

# Effects of Phthalate Esters on Lipid Metabolism in Various Tissues, Cells and Organelles in Mammals

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The effect of phthalate ester plasticizers on a variety of enzyme systems was studied in rats, rabbits and pigs. The feeding of di(2-ethylhexyl) phthalate (DEHP) to animals at levels from 0.1% to 1.0% in the diet resulted in diverse biochemical effects, such as inhibition of cholesterologenesis in liver, testes, and adrenal gland; inhibition of cholesterologenesis in brain and liver of fetal rats from DEHP-fed dams; decreased plasma cholesterol levels; decreased synthesis of hepatic phospholipid and triglyceride; increased fatty acid oxidation in isolated liver mitochondria; and a transient decrease in fatty acid oxidation in isolated heart mitochondria. The addition of DEHP to preparations of rat liver *in vitro* resulted in inhibition of cholesterologenesis, and its addition to isolated mitochondria from rat heart produced an inhibition of adenine nucleotide translocase. DEHP-feeding to rats and rabbits, however, did not affect platelet function as judged by collagen- and ADP-induced aggregation. The studies presented here indicate that the exposure of animals to phthalate esters can result in a significant perturbation of normal metabolism in liver, heart, testes, adrenal gland and brain and can affect blood lipid levels.

## Introduction

A number of reviews (1-5) have thoroughly outlined the synthesis, properties, and industrial application of the phthalate esters in use today. The utility of the phthalates is so diverse that annual production in the USA alone is estimated at  $1-2 \times 10^9$  lb, with worldwide production reaching about  $4 \times 10^9$  lb (5). The widespread use of phthalates and phthalate-containing products in all segments of society has, within the span of a few decades, resulted in global contamination by this class of compounds. The presence of phthalate esters in virtually all ecosystems (5-17) and in the tissues of man (18-22), animals (6, 7, 9, 11, 16, 23, 24), and in the food supply of man (24-26) is a shocking revelation.

Phthalates were evaluated in the 1940s and 1950s by classical toxicological criteria and judged to have a low order of acute and chronic toxicity (27-30). It was not until the 1960s that the safety of the phthalates came into serious question. During the

1960s, scattered reports appeared in medical journals citing concern over the possible toxic effects of poly(vinyl chloride) (PVC) plastic medical devices which contained phthalate ester plasticizers (31-36). Concern over phthalate toxicity heightened with reports by Marcel and Noel (37, 38) and Jaeger and Rubin (39, 40) in 1970 that stored human blood was being contaminated with phthalate ester, namely, di(2-ethylhexyl) phthalate (DEHP), while stored in PVC plastic blood bags. It was subsequently shown that tissues from patients recently transfused and/or exposed to PVC plastic catheters accumulated detectable levels of DEHP in their tissues (18-21). During the same period (1970-1975), a wide variety of biological studies appeared in the literature which further challenged the safety of the phthalates (24, 41-50); these provided evidence that the phthalates could accumulate in mitochondria (24) and that phthalates were teratogenic (41-43), mutagenic (44), toxic to various types of cultured cells (45-47), capable of placental transfer (48), capable of altering hepatic ultrastructure (49) and capable of promoting hepatic lipid accumulation under certain conditions (50).

What was surprising to me, however, was that as

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late as 1975, the knowledge that had accrued concerning phthalate toxicity was devoid of studies on the effects of phthalate esters on biochemical pathways and events, which by necessity would underlie the qualitative toxic responses being observed. It was for this reason, in 1975, that efforts in my laboratory were directed towards investigating the possible effects of phthalate esters on lipid metabolism in various species. The observations presented in this paper summarize results of studies which were initiated in 1975 and which have continued up to the present time. The studies were conducted in rats, rabbits and pigs. Depending upon the nature of the study, phthalate esters were either fed as an admixture to the diets or added, *in vitro*, to tissue preparations. Details of the techniques and methods used throughout the studies have been described elsewhere and will be referenced in the table footnotes and figure legends. Statistical analyses were performed using Student's independent *t*-test.

