Environmental Health Perspectives
Vol. 45, pp. 19-25, 1982

Pharmacokinetics, Interactions with Macromolecules and Species Differences in Metabolism of DEHP

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The recent long-term carcinogenesis bioassay of di(2-ethylhexyl) phthalate (DEHP) in rats and mice reported by the National Toxicology Program was the first such bioassay to implicate DEHP as a hepatocarcinogen. At the levels of DEHP fed (up to 1.2% of the diet for two years), the livers of the rats would have been exposed to unhydrolyzed diester; this would not have been the case at lower dosages. Extrapolation to lower dosages is therefore questionable. We do not have sufficient pharmacokinetic data in mice to evaluate the dose relationships as yet. Rodents differ conspicuously from primates in their manner of metabolizing DEHP, both in terms of the demand made on the oxidation potential of the liver and in the chemical properties of the major metabolites. The relevance of these differences must be determined before rodent species can be considered models for the effects of DEHP in humans. Radioactivity from carbonyl-labeled DEHP did not associate with purified protein, RNA or DNA from rat liver *in vivo*. Label from 2-ethyl-(1-^{14}C)-hexyl-labeled DEHP or mono(2-ethylhexyl) phthalate (MEHP) did appear to associate strongly with purified DNA, but label from free ^{14}C-labeled 2-ethylhexanol did not. The apparent binding from DEHP and MEHP was not exchangeable, but was not proven to be covalent. This phenomenon needs additional study.

Introduction

The studies to be presented in this report were undertaken in response to the recent announcement by the National Toxicology Program (NTP) that di(2-ethylhexyl) phthalate (DEHP), fed at high levels in the diet to rats and mice for two years, was a hepatocarcinogen. We wanted to know something about the possible mechanism of carcinogenesis in rodent liver and also to explore the relevance of the extremely high doses used to produce it.

Materials and Methods

Dose Dependence of Distribution/ Elimination

The first study relates to the effect of the dosage of DEHP on its metabolism, distribution and excretion. Preliminary experiments with both Sprague-Dawley (CD) and Fischer 344 rats revealed that the maximum amount of DEHP that could be given as a single oral dose without significant excretion of unabsorbed DEHP in the feces was 200 mg/kg body weight. Pharmacokinetic studies using ^{14}C-labeled DEHP at doses above this level would rapidly become dominated by unabsorbed DEHP and could not be directly compared to rates of elimination at lower doses.

We chose to give DEHP by gavage in cottonseed oil rather than incorporated into the solid diet for several reasons. First, we wished to avoid the problem of determining the amount of food actually eaten, and the problem of achieving a comparable dose among replicates on an *ad libitum* schedule. Second, we wished to avoid the possibility of contamination of urine and feces with radioactivity from food crumbs. Most importantly, doctoring feed with high levels of substances that can be smelled alters feeding habits. Feed containing low levels of DEHP is consumed over a shorter time interval than food containing a high level of DEHP. In order

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to have DEHP dosage be the dominant variable, then, it was necessary to administer the compound by gavage.

To lessen the discrepancy between the NTP bioassay feeding conditions and the present study, the DEHP was given each day at 5 P.M., shortly before the beginning of the dark cycle. The rats usually began feeding shortly thereafter, so absorption occurred in the presence of the normal dietary nutrients.

The first study involved 36 male Fischer 344 rats, 12 in each of three groups (A, B and C). The rats received NIH 31 chow and water *ad libitum*. Feces and urine were collected daily at 4:30 P.M. and stored frozen. Group A rats received 1.8 mg of [7-^{14}C]-DEHP (carbonyl label) per kilogram body weight per day. Group B received 18 mg/kg per day, and group C received 180 mg/kg per day. All received the same amount of radioactivity, with different amounts of nonradioactive DEHP diluent. Rats received a total of 10 daily doses of DEHP.

