Introduction

The chemical industry has a long-term interest in the health and environmental effects of phthalic anhydride-derived plasticizers. Until the beginning of this decade it was felt that the large volume of production of these plasticizers (in excess of one billion pounds per annum) and their extensive use in items of commerce were without any adverse consequences for public health. In fact, the Chemical Manufacturers Association (CMA) Phthalate Esters Panel had by 1980 confined its attention mostly to the environmental behavior of phthalate esters.

This circumstance was changed by the findings in the National Toxicology Program (NTP) bioassay (1), that bis (or di-) 2-ethylhexyl phthalate (DEHP) was a hepatocellular carcinogen in the Fischer 344 rat and the B6C3F1 mouse. These results came as a considerable surprise; the past human use experience with DEHP, its structure–activity relationships, its relative chemical inertness, and its lack of biological activity at other than extremely high intake levels led one to predict that it would possess no tumorigenic potential. These factors lead all those concerned, including regulatory agencies and industry, to adopt a cautious attitude in interpreting the bioassay results. Additional factors included the absence of evidence for genotoxicity for both DEHP and the phthalate ester class as a whole, indications in the scientific literature that substantial differences exist between the metabolism of DEHP in the rodent and in other species (2), and the fact that DEHP is a member of the hypolipidemic class of rodent peroxisome inducers (3). The results of the NTP bioassay therefore prompted a serious effort by CMA to explore the genotoxic, metabolic, and biological properties of DEHP to understand better the relevance of the bioassay. Also, concern over the implications of the bioassay results for other phthalate esters in significant commercial use was raised by the U.S. Environmental Protection Agency (US-EPA).

As a result of negotiations between CMA and US-EPA, a voluntary program of testing was undertaken by CMA to meet both regulatory and industrial concerns. The objectives of the voluntary program are (a) to confirm the absence of genotoxicity due to DEHP; (b) to stand-
Table 1. Phthalate esters and related compounds in the CMA voluntary test program (Production volume >10^6 lb/yr).

| Genotoxicity                        | Reference* | Test          | Peroxisome screening (phthalates) | Reference* | Test          |
|-------------------------------------|------------|---------------|-----------------------------------|------------|---------------|
| Di-2-ethylhexyl phthalate           | Di-2-ethylhexyl | Dimethyl      | Di-2-ethylhexyl                   | Di-2-ethylhexyl | Dimethyl      |
| Mono-2-ethylhexyl phthalate         | Di-2-ethylhexyl | Di-n-butyl    | Di-n-butyl                        | Di-2-ethylhexyl | Di-n-butyl    |
| 2-Ethylhexanol                      | Di-2-ethylhexyl | Diundecyl    | Diundecyl                         | Diundecyl   | Diundecyl    |
|                                    | Diisodecyl  | Diisodecyl    | Diisodecyl                        | Diisodecyl  | Diisodecyl    |
|                                    | Diisononyl  | Diisononyl    | Diisononyl                        | Diisononyl  | Diisononyl    |
|                                    | Dihexyl, octyl, decyl | Dihexyl, octyl, decyl | Dihexyl, octyl, decyl | Dihexyl, octyl, decyl | Dihexyl, octyl, decyl |
|                                    | Diheptyl, nonyl, undecyl | Diheptyl, nonyl, undecyl | Diheptyl, nonyl, undecyl | Diheptyl, nonyl, undecyl | Diheptyl, nonyl, undecyl |
|                                    | n-Butylbenzyl | n-Butylbenzyl | n-Butylbenzyl                     | n-Butylbenzyl | n-Butylbenzyl |

*Di-2-ethylhexyl-adipate included: results are reported elsewhere.

ardize test conditions for determining the genotoxic potential of representative phthalate esters, (c) to establish a standard peroxisome induction test based on rodent feeding, (d) to compare representative phthalate esters as a means of establishing the level of concern for potential health effects, (e) to investigate the metabolic behavior of DEHP under the conditions of the NTP bioassay in rats, and (f) to compare the metabolism of DEHP in rodents and a primate under standard conditions. This paper reports studies on the genotoxicity of DEHP and progress in setting up a standard peroxisome induction test. It provides new information on the metabolism of DEHP in the rat and a comparative study on the metabolism of DEHP in the Fischer 344 rat, the B6C3F1 mouse, and the cynomolgus monkey.