## Results and Discussion

### Effects of Phthalate Esters on Sterol Metabolism

The reports that phthalate esters possess teratogenic and mutagenic potential (41-44) suggested the possibility of their interference with sterologenesi which is a key process in mammalian development. This, in fact, proved to be the case, and the evidence was first published in 1976 (51, 52).

**Liver (Male Rats).** An inhibition of cholesterol synthesis from  $^{14}\text{C}$ -acetate is apparent within 48 hr after feeding 0.5% DEHP to male rats (Table 1). The inhibition is progressive and rises from 14% at 2 days to 70% by 11 days. Inhibition of labeled mevalonate incorporation into cholesterol by 0.5% DEHP feeding also occurs but is slower to develop; inhibition is not apparent until after 6 days of DEHP exposure. By 11 days, cholesterologenesis from  $^3\text{H}$ -mevalonate is inhibited about 40%. The lag period in the development of inhibition of hepatic cholesterologenesis from mevalonate relative to acetate has been observed under other conditions which result in inhibition of cholesterologenesis (54). The incorporation of mevalonate into cholesterol can eventually be suppressed to the same extent as acetate incorporation, as revealed by livers from rats fed DEHP for 18 days (Table 1). The data in Table 1 suggest that the site of action of phthalate is at the level of HMG CoA reductase (3-hydroxy-3-methylglutaryl CoA reductase, EC 1.1.1.34) (55), the first rate-limiting step in hepatic sterol synthe-

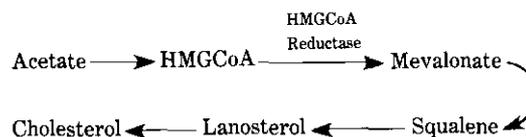
**Table 1. Effect of DEHP feeding on cholesterologenesis from  $^{14}\text{C}$ -acetate and  $^3\text{H}$ -mevalonate in liver minces from male rats.<sup>a</sup>**

Level of DEHP in diet, %	Duration of feeding, days	Incorporation into cholesterol, % of control	
		$^{14}\text{C}$ -Acetate	$^3\text{H}$ -Mevalonate
0.5	0	100	100
	2	86	104
	4	63	100
	6	39	91
	11	30	61
1.0	18	29	22

<sup>a</sup>Male Sprague-Dawley rats (300-325 g) were fed Purina Chow (control) or Purina Chow containing di(2-ethylhexyl) phthalate (DEHP) (52, 53), at levels of 0.5% or 1.0% (w/w). Liver minces were prepared and incubated for 3 hr in 4.0 ml (0.5% study) or 3.7 ml (1.0% study) of Krebs-Ringer-bicarbonate buffer, pH 7.4, which contained 4  $\mu\text{Ci}$  sodium acetate-1- $^{14}\text{C}$  (SA 58 mCi/mmole) and 2.4  $\mu\text{Ci}$  DL-mevalonic-5- $^3\text{H}$  acid (DBED salt, SA 6.7 Ci/mmole) (52). After incubation, the samples were hydrolyzed (saponified) with alcoholic KOH, and then the neutral sterols were extracted with *n*-hexane and subsequently fractionated by thin-layer chromatography (52). Values are means of two or three DEHP-fed animals expressed relative to control values obtained in parallel experiments.

sis (56) (Fig. 1). Inhibition at this site would first affect the flow-through of acetate to cholesterol as demonstrated in Table 1 (and diagrammed in Fig. 1) and permit the inhibition to be circumvented initially by use of exogenous mevalonate as a substrate. The inhibition of sterologenesi from labeled mevalonate which develops later (after 6 days, Table 1) may be explained if one or more of the enzymes between mevalonate and cholesterol are substrate-induced and thereby decline in activity as mevalonate production diminishes.

