, sister chromatid exchanges, micronuclei and dominant-lethal events. This classification does not imply that some chromosomal effects are not mutational events.

Depending upon the test results, the evidence is categorized as follows:

(i) Sufficient evidence is provided by at least three positive entries, one of which must involve mammalian cells in vitro or in vivo and which must include at least two of three end-points - DNA damage, mutation and chromosomal effects.

(ii) Limited evidence is provided by at least two positive entries.

(iii) Inadequate evidence is available when there is only one

positive entry or when there are too few data to permit an evaluation of an absence of genetic activity or when there are unexplained, inconsistent findings in different test systems.

(iv) No evidence applies when there are only negative entries; these must include entries for at least two end-points and two levels of biological complexity, one of which must involve mammalian cells in vitro or in vivo.

Using the IARC format, and summarizing the results of Table IV-4, we have the following:

Overall Assessment of Data from Short-Term Tests for Genetic Activity of DEHP

	Genetic Activity		
	DNA damage	Mutation	Chromosomal effects
Prokaryotes	-	-	
Fungi/Green plants	?	?	
Insects	-	-	-
Mammalian cells (in vitro)	-	-	-
Mammals (in vivo)	-	?	-
Humans (in vivo)			-

which leads to the conclusion that there is "no evidence" for genetic activity.

b. The Decision Point Approach

The decision point approach (Weisburger and Williams, 1981) relies on the results of a battery of assays: a DNA damage test in mammalian cells, gene mutation tests in bacterial and mammalian cells, and a mammalian cell chromosome test. A mammalian cell transformation assay is used as a supplementary test. Different activation systems should be used; these include subcellular fractions as well as intact cells.

Weisburger and Williams (1981) assigned different degrees of reliability to predict carcinogenicity to each set of tests. These were based upon analyses of the structural features of known carcinogens.

In this approach, a positive result in all four endpoints tested indicates with almost certainty a genotoxic mechanism of carcinogenicity. A positive test in only a single type of test is taken as equivocal.

In view of the fact that DEHP (Tables IV-1 and IV-4) gives negative responses in mammalian tests for DNA damage (i.e. UDS, alkaline elution, etc.), gene mutation in bacterial (Salmonella, E. coli) and mammalian cells (mouse lymphoma, Chinese hamster, etc.) and in mammalian chromosome tests (chromosomal aberrations, sister chromatid exchanges, micronuclei, etc.) it can be concluded that DEHP is not a genotoxic carcinogen.

c. Carcinogenicity Prediction and Battery Selection (CPBS) Method

The CPBS method was devised to evaluate the probability that a chemical is, or is not, a carcinogen based upon the results of short-term assays (Rosenkranz et al 1984a; Chankong et al 1985). The procedure uses Bayes' decision theory to calculate the probability that a test chemical is, or is not, a genotoxic carcinogen, taking advantage of the known performance characteristics of the various assays. Application of the CPBS method to DEHP leads to a 99.95% probability that DEHP is not a genotoxic carcinogen (see Appendix I).

3. Conclusions

Three independent analyses suggest that the basis of the carcinogenicity of DEHP is not based upon a DNA-damaging effect. In

this connection, it should be noted that it has been suggested that DEHP derives its carcinogenicity from an ability to induce the proliferation of peroxisomes, and that these, in turn, generate free radicals and peroxides which are endowed with genotoxic properties. It is, therefore, of interest that known inducers of peroxisome proliferation have been reported to be unable to induce mutations in Salmonella (Reddy and Lalwani 1983), or sister chromatid exchanges (Linnainmaa 1984), nor do they bind covalently to hepatic DNA (Von Daniken et al 1981, Gupta et al 1985, Goel et al 1985, Reddy et al 1985).

The lack of genotoxicity of DEHP is reinforced by the findings suggesting a non-genotoxic basis of action, i.e., aneuploidy/mitotic spindle abnormality, inhibition of metabolic cooperation and the enhancement of hepatic hyperplastic foci in the mouse, which together with the cell transformation assays imply either a non-genotoxic mechanism and/or the ability to act as a promoter.

The fact that the induction of ornithine decarboxylase, an event associated with tumor promotion (O'Brien et al 1975, Olson et al 1980) is mediated by drugs with peroxisome proliferative activity (Isumi et al 1981), supports the non- genotoxic nature of DEHP carcinogenesis.

D. Mono(-2-ethylhexyl)phthalate

1. Data Evaluation

Because MEHP is a major metabolite of DEHP, an examination of its genotoxicity is of interest. This chemical has not been tested as extensively as DEHP (see Table IV-2). Although the Salmonella assay results indicate a lack of mutagenicity, the results with the E. coli mutagenicity assay and the B. subtilis repair assay suggest mutagenic

activity. It should be noted, however, that in both assays, the positive responses are questionable (see Table IV-2). The bulk of the responses indicate a lack of genotoxicity. Assays that might indicate non-genotoxic mechanisms, i.e., aneuploidy or inhibition of metabolic cooperation, were not performed. It should be noted, however, that the transformation assay with Syrian hamster embryo cells was positive.

a. IARC Criteria

Scoring some of the questionable results (i.e lack of data, or no dose response) as being positive, the following data base was obtained:

Overall Assessment of Data from Short-Term Tests for Genetic Activity of MEHP

	Genetic Activity		
	DNA damage	Mutation	Chromosomal effects
Prokaryotes	+	+	
Fungi/Green plants			
Insects			
Mammalian cells (in vitro)	-	-	+
Mammals (in vivo)	-	-	+
Humans (in vivo)			

which indicates that by the IARC criteria MEHP would be classified as demonstrating "limited evidence" of genetic activity.

b. Decision Point Approach

By the criteria of the decision point approach analysis, even accepting the questionable E. coli mutagenicity results, the results would be classified as equivocal for genotoxicity in

view of the absence of a mammalian DNA damage assay, the primarily negative mammalian gene mutation assays and the questionable positive chromosomal assays.

c. CPBS Method

Even though 4 of the 12 assay results that were analyzed by the CPBS method (Appendix I) were positive, it was calculated that MEHP only had a 7.0% probability of being a genotoxic carcinogen (i.e. 93.0% probability of non-genotoxic carcinogenicity) (see General Remarks, below).