CMA Voluntary Test Program

The program was laid out as follows: (1) validation and optimization for (a) genotoxicity screening and (b) biological test screening; (2) fate and metabolism studies on DEHP regarding (a) dose and prefeeding response and (b) comparative metabolism; (3) screening of representative phthalate esters. A clear requirement (step 1) was the selection, validation, and optimization of standard test procedures for DEHP, certain related compounds, and representative phthalate esters. The validation and optimization step involved the selection of appropriate parameters for screening for peroxisome-inducing activity, and selecting established protocols for genotoxicity testing. Representative esters were selected on the basis of production volume and structure. The volume cut-off point was 10 million pounds per year. The factors in structural consideration were molecular weight (high or low), linearity or branching of the alcohol side chain, odd or even number of atoms in the side chain, and whether the side chains in an ester were identical or dissimilar.

These factors taken together resulted in the selection of eight representative test compounds (Table 1), including additional dimethyl phthalate genotoxicity testing because of recent inconclusive findings of its weak mutagenicity. Optimization and validation for both genotoxicity and peroxisome induction were performed with DEHP and di(2-ethylhexyl) adipate (DEHA) as reference compounds. Definitive information about the genotoxicity of DEHP was required for interpretive background to the bioassay results, and the primary metabolites of DEHP, i.e., mono(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol (2-EH), were also included in the prototype genotoxicity test battery.

Genotoxicity Testing

The proposed genotoxicity test battery was set up to complete the DEHP genotoxic profile and to establish standard protocols for representative phthalate ester testing. As initially set out, this consisted of the standard Ames Salmonella typhimurium test, the mouse lymphoma L5178Y thymidine kinase assay, the primary rat hepatocyte unscheduled DNA synthesis test, the mouse micronucleus (chromosomal aberration) test, and a cell transformation test using the BALB/3T3 cell line. All tests included activation where feasible. The rodent S9 fraction used for activation was derived from the Sprague-Dawley Aroclor-treated rat. The test protocols were those provided for standard tests performed by Litton Bionetics Inc., Kensington, MD, U.S.A.

The results obtained by running DEHP through this test battery are given in Table 2. It is seen that over the very wide dose ranges used in all of the tests employed, no positive results were obtained with DEHP. It is probably safe to say, that up to the limits of solubility for DEHP that can be obtained in any of these tests, it is not genotoxic. In addition, MEHP and 2-EH were tested in the CMA program in the Ames Salmonella typhimurium test, with and without activation, the mouse micronucleus test, and the BALB/3T3 cell transformation test, with and without rat primary hepatocyte activation. Both compounds were negative in all tests. Combined with results in the mouse lymphoma test and the unscheduled DNA synthesis test reported earlier (6) and other studies (7), these results strongly suggest that DEHP and its primary metabolites in the rodent, MEHP and 2-EH, are not genotoxic.

The components of the test battery selected by CMA, on the basis of these results, were the Ames Salmonella typhimurium test, the CHO-HGPRT test, and the BALB 3T3 cell transformation test. CMA is presently testing the representative esters in this modified battery. However, the Ames test will be omitted from the CMA
battery because the identical compounds are under test by the NTP (5). The mouse lymphoma test was replaced by the CHO-HGPRT test because of the lesser incidence of false positives in the latter test, and fewer confusing problems with interpretation at high levels of cell lethality.

**Biological Screening**

In rodents, DEHP, MEHP, 2-EH, and DEHA are known to depress serum triglyceride and cholesterol levels, produce liver enlargement, elevate hepatic catalase activity, and induce certain microbodies in the liver known as peroxisomes (8). They are thus thought to belong to a class of hypolipidemic compounds that were identified by Reddy and his associates as being both non-genotoxic and having the potential to induce liver tumors in rodents (8).