In all studies, the decreased synthesis of cholesterol was paralleled by decreases in synthesis of lanosterol and squalene (52), as would be anticipated from Figure 1. Additionally, the cholesterol content of livers from rats fed 0.5% DEHP for 6 days was not different from that of control rats (52),



**FIGURE 1.** Cholesterol, the principal  $\text{C}_{27}$  sterol of liver, is derived from units of acetyl CoA via a sequence of 20 or more intermediates (57) which include 3-hydroxy-3-methylglutaryl CoA (HMG CoA), mevalonic acid, squalene (a  $\text{C}_{30}$  hydrocarbon) and lanosterol (a  $\text{C}_{30}$  sterol). The conversion of HMG CoA to mevalonate by HMG CoA reductase (EC 1.1.1.34) is the primary rate-limiting step in the formation of squalene and sterols (56). Secondary control sites beyond mevalonate may also function (54, 56-58).

**Table 2.**  $^{14}\text{C}$ -Acetate incorporation into sterols of normal male rat liver minces: inhibition by exogenously added DEHP.<sup>a</sup>

DEHP concn, <i>mM</i>	<i>n</i>	$^{14}\text{C}$ -Acetate incorporation into digitonin-precipitable sterols, % of control
0.0	9	100 ± 0
0.25	6	92 ± 15
0.50	6	75 ± 12
1.0	4	87 ± 7
2.5	8	54 ± 6

<sup>a</sup>Livers from normal male Sprague-Dawley rats (275-300 g, maintained on Purina Chow) were used to prepare minces (500 mg) (59) which were incubated for 3 hr in 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4. Assays were initiated by the addition of 2  $\mu\text{Ci}$  sodium acetate- $^{14}\text{C}$  (SA 58.3 Ci/mole) and DEHP. DEHP was added as a Tween-20-stabilized dispersion (60). After incubation, the samples were hydrolyzed with alcoholic KOH (52). The nonsaponifiable lipids were recovered by extraction with *n*-hexane (52) and the  $3\beta\text{-OH}$  sterols (principally cholesterol) were precipitated with digitonin and assayed for radioactivity (52, 59). Values are means  $\pm$  SEM of the number of animals designated by *n*.

thus indicating that DEHP-induced inhibition of hepatic cholesterologenesis is unlikely to be attributed to end product inhibition. In fact, feeding of phthalates for longer periods (21 days) tends to reduce hepatic cholesterol (55). End product inhibition is also inconsistent with the observation that DEHP inhibits  $^{14}\text{C}$ -acetate incorporation into sterols when added directly to normal male rat liver minces (Table 2).

**Liver (Female Rats).** Studies in female rats fed 1.0% DEHP for 13 days (61) indicated that inhibition of hepatic sterologogenesis is not sex-specific (Table 3).

**Liver (Male Rabbits).** Inhibition of sterologen-

**Table 3.** Effect of DEHP feeding on the incorporation of  $^{14}\text{C}$ -mevalonate into sterols and squalene by liver minces from female rats.<sup>a</sup>

	Incorporation, dpm/g wet wt	
	$\text{C}_{27}$ + $\text{C}_{30}$ sterols	Squalene
Control	40520 $\pm$ 2440	56870 $\pm$ 5175
1% DEHP	29635 $\pm$ 3645	24355 $\pm$ 2490
	$p < 0.05$	$p < 0.001$

<sup>a</sup>Female Sprague-Dawley rats (245-255 g) were fed either Purina Chow or Purina Chow supplemented with 1.0% DEHP (w/w) for 13 days. Liver minces (500 mg) were incubated for 3 hr at 37°C in 3.4 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 1  $\mu\text{Ci}$  DL-mevalonic- $^{14}\text{C}$  acid (DBED salt, SA 40.8 Ci/mole). After incubation, the samples were hydrolyzed with alcoholic KOH, the nonsaponifiable lipids were extracted with *n*-hexane (52) and fractionated by thin-layer chromatography to yield  $\text{C}_{27}$  sterols (principally cholesterol),  $\text{C}_{30}$  sterols (principally lanosterol) and squalene (52). Values are means  $\pm$  SEM of six animals per group.