Three rats from each group were sacrificed 1, 3, 10 or 12 days after receiving their first dose of DEHP. Various tissues and feces were radioassayed using a tissue oxidizer. Urine was radioassayed directly in liquid scintillation fluid. The profiles of radioactive metabolites in urine were determined by HPLC as described previously (*1*). Lipid-soluble metabolites were extracted from liver (*2*) and chromatographed in Florisil with 15% diethyl ether in hexane. Unhydrolyzed DEHP was measured by radio-TLC of the eluate from Florisil on silica gel GF in hexane:diethyl ether:acetic acid, 120:30:1.5 (v/v).

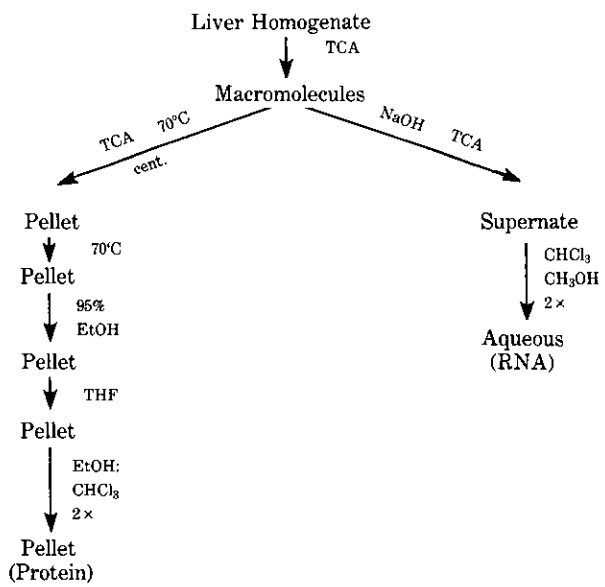


FIGURE 1. Outline of purification procedures for protein and RNA from rat liver. TCA = trichloroacetic acid.

Absorption Thresholds

In a separate experiment rats and mice were, in triplicate, given single oral doses of DEHP in cottonseed oil ranging from 1.8 mg/kg body weight to 1 g/kg. The animals were sacrificed 6 hr later, as this was the time after dosing at which the maximum concentration of ^{14}C was found in the livers. The livers were assayed for intact DEHP as described above. The results of this experiment were compared to the level of DEHP found in the livers of rats fed a diet containing 1% DEHP until the level reached a steady state ($\leq$ 6 days).

Species Dependence

The distributions of DEHP-derived metabolites in urine of Fischer 344 rats, CD mice, Syrian golden hamsters, and Hartley albino guinea pigs were determined following single oral doses of DEHP in cottonseed oil by techniques applied previously to urine from CD rats (*1*, *3*, *4*,), African Green monkeys (*5*) and humans (*6*). In general, the distributions were independent of dose up to the amount of DEHP that caused intact ^{14}C-DEHP to appear in the feces.

Labeling of Macromolecules

In the last type of experiment to be reported here, male Fischer 344 rats were fed diets containing either 0.01% or 1% DEHP, unlabeled, *ad libtum* for 11 days. The rats were given a tracer dose of labeled DEHP or one of its metabolites on day 1,

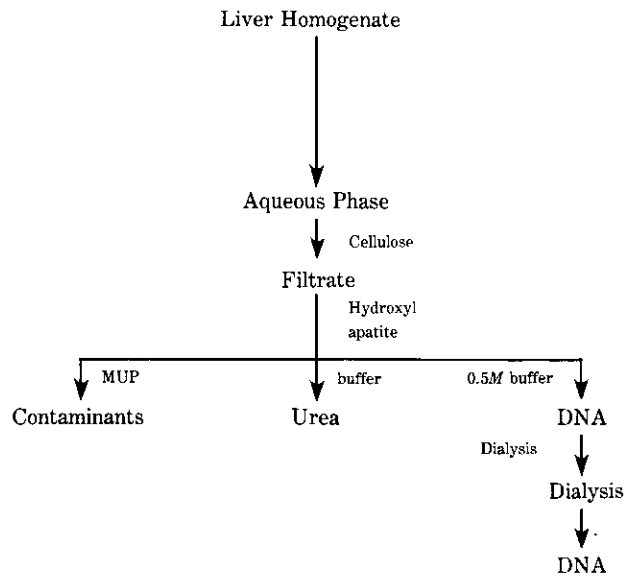


FIGURE 2. Outline of purification procedures for DNA from rat liver. MUP = buffer containing urea. For details see Markov and Ivanov (*7*).