2. Conclusions

Although there are some borderline positive results, the bulk of the evidence suggests that MEHP lacks genotoxic activity.

Note should be taken that MEHP was capable of transforming Syrian hamster embryo (SHE) cells; however, the test was not carried out as rigorously as some of the SHE transformation assays performed for DEHP.

E. 2-Ethylhexanol

2-Ethylhexanol was not tested extensively, however, there were no strong positive test results (Tables IV-3 and IV-4). By the IARC criteria, this chemical would be characterized as possessing "no evidence" of genetic activity.

Overall Assessment of Data from Short-Term Tests for Genetic Activity of 2-Ethylhexanol

	Genetic Activity		
	DNA damage	Mutation	Chromosomal effects
Prokaryotes	?	-	
Fungi/Green plants			
Insects			
Mammalian cells (in vitro)	-	-	-
Mammals (in vivo)			-
Humans (in vivo)			

The decision point approach indicates a lack of genotoxicity and CPBS predicts a 99.97% probability of non-carcinogenicity.

F. General Remarks

It has been suggested (see Astill et al 1985) that the carcinogenicity of DEHP is due to the induction of peroxisome proliferation which, in turn, lead to the generation of peroxides and free radicals. Such agents are expected to be mutagenic, genotoxic, as well as DNA damaging. On the other hand, no major genetic or genotoxic effect can be ascribed to DEHP. Recently Gupta et al (1985) using the exquisitely sensitive P32-postlabelling method, which is capable of detecting a base change in 10^{10} nucleotides, reported that neither DEHP nor other inducers of peroxisome proliferation showed any DNA damaging activity in rat liver, the site of peroxisome proliferation. These findings suggest either that DEHP or the peroxisomes induced by it do not mediate a genotoxic event or that the experimental conditions (including the duration of exposure) used to measure putative DEHP-induced genotoxicity were not appropriate for sufficient peroxisome proliferation. However, two recent series of experiments which simultaneously measured peroxisome proliferation and DNA damage (unscheduled DNA synthesis and alkaline elution) appear to dissociate the two events (Butterworth et al 1984, Elliott and Elcombe 1985).

In view of the fact that the generation of free radicals and peroxides has been associated with DEHP-induced peroxisome proliferation, it should be noted that negative responses were also obtained when DEHP was tested in the newer Salmonella tester strains which respond to free radicals, peroxisomes and aldehydes (Baker and Bonin 1985; Matsushima et al 1985).

With respect to the short-term tests used, obviously some are more accurate predictors than others. As a matter of fact some of the tests listed in Tables VI.1 to IV.4 have not been validated (eg. yeast assays). The CPBS method of analysis (see Appendix I) seeks to remedy this by (a) not using the results of assays of unknown selectivity and (b) placing different emphasis on each assay depending upon its performance characteristics with known carcinogens and non-carcinogens.

Finally, it should be mentioned that the positive test results appear to be confined to only a few laboratories, thus Tomita et al - (1982) report DEHP and its derivatives to respond positively in most assays. This is the chief reason for the fact that in the CPBS method the predicted (genotoxic) carcinogenicity of MEHP (7%) is higher than that of DEHP (0.2%) and 2-ethylhexanol (0.03%) and for designating MEHP as possessing "limited evidence" of genetic activity in the IARC scheme.

Although the preponderance of results indicate that neither DHEP nor its metabilites MEHP and 2-ethylhexanol induce chromosomal damage in mammalian cells, the report of DEHP-induced aneuploidy deserves mention, as it may indicate a heretofore unsuspected mechanism for DEHP carcinogenesis. The report of DEHP-induced disturbance of the mitotic spindle may provide the basis for this aneuploidy. However, it should be noted that both of the above findings, as well as the DEHP-induced mitotic aneuploidy in yeast, were reported by the same laboratory (Parry and Eckhardt 1985b; Danford 1985; Parry 1985). The aneuploidy in yeast was not confirmed by another laboratory that used the same DEHP specimen (Zimmerman et al 1985).

G. Conclusions

The mutagenicity and genotoxicity of DEHP and MEHP have been studied adequately in many laboratories. The vast majority of the results indicate a complete lack of genotoxicity. Most results were replicated in several laboratories.

The data indicate that DEHP possesses in vitro neoplastic transformation and cancer-enhancing activity (see also Chapter V).

Although the majority of the short-term assays have been carried out in systems which presumably do not induce the proliferation of peroxisomes, in several experiments using DEHP and MEHP in rat liver systems both in vivo and in vitro, the proliferation of peroxisomes was documented. These assays did not demonstrate genotoxicity either. Although the assays used (unscheduled DNA synthesis, alkaline elution, p^{32}-postlabelling) have not been studied rigorously with respect to response to peroxisome-mediated DNA damaging agents (i.e. H_2O_2, peroxides, free radicals), it should be stated that hamster V79 cells exposed to hydrogen peroxides, free radicals, and active oxygen, do exhibit DNA damage as measured by alkaline elution (Erickson et al 1980; Bradley and Erickson 1981), and exposure of cells to hydrogen peroxide has been reported to result in the induction of unscheduled DNA synthesis (Stich et al 1978).*

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* In a telephone conversation with a CHAP member (May 12, 1985), Dr. Gupta, the co-originator of the p^{32}-postlabelling technique, stated that he expected that H_2O_2-induced DNA damage would be detectable by the p^{32}-postlabelling procedure.