This hypothesis that peroxisome induction and rodent tumorigenesis are related has been extensively discussed at this and at other meetings on phthalate esters. While there is much evidence in favor of the correlation, at present no mechanism has been discovered that directly relates the observed physiological changes with liver tumorigenesis. As has been pointed out, the phenomenon may not occur in primates.

The primary biochemical and morphological change in the target organ for DEHP oncogenesis, the liver, is a derangement of lipid metabolism as manifested by the changes discussed above. In screening the selected representative esters for their possible oncogenic potential, CMA proposed a research program to determine if, in fact, phthalate esters could be graded for their effects on rodent hepatic lipid metabolism. The parameters established for the exploratory screening tests are hepatic catalase, hepatic acetyl carnitine transferase, cytochemical and morphological parameters on liver, serum triglyceride, serum cholesterol, and serum total lipids. The hepatic catalase and hepatic acetyl carnitine transferase have been replaced by cyanide-insensitive palmitoyl coenzyme A oxidation and lauryl hydroxylase (ω, ω - 1). It was the intention to see if these could be incorporated into a short feeding test with the Fischer 344 rat, using DEHP and DEHA as reference compounds. The initial parameters were liver size, serum lipid and cholesterol levels, liver catalase and acetyl carnitine transferase activities, and cytochemical and morphological measurements on liver cells. The enzyme systems were those considered most representative of liver peroxisome activity at the time of setting up the experiment.

The prototype feeding study employed three dietary dose levels and a control level, with five each of male and female rats at each level. Sacrifice points for measurements were at 1 or 3 weeks after feeding with the reference compound, and after 3 weeks of feeding followed by a 2-week recovery period. The intake levels were those employed in the NTP bioassay with an additional lower

| Test                                      | Dose range      | Results                                      |
|-------------------------------------------|-----------------|----------------------------------------------|
| Ames, activation                          |                 |                                              |
| No                                        | 0.15–150 μL/plate| No revertants over background               |
| Yes                                       | 0.15–150 μL/plate|                                              |
| Mouse lymphoma activation                 |                 |                                              |
| No                                        | 7.8–250 nL/mL   | No increase in mutant frequency              |
| Yes                                       | 7.8–125 nL/mL   |                                              |
| Mouse micronucleus                        |                 |                                              |
| Single dose                               | 5 g/kg          | No increase in % micronucleated cells        |
| Multiple dose                             | 5 g/kg/day      |                                              |
| Unscheduled DNA synthesis                 | 5–1000 nL/mL    | No significant increase in net nuclear grain counts |
| Cell transformation                       | 0.875–21 nL/mL  | No significant increase in foci              |
| Cell transformation with                  | 6.25–100 nL/mL  |                                              |
| primary hepatocytes                       |                 |                                              |

### Table 2. Genotoxicity data on DEHP (CMA Program).

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**FIGURE 1.** Relative liver weights. Fischer 344 rats received DEHP in the diet at the dose level indicated for 1 and 3 weeks, and 3 weeks followed by a 2-week recovery.
intake level of 0.1%. These studies were performed for CMa by the Midwest Research Institute, Kansas City, MO.

This communication will describe the results with DEHP only. Cumulative liver weights in serially sacrificed animals are given in Figure 1. It is clear that at the dose levels in the NTP study, dramatic increases in liver weight appear for both sexes within a few days of the onset of feeding. These increases are reversible, as they rapidly decline after the cessation of feeding of DEHP. In addition, the 0.1% dose level did not induce significant liver enlargement in female rats, and the increased weight at the 3-week point in the male rat may be statistically significant because of the drop in control liver weights.

The effect of this regimen on serum lipids is presented in Figure 2. All three dose levels depressed serum triglyceride levels, while the effect on cholesterol levels in males and females was somewhat less marked. However, in the withdrawal period, all levels reverted to normal.