day 6 or day 10. Rats given labeled DEHP on either day 1 or day 10 were sacrificed on day 11; those given labeled DEHP on day 6 were sacrificed on day 7 (24 hr later). Livers were homogenized, after which protein, RNA and DNA (*7*) were isolated in highly purified form. The purification procedures are briefly diagrammed in Figures 1 and 2. The fractions were tested for cross contamination by spectrophotometric assays (*7-9*), after which they were radioassayed, as were aliquots of the original homogenate, by digestion in NCS and dispersion in Aquasol.

Results and Discussion

Dose Effects

The cumulative excretion of ^{14}C expressed as a percentage of the cumulative dose is diagrammed in Figure 3. After an "adaptation period" of about 4 days, excretion became quite independent of the dose. On an absolute basis, rats in group C were eliminating ^{14}C at a rate 100 times that of the group A rats. Up to a dose rate of 180 mg/kg per day, then, there was no indication of even beginning to saturate the overall elimination mechanism.

Looking more specifically at the liver, we saw (Table 1) that the percentage of the dose of ^{14}C retained in the livers tended to decrease with time and also with increasing dose. The mean rates of dropoff in radioactivity after dosing was stopped were not statistically significantly different in the three groups. There was no evidence, then, for accumulation in this organ.

Essentially the same observations applied to the testes as were noted for liver (Table 2), except that

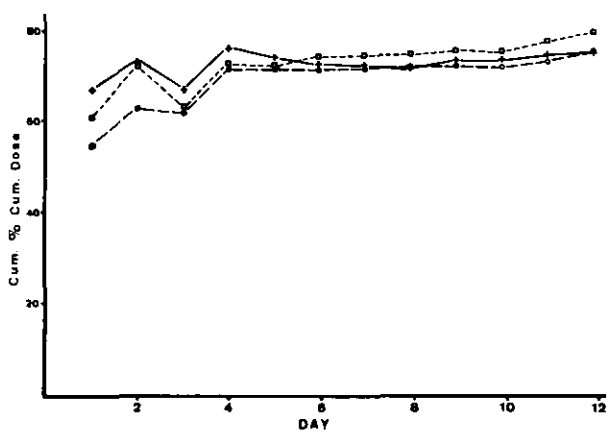


FIGURE 3. Cumulative excretion of ^{14}C in urine and feces as a percentage of the cumulative dose for male rats receiving (○) 1.8, (□) 18, or (+) 180 mg of [7-^{14}C]-DEHP per kg body weight per day by gavage in cottonseed oil, for 10 successive days.

Table 1. Percentage of ^{14}C fed left in liver (average of three).

	^{14}C left in liver, %		
Day	1.8 mg/kg/day	18 mg/kg/day	180 mg/kg/day
1	0.64 ± 0.31	0.45 ± 0.16	0.25 ± 0.08
3	0.44 ± 0.27	0.35 ± 0.03	0.15 ± 0.01
10	0.13 ± 0.05	0.15 ± 0.01	0.12 ± 0.02
12	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
10–12 % hr	0.93 ± 0.27	0.53 ± 0.07	0.62 ± 0.14

Table 2. Percentage of ^{14}C fed left in testes (average of three).[a]

	^{14}C left in testes, % (± SD)		
Day	1.8 mg/kg/day	18 mg/kg/day	180 mg/kg/day
1	0.0142(0.0028)	0.0148(0.0065)	0.0094(0.0049)
3	0.0060(0.0017)	0.0065(0.0005)	0.0064(0.0020)
10	0.0020(0.0004)	0.0019(0.0010)	0.0015(0.0006)
12	0.0037(0.0008)	0.0025(0.0010)	0.0032(0.0005)

[a]ANOVA; $F_c = 1.0159$, $p = 0.4169$; $F_R = 31.30$, $p = 0.0005$.