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I. Tables

Table IV-1.a
Summary of the Data of Short-Term Test Results
of Di(2-ethylhexyl)phthalate

Assay System	Result	Reference
Salmonella/Microsome	-[a]	Astill et al 1985
	-	Kirby et al 1982, 1983
	-	Rubin et al 1979
		Kozumbo et al 1982
	-	Yoshikawa et al 1983
	-	Zeiger et al 1982
	-	Norpoth et al 1980
	-	Baker and Bonin 1985
	-	Matsushima et al 1985
	-	Zeiger et al and Hawarth 1985
	-	Rexroat and Probst 1985
	-	Robertson et al 1983
	-	Warren et al 1982
	+[b]	Tomita et al 1982
	-[a]	Simmon et al 1977
	-	Zeiger 1985
Salmonella, forward assay	-	Liber 1985
	-[a]	Seed 1982
E. coli WP2	-	Yoshikawa et al 1983
	-	Yagi et al 1976
B. subtilis rec assay	-	Tomita et al 1982
	-	Yagi et al 1976
Body fluid/Salmonella	-	diVincenzo et al 1983,1985
Yeast and Aspergillus Assays		
Saccharomyces cerevisiae Mitotic crossing-over/		
Strain D7	-	Arni, 1985
	-	Parry and Eckardt 1985a
Strains D6 and D61.M	-	Parry and Eckardt 1985b

Table IV-1.b

Assay System	Result	Reference
Saccharomyces cerevisiae (cont'd)		
Mitotic gene conversion		
Strain D7	+	Arni 1985
Strain D7	-	Parry and Eckardt 1985a
Strain D7 +		Mehta and von Borstel 1985
Strain JD1 (stationary)	-	Brooks et al 1985
Strain JD1 (growing)	-	Brooks et al 1985
Strain PV	-	Inge-Vechtomov et al 1985
Mutation		
Strain D7	-	Arni 1985
Strain D7	-	Parry and Eckardt 1985a
Strain RM52	+	Mehta and von Borstel 1985
Strain XV185-14C	+	Mehta and von Borstel 1985
Strain D6	-	Parry and Eckardt 1985b
Strain PV-1	-	Inge-Vechtomov et al 1985
Mitotic aneuploidy		
Strain D61.M	-	Zimmerman et al 1985
Mitotic aneuploidy		
Strains D6 and D61.M	+	Parry and Eckardt 1985b
Schizosaccharomyces pombe		
Mutation	-	Loprieno et al 1985
Aspergillus nidulans		
Mitotic crossing-over	-c	Carere et al 1985
Mitotic aneuploidy	-c	Carere et al 1985
In Vitro Cytogenetics		
Chinese hamster ovary	-	Phillips et al 1982
	-	Douglas et al 1985a
	-	Natarajan et al 1985
	-	Palitti et al 1985

Table IV-1.c

Assay System	Result	Reference
In Vitro Cytogenetics (cont'd)		
	-	Gulati et al 1985
	-	Ishidate and Sufani 1985
	-	NTP Lab A 1985
	-	NTP Lab B 1985
	-	NTP Lab C 1985
Chinese hamster DON	-	Abe and Sasaki 1977
Chinese hamster lung cells	-	Ishidate and Odashima 1977
Chinese hamster liver fibroblast	-	Danford 1985
Rat liver epithelial-like	-	Priston and Dean 1985
Human lymphocytes	-	Howard et al 1985
Human leucocytes	-	Stenchever et al 1976
	-	Turner et al 1974
Human fetal lung	-	Stenchever et al 1976
Sister Chromatid Exchanges		
Chinese hamster ovary	-	Douglas et al 1985a,b
	- 1	Gulati et al 1985
	-	NTP Lab A 1985
	-	NTP Lab B 1985
Chinese hamster DON	- (?)d	Abe and Sasaki 1977
Rat liver epithelial-like	-	Priston and Dean 1985
Human lymphocytes	-	Obe et al 1985
Polyploidy - (in vitro)		
Chinese hamster lung cells	-	Ishidate and Sofuni 1985
Chinese hamster liver fibroblasts	-	Danford 1985
Rat liver epitheleal-like	-	Priston and Dean 1985
Aneuploidy (in vitro)		
Chinese hamster liver fibroblasts	+	Danford 1985

Table IV-1.d

Assay System	Result	Reference
Mitotic Spindle Abnormality		
Chinese hamster liver fibroblasts	+	Parry 1985
In Vivo Cytogenetics		
Human lymphocytes	-	Theiss and Fleig 1978
In Vivo Cytogenetics (cont'd)		
Rat bone marrow	-	Putnam et al 1982, 1983
Syrian hamster/Transplacental	+	Tomita et al 1982
Micronucleus		
Mouse bone marrow	-a	Astill et al 1985
Mouse peripheral blood	-	Douglas et al 1985a,b
Unscheduled DNA Synthesis		
Rat hepatocytes - in vitro	-a	Astill et al 1985
	-	Hodgson et al 1982
	-	Butterworth et al 1984
	-	Kornbrust et al 1984
	+ (?)e	Glauert et al 1985
	-	Probst and Hill 1985
	-	Williams et al 1985a
Human hepatocytes - in vitro	-	Butterworth et al 1984
Human HeLa cells	-	Martin and Campbell 1985
Rat hepatocytes in vivo/in vitro	-	Butterworth et al 1984
	-	Kornbrust et al 1984
DNA Damage		
Rat hepatocytes, in vitro alkaline elution	-	Butterworth et al 1984
	-	Bradley 1985
Rat hepatocytes, P32-postlabelling	-	Gupta et al 1985