**Table 4.** Effect of DEHP feeding on the incorporation of  $^3\text{H}$ -mevalonate into sterols and squalene by rabbit liver minces.<sup>a</sup>

	Incorporation, dpm/g wet wt		
	$\text{C}_{27}$ sterol	$\text{C}_{30}$ sterols	Squalene
Control	28890 $\pm$ 6880	9115 $\pm$ 2610	33080 $\pm$ 11360
1% DEHP	12140 $\pm$ 2815	3945 $\pm$ 655	13675 $\pm$ 5490
	$p < 0.05$	$p < 0.01$	

<sup>a</sup>Male New Zealand rabbits (2.5-3 kg) were fed Purina Chow Pellets or 1% DEHP (w/w) as an admixture in Purina Chow Pellets for 28 days. Liver minces (500 mg) were prepared and incubated for 3 hr at 37°C in 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, which contained 0.25  $\mu\text{Ci}$  DL-mevalonic- $^3\text{H}$ (N) acid (DBED salt, 5 Ci/mole). After incubation, the samples were treated with alcoholic KOH to hydrolyze the tissue and to saponify the lipids. The neutral sterols and squalene were extracted with *n*-hexane and subsequently fractionated by thin-layer chromatography. Values are means  $\pm$  SEM of six animals.

esis in liver from rabbits fed DEHP (59) also dismissed the possibility that the effect of DEHP is species-specific for the rat (Table 4).

**Adrenal and Testes.** Inhibition of sterologogenesis in DEHP-fed animals is not restricted to the liver. The results of Table 5 show that adrenal glands and testes are similarly affected (59, 61), with inhibition reaching 20-25% of normal synthesis. Impaired sterologogenesis in adrenal and testicular tissue may, in part, be responsible for the developmental abnormalities (62) reported to occur in fetuses from phthalate-treated dams (41, 42, 46) and for testicular atrophy in young and adult animals (63-67).

**Developing Fetuses and Suckling Offspring.** Placental transfer of DEHP has been reported in rats (48). The exposure of the developing fetus to DEHP by this route may have important implications in the development of the fetus. The results of Table 6 indicate that fetuses taken by cesarean section from pregnant rats fed DEHP beginning 5 to 10 days after conception have an impairment of sterologogenesis in brain and liver (68). Furthermore, if dams exposed to DEHP during gestation, and during the postnatal period as well, are permitted to nurse their naturally born offspring, hepatic sterologogenesis in the pups is reduced below control values (Table 7). In 8-day suckling rats, hepatic sterologogenesis was reduced 20% below control values and was accompanied by increased liver size and decreased body weight (Table 7). The decreased body weight seen in the 8-day-old pups was not apparent in litters permitted to reach weaning age (21 days) (Table 8). DEHP feeding to the dams resulted in a significant lowering of plasma cholesterol ( $p < 0.02$ ) but did not affect plasma cholesterol levels in the pups (Table 8).

**Table 5. Effect of DEHP feeding on the incorporation of  $^{14}\text{C}$ -mevalonate into  $\text{C}_{27}$  +  $\text{C}_{30}$  sterols and squalene in rat adrenal gland and testis.<sup>a</sup>**

Organ	Diet	Duration of study, days	Incorporation, dpm/100 mg wet wt	
			Sterols	Squalene
Adrenal (♀)	Control	13	352 ± 23 <i>p</i> < 0.01	124 ± 10 NS
	1% DEHP	13	266 ± 8	105 ± 7
Adrenal (♂)	Control	73	322 ± 20 <i>p</i> < 0.02	84 ± 4 NS
	1% DEHP	73	264 ± 12	66 ± 10
Testis	Control	28	233 ± 15 <i>p</i> < 0.02	212 ± 9 <i>p</i> < 0.01
	0.5% DEHP	28	175 ± 12	149 ± 13