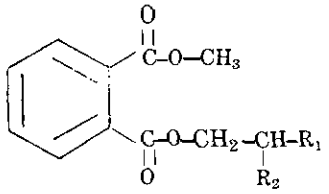
testes never reached as high concentrations of DEHP metabolites as did liver.

The structures of the urinary metabolites of DEHP that still retain aromaticity are shown in Table 3. These compounds are grouped according to functionality, or roughly according to the extent of oxidative metabolism needed to produce them, in Table 4. This table indicates that the only statistically significant effect of the dosage of DEHP on metabolite distribution in rat urine was a slight increase in free phthalic acid with increasing DEHP dosage. This probably reflects a high K_m for mono-(2-ethylhexyl) phthalate (MEHP) of the lipase that produces phthalic acid. A previous study of lipases and esterases in various rat tissues (*10*) indicated that the only detectable MEHP-hydrolase was located in liver microsomes. It is also possible that this enzyme was induced at high levels of DEHP, but there is no direct evidence at the present time.

Absorption Threshold

As the size of a single oral dose of DEHP was increased, a threshold was reached above which there was a steady climb in the amount of unhydrolyzed DEHP reaching the liver of rats. This is diagrammed for Fischer rats in Figure 4. Fischer rats on a diet of NIH 31 chow fortified with DEHP by weight reached a steady-state level of intact DEHP in their livers corresponding to the peak level predicted in Figure 4. That is, by keeping track of the amount of feed eaten per day per kg body weight, and treating the amount of DEHP

Table 3. Structures of DEHP metabolites after treatment with diazomethane.



Metabolite	R_1	R_2
I	$-CH_2-CO_2-CH_3$	$-CH_2-CH_3$
II	$-(CH_2)_3-CH_3$	$-CO_2-CH_3$
III	$-(CH_2)_2-CO_2-CH_3$	$-CH_2-CH_3$
IV	$-(CH_2)_3-CH_3$	$-CH_2-CO_2-CH_3$
V	$-(CH_2)_2-CO_2-CH_3$	$-CH_2-CH_3$
VI	$-(CH_2)_2-CO-CH_3$	$-CH_2-CH_3$
VII	$-(CH_2)_3-CH_3$	$-CH_2-CH_2OH$
VIII[a]	$-CH_2-CHOH-CH_2-CH_3$	$-CH_2-CH_3$
IX	$-(CH_2)_2-CHOH-CH_3$	$-CH_2-CH_3$
X	$-(CH_2)_3-CH_2OH$	$-CH_2-CH_3$
XI[b]	$-(CH_2)_3-CH_3$	$-CH_2-CH_3$

[a]Absent in the rat.
[b]Mono(2-ethylhexyl) phthalate.

thereby ingested as though it were a single dose, the concentration of DEHP in liver predicted in Figure 4 was in excellent agreement with what was found on analysis (123 μg/g vs 121 μg/g found).

Making the approximation that rats consume feed to the extent of 10% of their body weight daily, then intact DEHP will reach their livers whenever its concentration exceeds 0.43% of the diet. Levels above and below the threshold are therefore qualitatively different relative to what chemical compounds reach the liver. However, it must be kept in mind that when the diet contains more than about 0.5% DEHP, rats tend to reduce their food consumption.

In contrast to the results seen in rats, we were unable to detect an absorption threshold in either CD-1 or C3B6F1 mice for doses up to 1 g of DEHP/kg body weight. This may reflect the higher level of DEHP-hydrolase in the intestines of mice than in the intestines of rats (*10*).