Table IV-1.e

Assay System	Result	Reference
DNA Damage (cont'd)		
Hamster ovary cells/		
alkaline sucrose gradient	-	Douglas et al 1985a,b
	-	Lakhanisky and Hendrickx 1985
Rat, in vivo/alkaline elution	-	Butterworth et al 1984
	-[a]	Elliott and Elcombe 1985
DNA Binding-Adduct Formation/		
Rat liver, in vivo	-	von Daniken et al 1983 1984; Lutz 1985
	-	Jackh et al 1984
	+f (?)	Albro et al 1982 1983 -
P32-Postlabelling	-	Gupta et al 1985
Gene Mutations - In vitro		
Mouse lymphoma	-	Kirby et al 1982 1983
	-	Amacher and Turner 1985
	-	Garner and Campbell 1985
	-	Lee and Webber 1985
	-	Myrh et al 1985
	inconclusive	Oberly et al 1985
		Garner et al 1985
	-	Styles et al 1985
	-[a]	Astill et al 1985
Chinese hamster V79	-	Lee and Webber 1985
	-[a]	Malcolm and Mills 1982
Chinese hamster ovary	-	Zdzienika and Simons 1985
Human lymphoblasts TK	-	Crespi et al 1985
Human lymphoblasts AHH-1	-	Crespi et al 1985
Mouse Balb/c3T3	-	Matthews et al 1985

Table IV-1.f

Assay System	Result	Reference
Dominant Lethal Mutation		
Mice	+	Singh et al 1974, 1975
		Dillingham and Autian 1973
	-	Rushbrook & Jorgenson 1981
		Rushbrook et al 1982
	-[a]	Hamano (cited by IARC 1982)
	-[a]	cited by Butterworth 1984
	-	Bishop 1984
Sperm Morphology		
Mice	-	Douglas et al 1985a
Rats	-	Douglas et al 1985a
Drosophila		
Sex-linked recessive lethal	-	Yoon et al 1985
Recombination/Somatic Mutations/ Aneuploidy	-	Wurgler et al 1985
		Vogel et al 1985
Somatic Mutations/Deletions	marginal	Fujikawa et al 1985
		Vogel et al 1985
	marginal	Vogel 1985; Vogel et al 1985
Recombination	-	Vogel 1985; Vogel et al 1985
Cell Transformation Assays		
Syrian hamster embryo	+	Tomita et al 1982
	+	Barrett and Lamb 1985
	+	Sanner and Rivedal 1985
Mouse embryo fibroblasts		
C3H/10T½	+	Lawrence and McGregor 1985
	-[a]	cited by Butterworth 1984
Mouse BALB/c3T3		
	-[a]	Astill et al 1985
	-	Matthews et al 1985
Mouse BALB/c3T3/Rat Hepatocyte	-[a]	Astill et al 1985
	-	Matthews et al 1985

Table IV-1.g.

Assay System	Result	Reference
Cell Transformation Assays (cont'd)		
Syrian hamster embryo viral enhancement (SA7)	+	Hatch and Anderson 1985
Rat embryo viral enhancement of survival (RLV)	+[g]	Suk and Humphreys 1985
Tests for Promotion		
In Vitro		
Metabolic Cooperation		
Chinese hamster V79	-[h]	Kornbrust et al 1984
	+[h]	Malcolm and Mills 1983
	+[i]	Elmore et al 1985
	-[i]	Umeda et al 1985
Mouse Epidermis/Anchorage independence	+	Diwan et al 1983 Ward et al 1985
Promotion of C3H/10T½ transformations	-[a]	cited by Butterworth 1984
In Vivo		
Hepatocellular hyperplastic foci, B6C3F1 mice	+	Diwan et al 1983 Ward et al 1983 Ward et al 1985
Fischer 344 Rats	- -[a]	Popp et al Ward et al cited by Butterworth, 1984
Sprague-Dawley Rats	-[k]	DeAngelo and Garrett 1983
Skin painting,		
SENCAR mouse	+ (weak)	Ward et al 1985
CD-1 mouse	-	Ward et al 1985 Diwan et al 1983

Table IV-1.h.

a No data given.

b Positive at one very high dose, only.

c The evaluation group felt that due to the fact that sufficient concentrations might not have been achieved, the negative results should be regarded as provisional (Parry et al 1985).

d Not a dose-related effect.

e Not a dose related effect, see Williams et al 1985b.

f These investigators did not demonstrate that the radioactivity of DEHP was in an altered base. Hence, results could be consistent with those of von Daniken et al (1983, 1984) who demonstrated that radioactivity was derived from the intermediary metabolism pool. Albro (CHAP meeting of 4/3/85) indicated that no DNA adducts were found on DEHP treated animals.

g The final evaluation of the results of this assay were considered questionable by the concensus panel (Ashby et al 1985; McGregor et al 1985).

h However, see also Malcolm and Mills 1982 who report negative results.

i The evaluation group judged this result to be weakly positive or a suspect positive (Elmore and Nelmes 1985).

k There was an actual inhibition of the formation of preneoplastic lesion by DEHP.

l Subsequent statistical analyses of these data as well as of the results of three additional samples of DEHP indicated a reproducible, although weak, dose-related increase in SCE at high doses (Gulati et al 1985; Shelby MD, Personal communication, Aug. 15, 1985).