Effects on marked enzyme activities are given in Figures 3 and 4. It is seen that in general, carnitine acetyl transferase showed marked increases in specific activity for all dose levels at 1 week after the onset of feeding (Fig. 3). This effect was maintained for the uppermost dose levels after 3 weeks of feeding. This enzyme returned to nearly normal values during the withdrawal period. Hepatic catalase was evidently less responsive to DEHP feeding (Fig. 4). It showed a fairly high normal specific activity that was widely variable. Increases produced by feeding were usually less than double the control level. The range of variability from rat to rat for a given data point was also unusually wide. Although some dose-associated elevations occurred and specific activities apparently reverted to normal when elevated, it was felt that the catalase activity determination was of qualitative value only. The test laboratory also reported cyto-

![Figure 3](image-url) **Figure 3.** DEHP and hepatic carnitine acetyltransferase (CAT). Fischer 344 rats received DEHP in the diet at the dose level indicated for 1 and 3 weeks, and 5 weeks followed by a 2-week recovery.

![Figure 4](image-url) **Figure 4.** DEHP and hepatic catalase. Fischer 344 rats received DEHP in the diet at the dose level indicated for 1 and 3 weeks, and 3 weeks followed by a 2-week recovery.
induction than the earlier enzyme markers (9,10). Work in progress at the British Industrial and Biological Research Association (BIBRA) has in fact confirmed the original validation study performed at the Midwest Research Institute, established the correlation of dose levels with the new enzyme markers, and allowed us to establish dose ranges for testing the selected representative phthalate esters. Testing of these is now underway at BIBRA in the screening system.

Metabolism and Pharmacokinetic Studies

Effects of Dose Variability and Prolonged Feeding

The extensive studies on the metabolism of DEHP in the rat after administration by gavage have been thoroughly reviewed at previous conferences on phthalate esters, and in this conference. The pioneering work of Albro (2,11), and the more recent work of Lhuguenot et al. (12), have established the salient features of DEHP absorption, metabolism, and elimination. The convention for numbering the metabolites as established by Albro has now become accepted as a shorthand way of referring to them rather than by their complex structural nomenclature.

Although these accounts of metabolism were reasonably complete, two important issues relating to the NTP bioassay needed clarification. First, most of the existing studies were performed by gavage intake with single doses. In fact, we need to know the effects of incorporating DEHP into the diet, of prolonged feeding, and of varying the intake, on the uptake and metabolism of DEHP. Second, an interspecies comparison using similar doses and dosing regimens was needed to confirm the indications in the literature that primates and rodents metabolized DEHP in a significantly different manner.

The procedures for the dietary feeding study are given in Table 3. This work was performed by Arthur D. Little Inc., Cambridge, MA. The study design called for up to 21 days feeding of unlabeled DEHP at three intake levels, the upper two of which were those used in the NTP bioassay. Labeled DEHP was administered in the diet at the corresponding intake level at days 1, 7, and 21. Excreta and expired air were collected for analysis for the 96-hr period following feeding of the labeled compound.

| Parameter                      | 14C-DEHP                         |
|-------------------------------|----------------------------------|
| Compound                      | F344 rat, M                      |
| Intake level                  | 2,000, 6,000, 12,000 ppm DEHP     |
| Duration                      | 21 days feeding                  |
| Dosing                        | Diet at 1, 7, 21 days            |
| Collections                   | 12, 24, 48, 96 hr. after labeled compound CO2, urine, feces |
| Sacrifice                     | Selected rats 1 day after each labeled dose |

These collections were made at discrete intervals, but collections were pooled for metabolic identification. Metabolites were detected and quantitated both before and after glucuronide and sulfate conjugate hydrolysis. The detection procedures included high-pressure liquid chromatography and gas chromatography-mass spectrometry.

A study of this type generates a large amount of data, and this report will highlight only the more significant findings. The distribution of the radiolabel in excreta is given in Table 4. The earlier gavage studies suggested that conversion of DEHP to MEHP in the gastrointestinal tract and uptake of DEHP were rate-limited or saturable steps. It is evident that when DEHP is administered in the diet these limiting mechanisms are not invoked, presumably because the amount of DEHP in the intestinal tract is too low. In fact, an apparently higher proportion of radioactivity is eliminated in the urine at the 12,000 ppm intake level than at the 1000 ppm level. The proportion of the dose eliminated in the feces also may drop with increased dose. At the dose levels encountered in the NTP bioassay, however, no dose-related changes in uptake or absorption were seen.