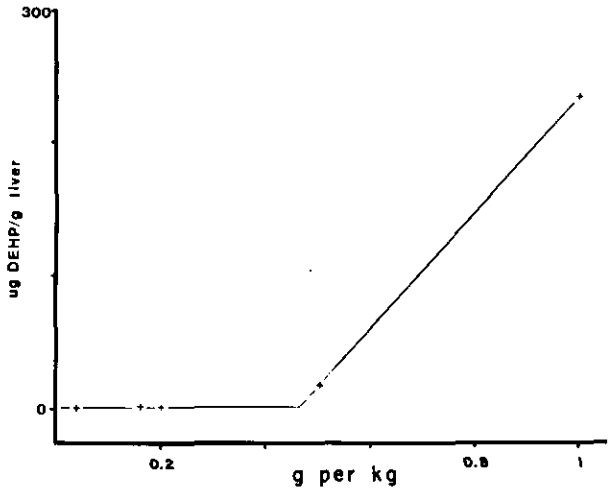


FIGURE 4. Level of intact DEHP in livers of male rats 6 hr after a single oral dose of [7-^{14}C]-DEHP in cottonseed oil.

Association with Macromolecules

Table 5 shows the amounts of radioactivity associated with liver homogenates, purified protein, RNA and DNA when Fischer 344 rats were given a tracer dose of ^{14}C-DEHP or its primary hydrolysis products. The rats had been eating NIH 31 chow fortified with unlabeled DEHP (1% by weight) for 6 days to build up the pools of metabolites and intermediates to a steady-state level; the ^{14}C tracer was given, and the rats sacrificed 24 hr later. When 100 μCi of carbonyl-^{14}C (7-^{14}C) DEHP was given, no detectable radioactivity was found associated with purified macromolecules. However, when the ^{14}C was in the ethylhexyl moiety of DEHP (1′-^{14}C), all three classes of macromolecules became labeled. If the [1′-^{14}C]-DEHP was saponified to phthalic acid plus two equivalents of [1′-^{14}C]-2-ethylhexanol before administration, protein was still labeled, but no detectable ^{14}C became associated with DNA. If [1′-^{14}C]-MEHP was given, DNA was again labeled,

Table 4. Metabolite distribution as a function of dose of DEHP.

		^{14}C in urine, %		
Dose, mg/kg	Day	-OH side chain	Diacids	MEHP+PA
1.8	1	33.1	66.5	0.4
	3	35.0	64.5	0.5
	10	40.0	58.9	1.1
18	1	38.7	61.0	0.3
	3	38.3	61.3	0.4
	10	36.1	62.8	1.1
180	1	38.1	61.0	0.9
	3	35.5	61.4	3.1
	10	36.1	57.1	6.8

but not as efficiently as from [1′-^{14}C]-DEHP. Protein was much less efficiently labeled from [1′-^{14}C]-MEHP than from [1′-^{14}C]-DEHP.

If the label associated with DNA was covalently bound, which cannot be proven without isolation and characterization of the adduct(s), it would appear that C-1 (at least) of 2-ethylhexanol is incorporated into DNA only when esterified ethylhexanol is given. In any event, it seems clear that the phthalate moiety of DEHP is not bound to either DNA, RNA or protein in rat liver under these conditions. Chemical analysis of the DNA isolated according to the procedure described detected less than 0.1% RNA and less than 0.01% protein.

Table 6 shows the decrease with time in the amount of radioactivity associated with macromolecules labeled from [1′-^{14}C]-DEHP. The decrease rate for protein was consistent with the average turnover rate of protein in rat liver (*11*) and much slower than would be expected for simple exchange with the unlabeled DEHP from the diet. Labeling of DNA was slightly higher for rats on the 1% DEHP diet than for those on the 0.01% DEHP diet, but there is insufficient information on pool sizes and turnover rates to speculate on this phenomenon. The decrease in labeling of DNA with time was faster for rats on the 0.01% DEHP diet than would be expected if the decrease was simply due to normal DNA turnover. This suggests that some sort of repair mechanism could be involved that was less effective at reducing DNA radioactivity in rats on the 1% DEHP diet. However, much more information would be needed to establish that repair was occurring. At best, this experiment suggests that further studies of the interaction of DEHP with DNA might be worthwhile.