Table IV-2.a
Summary of the Data of Short-Term Test Results
of Mono(2-ethylhexyl)phthalate

Assay System	Result	Reference
Salmonella/microsomes	-[a]	Astill et al 1985
	-	Kirby et al 1983
	+[b,c]	Tomita et al 1982
	-	Yoshikawa et al 1983
	-	Zeiger et al 1982
	-	Ruddick et al 1981
	-	Zeiger et al 1985
Salmonella, forward assay	-[a]	Seed 1982
E. coli WP2	+[b]	Tomita et al 1982
	-	Yoshikawa et al 1983
	+[a]	Yagi et al 1976
B. subtilis rec assay	+[a]	Yagi et al 1976
	+[b]	Tomita et al 1982
In Vitro Cytogenetics		
Chinese hamster ovary	+[d]	Phillips et al 1982, 1985
Rat liver cells	+	Phillips et al 1985
Sister Chromatid Exchanges		
Chinese hamster ovary	-	Phillips et al 1982
Chinese hamster V79	+[a]	Tomita et al 1982
In Vivo Cytogenetics		
Rat bone marrow	-	Putnam et al 1982, 1983
Syrian hamster/Transplacental	+	Tomita et al 1982
Micronucleus		
Mouse	-[a]	Astill et al 1985
Unscheduled DNA Synthesis		
Rat hepatocyte, in vitro	-[a]	Hodgson et al 1982
Human hepatocytes, in vitro	-	Butterworth et al 1984

Table IV-2.b

Assay System	Result	Reference
DNA Damage		
Alkaline elution/Rat hepatocyte in vitro	-[a]	Elliott and Elcombe 1985
Alkaline elution/Rat liver in vivo	-[a]	Elliott and Elcombe, 1985
DNA Binding/rat liver, in vivo	+[e](?)	Albro et al 1982, 1983
Gene Mutations - In vitro		
Mouse lymphoma	-	Kirby et al 1982, 1983
Chinese hamster ovary	-	Phillips et al 1982
Chinese hamster V79	marginal[a]	Tomita et al 1982
Dominant Lethal Mutations		
Mouse	-	Rushbrook & Jorgenson 1981, 1982
Cell Transformation Assay		
Syrian hamster embryo	+	Tomita et al 1982
Mouse embryo fibroblasts C3H/10T½	-[a]	cited by Butterworth 1984
Mouse BALB/C3T3	-[a]	Astill et al 1985
Test for Promotion In Vitro		
Promotion of C3H/10T½ transformation	-[a]	cited by Butterworth 1984
Mouse epidermis/Anchorage independence	+	Ward et al 1985

a No data given.

b Positive at one very high dose, only.

c Net increase in mutant number not observed.

d Phillips et al 1985 suggest that the clastogenic changes are possibly due to MEHP-mediated damage to lysosomal membranes and the resulting release of endonucleases.

e These investigators did not demonstrate that the radioactivity of DEHP was in an altered base. Hence, results could be consistent with those of von Daniken et al (1983, 1984) who demonstrated that radioactivity was derived from the intermediary metabolism pool.

Table IV-3.a

Summary of Data of Short-Term Test Results of 2-Ethylhexanol

Assay System	Result	Reference
Salmonella/Microsome	-[a]	Astill et al 1983
	-	Kirby et al 1983
	-	Warren et al 1982
	-	Zeiger et al 1985
Salmonella, forward assay	marginal[b]	Seed 1982
B. subtilis rec assay	+[a]	Tomita et al 1982
Body fluid/Salmonella	-	diVincenzo et al 1983, 1985
In Vitro Cytogenetics		
Chinese hamster ovary	marginal	Phillips et al 1982
	-a	Phillips et al 1982
In Vivo Cytogenetics		
Rat bone marrow	-	Putnam et al 1982, 1983
Micronucleus		
Mouse	-[a]	Astill et al 1985
DNA Damage		
DNA binding/rat liver,	-	von Daniken 1983, 1984
in vivo	+[c] (?)	Albro et al 1982
	-	Albro et al 1983
Unscheduled DNA Synthesis		
Rat hepatocyte, in vitro	-[a]	Hodgson et al 1982
Gene Mutation		
Mouse lymphoma	-	Kirby et al 1983
Dominant Lethal Mutations		
Mouse	-	Rushbrook & Jorgenson 1981, 1982

Table IV-3.b

Assay System	Result	Reference
Cell Transformation Assay		
BALB/c3T3/Rat hepatocyte	-	Litton-Bionetics 1983 Astill et al 1985
Test for Promotion		
In vitro mouse epidermis/ Anchorage independence	-	Ward et al 1985

a No data given.

b Net increase in mutant number not observed.

c These investigators did not demonstrate that the radioactivity of DEHP was in an altered base. Hence, results could be consistent with those of von Daniken et al (1983, 1984) who demonstrated that radioactivity was derived from the intermediary metabolism pool.