Profiles of the metabolites of DEHP were obtained for all the pooled urine collections. It was thus possible to ascertain any quantitative or qualitative changes in metabolic pattern with duration of feeding and size of dose. It will be recollected that in the Albro scheme of metabolism two important routes for side chain oxidation were suggested. One of these (Fig. 5) began with metabolite

![Diagram](https://via.placeholder.com/150)

**FIGURE 5.** Conversion of DEHP to metabolite I. Metabolites are numbered according to Albro based on the metabolism of DEHP in the rat.
X, which is formed from DEHP by hydrolysis and terminal common carbon oxidation. Metabolite X in turn is oxidized to metabolite V, which then enters the \( \beta \)-oxidation pathway to be converted to metabolite I. A second pathway (not pictured) also starts with MEHP, and by penultimate carbon oxidation yields metabolites VI and III.

Upon examination of the urinary metabolic profiles, no qualitative differences were seen which could be ascribed to variations in intake level or duration of feeding. However, qualitative differences in the percentages of metabolites I and V were found (Table 5). At 1000 ppm, the output of metabolite I, the end product of \( \beta \)-oxidation, approximately doubles by day 6 of feeding. The proportion of metabolite V is unchanged. At 6000 ppm, the proportion of metabolite V produced without prefeeding is increased from that seen at 1000 ppm. A striking increase in the amount of metabolite I is seen after 6 days of prefeeding, with a corresponding fall in the amount of metabolite V. At 12,000 ppm, the same phenomenon is seen, but with slightly larger amounts of production of metabolite V without prefeeding, and of metabolite I with prefeeding.

It is clear that nonlinear changes in the production of these metabolites associated with terminal carbon and \( \beta \)-oxidation are occurring as a result of prolonged feeding and increased DEHP intake. It is of interest that the penultimate C-atom route of oxidation does not appear to be affected by these procedures to the same extent as the terminal C-route.

The biological studies on DEHP described above indicated that the feeding of DEHP over a dose range and for a similar duration to that in these metabolic studies produced liver weight increases, serum lipid depression, increased activity of liver oxidative enzyme systems, and induction of peroxisomes. Selected animals were removed from these metabolic studies and sacrificed. The relative liver weights at sacrifice points and dose levels which correspond to those in the metabolic study are given in Table 6. These values agree with those found in the biological studies and also indicate a correlation between increased liver weight and the increased output of \( \beta \)-oxidation metabolites from DEHP.

This enhancement of beta oxidation may have considerable significance in suggesting a mechanism for DEHP hepatocellular carcinogenesis (Fig. 6). Under normal circumstances, metabolite V would proceed to metabolite I through the mitochondrial \( \beta \)-oxidation processes. However, at high doses, under conditions of prolonged feeding, the overload of metabolite V may pass through the peroxisomal \( \beta \)-oxidation pathway. This produces hydrogen peroxide, unlike the \( \beta \)-oxidation pathway in the mitochondria. This in turn may be regarded as a substance capable of producing further cellular damage, including alterations in DNA. While this hypothesis is unproven, it is reasonable to conclude that high doses and prolonged feeding of DEHP produce a marked disturbance in the lipid oxidation processes in the liver. As the liver is the only organ that shows a neoplastic response to DEHP in the rat, it is reasonable to suppose that the two phenomena may be related.

These relationships, as elucidated by the CMA study, are summarized graphically in Figure 7. This three-dimensional figure combines the prefeeding DEHP intake with the labeled dose intake and the duration of prefeeding. The shaded area indicates where increased \( \beta \)-oxidation of DEHP occurs. It is clear that the greatest amount of this activity was found at the DEHP intake level of 12,000 ppm for feeding periods in excess of 6 days. A differential metabolic response is clearly demonstrated for the dose levels employed in the NTP bioassay. The biological studies also target these doses as being marked by increased responses in the parameters associated with peroxisome induction.