Species Differences in Metabolism

The data in Table 7 are collected from several sources. Urine was collected at intervals after single oral (species other than primates) or IV [African Green monkeys (*5*) and human leukemia patients (*6*)] doses of [7-^{14}C]-DEHP. Urine containing 90% of the total radioactivity excreted was pooled for analysis. Table 7 represents the distributions of radioactive metabolites in pools of urine from three animals except monkeys (two) and humans (two, nonradioactive DEHP). Urine was hydrolyzed with β-glucuronidase for the data in Table 7. A compari-

Table 5. "Bound" radioactivity in macromolecules.

	Liver activity, DPM/g[a]			
	7-^{14}C-DEHP	1′-^{14}C-DEHP	1-^{14}C 2-Et Hexol	1′-^{14}C-MEHP
Homogenate	25918	200247	221600	16460
Protein	0	67805	45180	1331
RNA	0	63000	4350	1878
DNA	0	400	0	114

[a]Measured 24 hr after single oral dose of (a) i00 μCi of carbonyl-labeled (7-^{14}C) DEHP, ethylhexyl-labeled (1′-^{14}C) DEHP, or [1-^{14}C]-2-ethylhexanol + unlabeled phthalic acid, or 50 μCi of ethylhexyl-labeled (1′-^{14}C) mono-ethylhexyl phthalate (MEHP). In all cases the rats had feed containing 1% unlabeled DEHP, and water available *ad libitum*. One gram-equivalent of liver corresponds to approximately 190 mg of protein, 13.5 mg of RNA and 4 mg of DNA.

Table 6. Association of radioactivity with macromolecules.

	Time after [1′-^{14}C] – DEHP given			
	0.01% DEHP diet		1.0% DEHP diet	
	1 day	10 days	1 day	10 days
Liver activity, DPM/g				
Total	161835	9102	200247	14980
In protein	41171	4500	67805	7832
In RNA	40040	4402	63000	7471
In DNA	310	134	400	293
Protein activity, DPM/g				
Protein	230	25	340	42
As RNA	3337	358	5122	607
As DNA	77	33	100	73
Decrease/10 days, %				
Protein	89.1		88.4	
RNA	89.3		88.1	
DNA	56.8		26.7	

Table 7. Distribution of phthalate metabolites.

Metabolite	Percentage of total metabolites					
	Rat	Mouse	Guinea pig	Green monkey	Man	Hamster
Residual DEHP	—	0.5	—	2.2	—	0.3
MEHP	Trace	18.6	71.2	28.9	18.3	4.5
I	17.2	16.8	2.4	0.1	—	13.0
II	2.0	1.0	0.4	—	1.8	0.1
III	1.2	0.4	0.5	—	—	0.3
IV	3.3	0.8	0.4	—	1.2	0.4
V	51.3	1.1	6.9	4.2	5.3	14.0
VI	2.6	14.9	1.1	5.9	12.1	10.2
VII	2.6	7.2	0.8	7.0	11.9	4.9
VIII	—	—	—	5.7	8.1	—
IX	13.3	12.3	3.4	38.2	36.2	32.7
X	0.6	2.2	1.3	0.1	0.1	1.9
A, B, C	4.1	8.1	6.2	7.6	4.9	6.1
Phthalic acid	1.8	12.4	5.4	0.1	0.1	9.5

son of extracts from unhydrolyzed and glucuronidase-hydrolyzed portions of urine gave the results shown in Table 8. No conjugates other than glucuronides have been detected in any of the species tested; glycine, taurine and sulfate conjugates have been sought and found absent, in contrast to results reported for substituted benzoic and phenylacetic acid metabolites (*12*).