<sup>a</sup>*Sprague-Dawley rats of both sexes were fed Purina Laboratory Chow or the chow diet supplemented with DEHP. Adrenal glands were removed from females (body wt 251 ± 3 g, n = 12) and males (body wt 320 ± 20 g, n = 10), dissected free of adhering tissue and capsular material, and then incubated for 3 hr at 37°C in 1.52 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 1.0 μCi DL-mevalonic-2- $^{14}\text{C}$  acid (DBED salt, 40.8 Ci/mole). Testes (right side) from rats weighing 225-250 g were decapsulated and incubated as the whole gland for 3 hr at 37°C in 5.0 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 2.5 μCi DL-mevalonic-2- $^{14}\text{C}$  acid (DBED salt, 40.1 Ci/mole). After incubation, the tissues were treated with alcoholic KOH as described in the footnote to Table 1. The neutral sterols and squalene were extracted with *n*-hexane and subsequently fractionated by thin-layer chromatography. Values are means ± SEM (male adrenals, n = 5 per group; all others, n = 6 per group).*

**Table 6. DEHP feeding to rats during gestation: effect on the incorporation of radiolabeled acetate and mevalonate into cholesterol in fetal brain and liver.<sup>a</sup>**

Expt. no.	Treatment	Labeled compound	Incorporation of labeled compound into cholesterol		
			$^{14}\text{C}$ -Acetate dpm/brain	$^{14}\text{C}$ -Mevalonate, dpm/brain	$^3\text{H}$ -Mevalonate, dpm/mg dry wt of liver
1	Pregnant rats were fed 0.5% DEHP for 13 days beginning on the 5th day of gestation. Fetuses were taken by cesarean section on the 18th day of gestation. Brains were obtained from seven fetuses from seven different dams. Livers were obtained from fetuses from up to eight dams; not more than two fetuses per dam were used.	Control	—	13570 ± 700 <i>n</i> = (7)	6680 ± 300 <i>n</i> = (14)
		DEHP	—	11510 ± 520 <i>n</i> = (7) <i>p</i> < 0.05	5675 ± 220 <i>n</i> = (13) <i>p</i> < 0.02
2	Pregnant rats were fed 1.0% DEHP for 8 days beginning on the 10th day of gestation. Fetuses were taken by cesarean section on the 18th day of gestation. Brains and livers were obtained from fetuses from two to three different dams.	Control	25300 ± 1795 <i>n</i> = (8)	—	4990 ± 490 <i>n</i> = (9)
		DEHP	18375 ± 2240 <i>n</i> = (6) <i>p</i> < 0.05	—	3010 ± 170 <i>n</i> = (6) <i>p</i> < 0.01

<sup>a</sup>Livers and brains were incubated as the entire tissue for 3 hr at 37°C in 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4. Radioactive precursors (acetate-1- $^{14}\text{C}$ , sodium salt, SA 59 Ci/mole, DL-mevalonic acid-2- $^{14}\text{C}$ , DBED salt, SA 46 Ci/mole, and DL-mevalonic acid-5- $^3\text{H}$ , DBED salt, SA 5 Ci/mole) were present at a level of 2 μCi.

**Plasma Cholesterol.** Certain phthalate esters evoke a hypocholesterolemic response in rats of either sex (Tables 8 and 9). At levels of 0.5% and 1.0% in the diet, DEHP reduces plasma cholesterol about 20% in the male rat (Table 9) (55, 59, 69); this reduction appears to represent a maximum response inasmuch as 0.5% and 1.0% DEHP appear to have equivalent effects (55, 59, 69). The maximum cholesterol-lowering effect occurs within the first week of DEHP feeding (55). Since a hypocholesterolemic effect of phthalates has also been observed in mice (70) but not in rabbits (59, 69), this response appears to have some degree of species specificity.