**Comparative Metabolism of DEHP**

A second major aspect of the CMA metabolic studies concerned the definition of any species differences which

| Dose, ppm | 1 day | 7 days | 21 days |
|----------|-------|--------|---------|
| 1000     | 7.24  | 8.62   | 9.06    |
| 6000     | 7.08  | 9.50   | 10.98   |
| 12000    | 6.87  | 10.51  | 12.16   |

![Figure 6](image_url)
exist in the metabolism of DEHP. Clearly, these differences can play a key role in understanding the significance of the bioassay findings for man.

Previous studies by Albro (11) had indicated that DEHP metabolites in the rat were mostly dicarboxylic acids with a minor glucuronide component. In man and the monkey, metabolites tended to be alcohols and were extensively conjugated as glucuronides. However, considerable differences in routes of administration and dose levels exist for these studies which make direct comparison difficult.

The protocols proposed by CMA (Table 7) provide for comparison of the metabolism of a single dose of labeled DEHP in male Fischer 344 rats, the cynomolgous monkeys and B6C3F1 mice. A 100 mg/kg dose was given by gavage, and urine and feces were collected periodically.

Metabolite identification was performed on the pooled 96-hr urine using the techniques already worked out in the dose-response and feeding study described above.

Similar qualitative metabolic profiles were found in all three species (Table 8), and, because of the complexity of these pathways, only the salient details are given. Under conditions of oral gavage, it is evident that all three species produce significant amounts of acidic metabolites and that conjugation is not the major pathway in any of them. Furthermore, all three species extensively hydrolyse DEHP in the gastrointestinal tract, and their major metabolites arise from MEHP. The key difference appears to lie in the negligible output of metabolite I in the monkey as compared with the rat and the mouse. In fact, in the important sequence of metabolites X, V, and I, there appears to be a buildup of metabolite V in the monkey with almost no metabolite I. In contrast, both the rat and the mouse were able to produce metabolite I.

These results suggest that DEHP is not handled by the β-oxidation pathway in the monkey and, by extension, in other primates. This may represent a key distinction between the primate and the rodent of importance in assessing the results of the bioassay. It should be pointed out, however, that we do not know whether prolonged feeding of a high dose of DEHP to the monkey would produce the stimulation of this pathway as seen in the rodent. Suggestive evidence that DEHP is not a potent peroxisome inducer in subhuman primates such as the marmoset (13) would indicate that this is not likely to be the case.

### Table 8. Comparative metabolic studies on DEHP (CMA): 100 mg/kg 14C-DEHP, % of radioactivity.

| Metabolite        | Cynomolgous monkey % | Rat % | Mouse % |
|-------------------|----------------------|-------|---------|
| Urine MEHP        | 11                   | —     | 17      |
| Phthalic acid     | 2                    | 2     | 13      |
| Metabolite I      | 0.5                  | 11    | 13      |
| Metabolite V      | 25                   | 29    | 1       |
| Metabolite X      | 9                    | 4     | 2       |
| Conjugates        | 15                   | 1     | 5       |
| Feces DEHP        | 34                   | 20    | 16      |
| MEHP              | 3                    | 8     | 24      |

**Conclusions**

This survey of the interim results of the CMA program, while necessarily incomplete in many details, indicates that satisfactory progress is being made in achieving the objectives of the voluntary test program. It is now clear that DEHP is nongenotoxic, and that under in vivo conditions its major initial metabolites are also not genotoxic. Satisfactory experimental conditions have been worked out for screening representative phthalate esters for their effects on the oxidative processes of the rat liver. A differential metabolic response has been demonstrated with prolonged feeding over a range of intake levels in DEHP in the rodent. It is interesting that this response is associated with the disturbances in lipid metabolism already demonstrated in our biological studies and those of others. Finally, while there are no qualitative species differences in the metabolism of DEHP, quantitative differences which do ex-
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ist between primates and rodents lie in the area of metabolite oxidation.

It would be desirable to see further studies on the relationship between peroxisome proliferation and rodent tumorigenesis and on the effects of lipid oxidation processes in the monkey of prolonged feeding of a high dose of DEHP.

These studies were carried out under the supervision of the Toxicology Research Task Group, Phthalate Ester Panel, Chemical Manufacturers Association, Washington, DC, U.S.A. They were performed at Arthur D. Little, Inc., the Midwest Research Institute, and Litton Bionetics, Inc.

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