Most of our investigations of urinary metabolites have involved rats. The metabolite distributions in rats, examined over the past 8 years, have been surprisingly reproducible and independent of factors such as dose of DEHP and age of animal. Rats do not excrete conjugates of DEHP metabolites, an observation that has been confirmed in at least three strains (*5*, *13*). In contrast, each of the other five species examined excretes glucuronide conjugates (Table 6). In most cases, conjugates are the major urinary metabolites.

Whereas primates excrete predominately glucuronides of MEHP, whose formation does not require oxidative metabolism, and metabolites with hydroxyl side chains which require only one round of oxidative metabolism, rats excrete predominately metabolites having carboxyl groups on the side chain (metabolites I-V). These diacids require from three to six oxidative steps for their formation. Carrying oxidative metabolism all the way to the highly water-soluble diacids may be necessary to compensate, in rats, for not making glucuronides.

If formation of hydroxyl side chains involves, by analogy with fatty acid ω-oxidation, a mixed function oxidase reaction as postulated earlier (*3*), one would expect a net conversion of NAD(P)H to NAD(P). The additional steps, from alcohol to aldehyde (or ketone) and from aldehyde to acid, as well as the apparent α- and β-oxidations needed to produce metabolites I-III, would all be associated with net conversion of NAD(P) to NAD(P)H. Thus the overall demand on the oxidation potential of the liver when high doses of DEHP are given would be in opposite directions for rat and primate. To the extent that metabolism of DEHP is involved in its biological activity, then, one must question seriously whether rats can be accepted as a model for man.

Table 8. Forms of excretion products in urine.

Species	Excretion products, %	
	Free	Conjugated
Rat[a]	100	0
Mouse, CD	36	64
Guinea pig	35	65
Green monkey	20	80
Man	20	80
Hamster	85	15

[a]Three strains

Summary

The recent long-term carcinogenesis bioassay in rats and mice as reported at this conference by the NTP is the first such bioassay to report carcinogenesis by DEHP. It is also the first such bioassay to have involved a level of DEHP in the diet high enough to exceed the absorption threshold for unhydrolyzed DEHP. The livers of the rats in the NTP study would have been exposed to moderate levels of intact diester, whereas at levels half those tested or below this would not have occurred. This qualitative difference makes any attempts to extrapolate the hepatocarcinogenesis observed in rats to lower dosage levels questionable. We do not have enough pharmacokinetic data from mice to evaluate the dose relationships as yet.

While we were unable to obtain any evidence for

covalent binding of the phthalate moiety of DEHP to macromolecules in rat liver, and there was also no apparent binding of radioactivity from [1′-^{14}C]-2-ethylhexanol to DNA *in vivo*, the labeled carbon atom from either di-([1′-^{14}C]-2-ethylhexyl) phthalate or the corresponding monoester did appear to bind to DNA in rat liver *in vivo*. The diester was a more efficient labeling precursor than the monoester. Disappearance of ^{14}C from the labeled DNA had a time course consistent with the operation of a repair mechanism. If so, repair was less effective when there was a high level of DEHP in the diet. That the apparent binding to DNA was truly covalent has not been proven; however, it was not exchangeable.

We found no saturation of DEHP metabolic pathways in rats over the 100-fold range of dosages between 1.8 mg DEHP/kg body weight per day and 180 mg/kg per day. There was no apparent reason to suspect metabolic saturation in the biological activity of high DEHP dosages. Levels of metabolites in liver and testes fluctuated for about four days on a regimen of daily dosing, then slowly but smoothly declined. This hinted at the operation of an adaptation mechanism, and argued against tissue accumulation.

Rats were unique among six species (including man) tested, in that they did not excrete glucuronide conjugates of DEHP metabolites in urine. Rodent species in general carried oxidative metabolism of DEHP much farther than did primates; the former should experience a net decrease and the latter a net increase in the ratio of NAD(P) to NAD(P)H in liver during exposure to high levels of DEHP. Whereas the major metabolites of DEHP in rat urine are diacids, the major metabolites in urine of primates are glucuronides of hydroxyacids. These species differences in the metabolism of DEHP cast doubts upon the validity of using rats as a model for the effects of DEHP in man.

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