Table IV-4.a

Results of Short-Term Assays

Description of Test	DEHP		MEHP		2-Ethylhexanol	
	Positive (N)	Negative (N)	Positive (N)	Negative (N)	Positive (N)	Negative (N)
Bacteria						
Salmonella mutagenicity	1	16	1	7	1m	4
E. coli mutagenicity	0	2	2	1		
B. subtilis DNA repair	0	2	2	0	1	0
Yeast						
Saccharomyces cerevisiae						
Mutation	2	4				
Crossing-over	0	3				
Gene Conversion	2	4				
Aneuploidy	1	1				
Schizosaccharomyces pombe						
Mutation	0	1				
Aspergillus						
Crossing-over	0	1				
Aneuploidy	0	1				
In Vitro Mammalian Cells - Genotoxicity						
Chromosomal damage	0	17	2	0	1m	0
Sister chromatid exchanges	0	7	1	1		
Polyploidy	0	3				
Aneuploidy	1	0				

Table IV-4.b

Description of Test	DEHP Positive (N)	DEHP Negative (N)	MEHP Positive (N)	MEHP Negative (N)	2-Ethylhexanol Positive (N)	2-Ethylhexanol Negative (N)
In Vitro Mammalian Cells - Genotoxicity (cont'd)						
Mitotic spindle	1	0				
Unscheduled DNA synthesis	1 (?)	8	0	2	0	1
DNA damage	0	5	0	1		
In Vitro Mammalian Cells - Gene Mutations						
Mouse lymphoma	1i	7	0	1	0	1
Mouse Balb/c3T3	0	1				
Chinese hamster ovary	0	1	0	1		
Chinese hamster V79 0		2	1m	0		
Human lymphoblasts TK	0	1				
Human lymphoblasts AHH	0	1				
In Vivo Mammalian Genotoxicity						
Micronucleus test	0	2	0	1	0	1
Human lymphocyte chromosome damage	0	1				
Sperm morphology - mouse	0	1				
Sperm morphology - rat	0	1				
Rat bone marrow chromosome damage	0	1	0	1	0	1
Syrian hamster transplacental chromosome damage	1	0	1	0		
Body fluids/Salmonella	0	1		0	1	
Unscheduled DNA synthesis	0	2				
DNA damage	1?	5	1i	1	1?	2

Table IV-4.c.

Description of Test	DEHP Positive (N)	DEHP Negative (N)	MEHP Positive (N)	MEHP Negative (N)	2-Ethylhexanol Positive (N)	2-Ethylhexanol Negative (N)
In Vivo Mammalian Mutations						
Dominant lethality in mice	1	4	0	1	0	1
In Vitro Mammalian Cell Transformation						2
Syrian hamster embryo	3	0	1	0		
Mouse C3H	1	1	0	1		
Mouse BALB/e 3T3	0	3	0	1	0	1
Virus-enhanced transformation	1	0				
Virus-enhanced survival	1	0				
Drosophila						
Sex-linked recessive lethal	0	1				
Recombination	0	1				
Somatic mutations/deletions	1m					
Recombination/somatic mutation/aneuploidy	0	1				2
Tests of Promotion In Vitro						
Metabolic cooperation	2 + 1m	1				
Anchorage independence	1	0	1	0	0	1
Promotion of Cell Transformation	0	1	0	1		

Table IV-4.d.

Description of Test	DEHP Positive (N)	DEHP Negative (N)	MEHP Positive (N)	MEHP Negative (N)	2-Ethylhexanol Positive (N)	2-Ethylhexanol Negative (N)
Tests of Promotion (cont'd)						
In Vivo						
Hepatocellular hyperplastic foci						
Mouse	1	0				
Rats	0	3				
Skin painting						
SENCAR mouse	1	0				
CD-1 mouse	0	1				

m = marginal
i = inconclusive

Table IV-5

Summary of Test Results for DEHP and Metabolites

Test Name	Abbreviation	DEHP	MEHP	EH	α^{+a}	α^{-}
Salmonella mutagenicity	Sty	-	-	-	0.612	0.806
E. coli mutagenicity	Ecw	-	+		0.612	0.857
B. subtilis DNA repair	Bsr	-	+	+	0.906	0.500
In Vitro cytogenetics	Cvt	-	+	-	0.890	0.667
Sister chromatid exchange	SCE	-	+*		0.890	0.667
Unscheduled DNA synthesis	UDS	-	-	-	0.612	0.806
DNA damage in vivo	Drp	-	-	-	0.890	?(0.5)
Specific gene mutation in mouse lymphoma L5178Y cells	Mly	-	-	-	0.836	?(0.5)
Specific gene mutation in Chinese hamster ovary cell	CHO	-	-		0.781	?(0.5)
Specific gene mutation in V79 Chinese hamster cells	V79	-			0.781	?(0.5)
Micronucleus test	Mnt	-	-	-	0.836	?(0.5)
In vivo bone marrow cytogenetics	Cbm	-	-	-	0.836	?(0.5)
In vitro leukocytes cytogenetics	Cle	-			0.836	?(0.5)
Body fluid/Salmonella	Bfl	-		-	0.757	?(0.5)
Transformation of Syrian hamster embryo cells	SHE	+	+		0.906	0.667
Transformation of murine C3H cells	C3H	+ *	-		0.890	?(0.5)
Transformation of murine Balb/C 3T3 cells	3T3	-	-	-	0.781	?(0.5)
Virus-enhanced transformation	VET	+			0.890	0.444
Drosophila sex-linked recessive lethal	DRL	-			0.836	0.806

* equivocal result

a values for alpha + and alpha- were estimated from the performance of the assays using the Gene-Tox data base (Palajda and Rosenkranz 1985; Pet-Edwards et al 1985a,b).