**Comparison of Dimethyl Phthalate (DMP), Di-*n*-butyl Phthalate (DBP) and Di-2-ethylhexyl Phthalate (DEHP).** In rats fed DMP, DBP or DEHP at a level of 25 mmole/kg diet (equivalent to 1.0% DEHP) for 21 days, DEHP and DBP reduced plasma cholesterol levels 16% and 2, respectively, and inhibited hepatic cholesterologenesis from labeled mevalonate by 67% and 30%, respectively (Table 9). It is possible that the hypocholesterolemic effect of DBP and DEHP is a direct consequence of the inhibition of hepatic sterologogenesis, although other mechanisms could be possible. Since inhibition of hepatic sterologogenesis from labeled mevalonate (Table

**Table 7. Incorporation of  $^{14}\text{C}$ -mevalonate into sterols in livers of 8-day-old suckling rats from dams fed 0.5% DEHP during gestation and throughout the postnatal period.<sup>a</sup>**

	Pup wt, g	Liver wt, % body wt	Incorporation into $\text{C}_{27} + \text{C}_{30}$ sterols, dpm/g wet wt $\times 10^{-2}$
Control	21.7 $\pm$ 0.3	2.75 $\pm$ 0.03	3906 $\pm$ 280
DEHP	18.8 $\pm$ 0.7	3.01 $\pm$ 0.06	3107 $\pm$ 208
	$p < 0.01$	$p < 0.01$	$p < 0.05$

<sup>a</sup>Pregnant rats were fed 0.5% DEHP in the diet (w/w) beginning on the 5th day of gestation and were permitted to deliver and to nurse the pups for 8 days; the DEHP diet was continued throughout the entire postnatal period. Liver minces were prepared from each pup and incubated 3 hr at 37°C in 3.5 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, which contained 2  $\mu\text{Ci}$  DL-mevalonic-5- $^3\text{H}$  acid, (DBED salt, SA 5 Ci/mole). Control pups ( $n = 10$ ) were from 1 dam; pups in the DEHP group ( $n = 12$ ) came in equal numbers from two dams.

**Table 8. Plasma cholesterol levels in DEHP-fed nursing dam rats and their suckling pups.<sup>a</sup>**

	$n$	Body wt, g	Total plasma cholesterol, mg/dl
Controls			
Dams	4	363 $\pm$ 6	61 $\pm$ 5 <sup>b</sup>
Pups	41	46 $\pm$ 1	102 $\pm$ 2
DEHP-fed			
Dams	4	353 $\pm$ 6	37 $\pm$ 6 <sup>b</sup>
Pups	26	48 $\pm$ 1	102 $\pm$ 4

<sup>a</sup>Female rats were fed Purina Laboratory Chow or the chow supplemented with 0.5% DEHP (w/w) from the first day of gestation until the pups were weaned at 21 days. Cholesterol was measured as described (59). Values are means  $\pm$  SEM of the number of animals designated by  $n$ .

<sup>b</sup>Significant,  $p < 0.02$ .

**Table 9. Effect of feeding various phthalate esters on lipid metabolism in the rat.<sup>a</sup>**

	Liver weight, % body wt	Total liver lipid, mg/g wet wt	Liver cholesterol, mg/g wet wt	Total plasma cholesterol, mg/dl	Incorporation of $^{14}\text{C}$ -mevalonate into cholesterol in liver, % of control
Control	4.0 $\pm$ 0.1	42.7 $\pm$ 1.4	4.5 $\pm$ 0.2	87 $\pm$ 5	100
DMP	4.1 $\pm$ 0.2	38.5 $\pm$ 1.2 <sup>b</sup>	3.1 $\pm$ 0.2 <sup>c</sup>	80 $\pm$ 3	114 $\pm$ 21
DBP	4.6 $\pm$ 0.1 <sup>c</sup>	47.3 $\pm$ 2.2	3.8 $\pm$ 0.2	69 $\pm$ 3 <sup>b</sup>	70 $\pm$ 6 <sup>b</sup>
DEHP	6.8 $\pm$ 0.2 <sup>c</sup>	44.2 $\pm$ 1.0	4.1 $\pm$ 0.1	73 $\pm$ 5 <sup>b</sup>	33 $\pm$ 8 <sup>c</sup>

<sup>a</sup>Male Sprague-Dawley rats were fed Purina Chow (control) or the chow containing dimethyl phthalate (DMP), di-*n*-butyl phthalate (DBP), or di(2-ethylhexyl) phthalate (DEHP); the phthalates were present at a level of 2.5 mmole/100g feed. The diets were fed for 21 days. Details of the analyses have been presented (55). All values are means  $\pm$  SEM of 5-10 observations.