K. Appendix I

The Carcinogenicity Prediction and Battery Selection Method (CPBS)

1. CPSB Method

The Carcinogenicity Prediction and Battery Selection (CPBS) method uses Bayes' decision theory (Kendall and Stuart 1967) to calculate the probabilities θ+ and θ- that a test chemical is or is not a carcinogen by taking advantage of the known performance characteristics of the various assays, i.e. their sensitivity (alpha + = Pr(+/CA)) and specificity (alpha - = Pr(-/NC)), where Pr, CA and NC signify probability, carcinogen, and noncarcinogen, respectively.

If assay A_i gives a positive result:

$$\theta_i^+ = Pr(CA/A_i = +)$$

$$\theta_i^+ = \frac{\theta_i^+ \text{-1 } Pr(A_i = +/CA)}{\theta_i^+\text{-1 } Pr(A_i = +/CA) + (1 - \theta_i^+\text{-1}) Pr(A_i = +/NC)}, \text{ and}$$

$$\theta_i^- = Pr(NC/A_i = +) = 1 - \theta_i^+$$

If assay Ai gives a negative result:

$$\theta_i^+ = Pr(CA/A_i = -)$$

$$\theta_i^+ = \frac{\theta_i^+\text{-1 } Pr(A_i = -/CA)}{\theta_i^+\text{-1 } Pr(A_i = +/CA) + (1 - \theta_i^+\text{-1}) Pr(A_i = -/NC)}$$

$$\theta_i^- = Pr(NC/A_i = -) = 1 - \theta_i^+$$

and we define

$$\theta_o^+ = Pr(CA) \quad \text{and} \quad \theta_o^- = Pr(NC)$$

where A_i is an assay and Pr(CA) and Pr(NC) refer to the expert's preknowledge regarding the possible carcinogenicity or noncarcinogenicity of the chemical under consideration. If on the basis of structural features or known metabolic transformation the expert believes that the chemical is likely to be a carcinogen or a noncarcinogen, the appropriate

term can be increased to exceed 0.5. If, on the other hand, there is no preknowledge regarding the carcinogenicity, or lack thereof, of a test chemical, then a value of 0.5 is used. With DEHP and its metabolites, no such preknowledge is assumed and a value of 0.5 is used.

The value of alpha + (sensitivity) is the ratio of the number of carcinogens found to be positive in an assay to the number of carcinogens tested, and for alpha - (specificity), it is the ratio of the number of noncarcinogens found to be negative in an assay to the number of noncarcinogens tested. These are estimated from the reported performance of the assay in the appropriate peer-reviewed Gene-Tox report (Palajda and Rosenkranz 1985). When there are not enough statistically significant test results for either an alpha + or alpha - to be estimated (as for alpha - in this report), a noninformative assumption is made, i.e., a lack of discrimination for noncarcinogens is assumed and a value of 0.5 is assigned (Pet-Edwards et al 1985a,b). As demonstrated below for DEHP and its derivatives, the effects are far smaller due to the unambiguous nature of the estimated values of $\theta+$ and $\theta-$.

2. Application of CPBS to DEHP

The various test results for which alpha + or alpha - values can be estimated from the Gene-Tox data base (Palajda and Rosenkranz 1985; Pet-Edwards et al 1985a,b) are listed in Table IV-5. To illustrate the CPBS method, a few detailed examples are given. First, using only data from assays for which both alpha + and alpha - values are known and using Bayes' equation and assigning a value of 0.5 to Pr(CA) and Pr(NC), in the absence of pre-knowledge, for UDS, which gave a negative test result:

$$\text{UDS}\quad \theta_1^+ = \frac{(0.5)\ (1 - 0.612)}{(0.5)\ (1 - 0.612) + (1 - 0.5)\ (0.806)} = 0.325 \quad (1)$$

That is, based on the results of a negative UDS test only, there is a 32.5% chance that DEHP is a genotoxic carcinogen, or a 67.5% probability of non- carcinogenicity.

Iterative use of the Bayes' equation gives for the combination UDS and SCE (the latter also gave a negative result):

$$\theta_2^+ = \frac{(0.325)\ (1 - 0.890)}{(0.325)\ (1 - 0.890) + (1 - 0.325)\ (0.667)} = 0.07355^* \quad (2)$$

and for the combination UDS, SCE and SHE, it is

$$\theta_3^+ = \frac{(0.07355)\ (0.906)}{(0.07355)\ (0.906) + (1 - 0.7355)\ (1 - 0.667)} = 0.1777 \quad (3)$$

For the combination UDS, SCE, SHE and VET, it is

$$\theta_4^+ = \frac{(0.1777)\ (0.890)}{(0.1777)\ (0.890) + (1 - 0.1777)\ (1 - 0.444)} = 0.2571 \quad (4)$$

In other words, by the above battery of four assays with known alpha + and alpha - values, two of which give positive responses and two negative responses, it can be estimated that DEHP has a 74% (i.e. 100 - 25.7) probability of not being a genotoxic carcinogen.

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* The computation procedure using Bayes' equation iteratively is numerically symmetrical, i.e. the same result would be obtained irrespective of the order in which the test results are computed.