<sup>b</sup> $p < 0.05$ , Student's independent  $t$ -test.

<sup>c</sup> $p < 0.001$ , Student's independent  $t$ -test.

9) and labeled acetate (55) is greater with DEHP than DBP and since DMP does not have this action (Table 9) (55), the presence of a two-carbon alkyl chain appears to be the minimum requirement for inhibition of sterologenesi.

DMP differed from DBP and DEHP in two other respects; it reduced liver total lipid and liver total cholesterol (Table 9), whereas DBP and DEHP showed no significant effect on these parameters. Additionally, DMP did not produce hepatomegaly as did DBP and DEHP (Table 9). The increased liver size in the DBP- and DEHP-fed animals appeared to represent a true increase in tissue mass rather than an edematous change since the ratio of lipid-free dry weight to wet weight of liver was similar to control values (55).

## Effects of DEHP Feeding on Biosynthesis of Fatty Acids and Glycerol Lipids in the Rat

Feeding DEHP to rats affects not only sterol synthesis as mentioned previously, but synthesis of other lipids as well. Interestingly, not all tissues appear to be affected to the same extent or in the same manner. The incorporation of 1- $^{14}\text{C}$ -acetate into fatty acids, phospholipids and triglycerides was not statistically significantly altered, *in vitro*, in heart and aorta of male rats fed 0.5% DEHP for 10 days (Table 10). By contrast, 1- $^{14}\text{C}$ -acetate incorporation into these same lipids was reduced about 40% in liver; total  $^{14}\text{C}$ -labeled lipid synthesis (includes sterols) was also reduced about 48% (Table

Table 10. Effect of DEHP feeding (0.5%) on the incorporation of  $^{14}\text{C}$ -acetate into fatty acids and glyceryl lipids in various tissues of the rat, *in vitro*.<sup>a</sup>

Tissue	Group	$^{14}\text{C}$ -Acetate incorporation, dpm/g wet weight			
		Phospholipid	Triglyceride	Free fatty acid	Total lipid
Aorta	Control	320 ± 105	295 ± 150	290 ± 120	1225 ± 440
	DEHP	205 ± 75	315 ± 175	200 ± 60	965 ± 350
Heart	Control	1765 ± 165	1095 ± 90	1895 ± 200	6755 ± 465
	DEHP	1500 ± 145	1085 ± 135	1515 ± 80	6245 ± 470
Kidney	Control	2470 ± 220	1945 ± 220 <sup>b</sup>	1775 ± 145	8400 ± 435
	DEHP	2060 ± 130	1315 ± 100 <sup>b</sup>	2185 ± 120	8095 ± 350
Liver	Control	695 ± 80 <sup>c</sup>	625 ± 105 <sup>b</sup>	945 ± 140 <sup>c</sup>	3525 ± 455 <sup>c</sup>
	DEHP	425 ± 40 <sup>c</sup>	370 ± 65 <sup>b</sup>	500 ± 60 <sup>c</sup>	1860 ± 165 <sup>c</sup>
		$p < 0.02$	$p < 0.05$	$p < 0.02$	$p < 0.02$

<sup>a</sup>Male Sprague-Dawley rats were fed Purina Chow (control) or the chow containing 0.5% DEHP (w/w) for 10 days. Tissue slices were prepared from liver, heart and kidney and incubated for 3 hr at 37°C in 5.0 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 4  $\mu\text{Ci}$  sodium acetate-1- $^{14}\text{C}$  (SA 58.6 mCi/mole) (53). Aortas were incubated under exactly the same conditions with the exception that the tissue was bisected longitudinally rather than sliced. All values are means  $\pm$  SEM of six animals.

<sup>b</sup>Significant,  $p < 0.05$ .

<sup>c</sup>Significant,  $p < 0.02$ .