Continuing with the other assays with known values for alpha + and alpha - with Sty we have:

$$\theta_5^+ = \frac{(0.2571)\ (1 - 0.612)}{(0.2571)\ (1 - 0.612) + (1 - 0.2571)\ (0.806)} = 0.1428 \quad (5)$$

adding Cvt:

$$\theta_6^+ = \frac{(0.1428)\ (1 - 0.890)}{(0.1428)\ (1 - 0.890) + (1 - 0.1428)\ (0.667)} = 0.0267 \quad (6)$$

adding Ecw:

$$\theta_7^+ = \frac{(0.0267)\ (1 - 0.612)}{(0.0267)\ (1 - 0.612) + (1 - 0.0267)\ (0.857)} = 0.01227 \quad (7)$$

finally including Bsr, we have:

$$\theta_8^+ = \frac{(0.01227)\ (1 - 0.906)}{(0.01227)\ (1 - 0.906) + (1 - 0.01227)\ (0.5)} = 0.00233 \quad (8)$$

or a 0.23% probability of genotoxic carcinogenicity, i.e., a 99.8% probability of non-genotoxic carcinogenicity. θ^+ is further decreased when the results of the assays for which alpha+ values are not known and which are assigned values of 0.5 (i.e. non-discrimination) are included in the estimates.

Similar calculations were performed for MEHP and 2-ethylhexanol using the values listed in Table IV-5.

The validation of the CPBS method as well as other applications have been described previously (National Academy of Sciences 1985; Pet-Edwards et al 1984a; Rosenkranz et al 1984b,c).

V. CARCINOGENICITY IN ANIMALS

A. Animal Bioassays for Carcinogenicity

Relatively few bioassays of DEHP in animals have been conducted: three in rats of different strains (one of which was a multigenerational study) and one in mice. Two of the assays were well done, however, and provide definitive evidence for the carcinogenicity of DEHP for rodents.

The first reported long-term bioassay of DEHP was a multigenerational study (Carpenter et al 1953). The parental generation consisted of groups of 32 male and 32 female Sherman rats that were administered diets containing 0.4, 0.13, 0.04 or 0% DEHP starting at 60 days of age. Offspring from rats that had been on the high dose level or the control diet for 120 days formed the F_1 generation. F_1 litters were reduced to 2 male and 2 female pups and groups of 32 male and female offspring were continued on the 0.4% DEHP or control diets. After one year on their respective diets, all but 8 in each parental group and all the F_1 rats were killed. The remaining groups of 8 parental rats were kept up to 2 years on the diet (790 days of age). Portions of 8 organs and abnormal tissues from each rat were examined microscopically.

Survival was poor by present standards. The mortality in the P_1 generation after one year of DEHP exposure was 64%, and 72% of the deaths were due to pneumonia. Mortality in the F_1 groups was 46% of dosed rats and 43% of control rats. In both the P_1 and F_1 generations liver and kidney weights were higher at the 0.4% dose level than in control rats. Tumors were found in 3 high dose, 4 mid-dose, 2 low dose, and 5 control P_1 rats and in 2 dosed and 1 control F_1 rats but the age at diagnosis, the locations of the tumors, and the diagnoses of tumor types were not given.

Data for the rats surviving more than one year were not given separately in the report.

The Panel, noting a number of deficiencies in this study including small group sizes, shortened survival from pneumonia, incomplete histopathologic examinations, and under-reporting of results, concluded that the study in rats was inadequate as a bioassay for carcinogenicity. One-year bioassays in guinea pigs and dogs, reported in the same paper, were also judged to be inadequate for conclusions to be drawn about carcinogenicity in these species because of small group sizes and the short duration of the experiments.

Another bioassay was conducted in Wistar rats (Harris et al 1956). Groups of 43 weanling rats of each sex were fed diets containing 0.5, 0.1, or 0% DEHP. After serial killing (4 from each group after 3 months, 4 after 6 months, and 10 after 12 months), 24 rats from each group were to be maintained on the diets for 24 months.

During the two-year period 85 to 96% of the rats in the various groups died. Surviving 24 months were 3 males and 4 females in the high dose group, 2 males and 2 females in the low dose group, and 1 male and 1 female in the control group. The only tumors reported were fibroliposarcomas of the hind feet in 2 high dose rats and in 1 control rat. Bronchopneumonia and chronic nephritis were common findings unrelated to compound administration.

The Panel noted that data on food consumption, body weights, and organ weights are difficult to interpret because of possible variations related to intercurrent diseases. Because of small group sizes, poor survival, and under-reporting, this study is inadequate for the evaluation of the carcinogenicity of DEHP.

More recently, definitive bioassays in rats and mice have provided evidence that DEHP is hepatocarcinogenic for rats and mice (NTP 1982; Kluwe et al 1982). Groups of 50 weanling F344 rats of both sexes were fed diets containing 12,000 or 6,000 ppm and groups of 50 B6C3F1 mice of both sexes were fed diets containing 6,000 or 3,000 ppm DEHP for 103 weeks. Untreated concurrent control groups of 50 rats and mice of each sex were used.

In rats, mean body weight gain was decreased in dosed males and in high dose females throughout the study but survival was comparable in-all groups. Food consumption was reduced slightly in dosed rats of both sexes. The incidences of hepatocellular carcinoma and neoplastic nodules (tumors of the liver in which the degree of malignancy cannot be established solely on a histologic basis) were increased in dosed rats of both sexes as compared to control rats (Table V-1). In addition, clear cell foci, which are generally considered to be preneoplastic, occurred with higher frequency in dosed male rats than in control rats. On the other hand, high dose male rats had lower incidences of pituitary tumors, thyroid C-cell tumors, and testicular interstitial cell tumors.

In mice, mean body weights were slightly lower in dosed male mice but considerably lower in dosed female mice in a dose-related manner. There were no apparent differences among the groups of mice in food consumption and no significant differences in survival. The incidences of liver tumors (adenomas and carcinomas combined) were increased in dosed mice (Table V-2). The liver tumors were more often multiple in dosed mice and were more often diagnosed as malignant in dosed female mice. Pulmonary metastases were found in more than a third of dosed tumor-bearing mice of both sexes but in no control mice.