10). In kidney,  $^{14}\text{C}$ -acetate incorporation in triglycerides was reduced 33%, whereas synthesis of the fatty acids and phospholipids was not significantly altered by DEHP feeding (Table 10). Fractionation of the hepatic phospholipids synthesized from  $^{14}\text{C}$ -acetate in rats fed 0.5% DEHP showed a significant reduction in the distribution of  $^{14}\text{C}$ -acetate into the phosphatidylcholine fraction of the liver phospholipids (53). In addition, feeding 0.5% DEHP to male rats for 9 days resulted in 37-44% reduction in the esterification of  $^3\text{H}$ -oleate into diglycerides and triglycerides in liver minces (55). The above studies suggest that DEHP can, in certain tissues, inhibit *de novo* fatty acid synthesis, decrease fatty acid esterification, and selectively affect the synthesis of specific phospholipid species; the latter has been confirmed chemically as well (17).

## Effects of DEHP on Mitochondrial Function

The ability of DEHP to accumulate in heart tissue of animals (23, 24) and man (21) and, more specifically, to accumulate in the mitochondrial fraction of heart tissue (23, 24) suggested a need to investigate possible biochemical effects of DEHP on mitochondrial function.

**Fatty Acid Oxidation in Heart and Liver Mitochondria from Rat, Rabbit and Pig.** DEHP feeding to rats, rabbits and pigs resulted in an increased capacity of their liver mitochondria to oxidize palmitic acid (72). In suitably prepared suspensions of isolated mitochondria, the oxidation of  $^{14}\text{C}$ -palmitoyl CoA was enhanced two- to threefold after several days' exposure to the DEHP (Table 11). The enhanced

capacity to oxidize  $^{14}\text{C}$ -palmitoyl CoA substrate was undiminished throughout the longest periods of exposure studied (i.e., 12 days in rabbits and 35 days in rats). In the rat, the maximum response appeared at a level of 0.1% DEHP in the diet (Table 11). The response of heart mitochondria to DEHP feeding was markedly different from that shown in liver. Ingestion of 0.1% DEHP for 2 days resulted in a 25% reduction in fatty acid oxidation by heart mitochondria. The inhibition increased to about 50% in 3 to 4 days (Table 11). The inhibition, however, appears to be a transient effect, since full oxidizing capacity is restored in 8 or 9 days even though DEHP-feeding is continued. The addition of DL-carnitine and cofactors to heart mitochondria from rats fed DEHP for 5 days resulted in a stimulation of  $^{14}\text{C}$ -palmitoyl CoA oxidation (Table 12). This suggests that the inhibition is not a result of functional change in the fatty acid oxidation system of the mitochondria.

### Mitochondrial Adenine Nucleotide Translocase.

Adenine nucleotide translocase is one of the mitochondrial anion translocators. It is located in the inner mitochondrial membrane and is responsible for affecting a mole:mole exchange of extramitochondrial ADP for intramitochondrial ATP. It is via this specific mechanism that ATP generated by oxidative phosphorylation can be transported into the cytoplasm to support cytosolic energy-requiring reactions. The addition of DEHP to suspensions of isolated heart mitochondria results in an inhibition of adenine nucleotide translocase. Activity of the transporter was reduced 37% by 300  $\mu\text{M}$  DEHP (Fig. 2). Such an effect of DEHP may be the mechanism underlying the death of myocardial cells cultured in the presence of DEHP (45, 74) and may

**Table 11. Effect of oral ingestion of DEHP on the oxidation of  $^{14}\text{C}$ -palmitoyl CoA in isolated heart and liver mitochondria.<sup>a</sup>**

Tissue	Species	Dose	Duration, days	Dpm $^{14}\text{CO}_2$ /mg protein, % of control	No. of observations		
Heart	Rat	0.1% in diet	2	77	1		
			3	55 ± 25	3		
			4	55	2		
	Rat	0.5% in diet	9	110	1		
			4	61	2		
			9	103 ± 14	6		
	Rat	1.0% in diet	9	98	2		
			16	105 ± 5	5		
			2	170	2		
Liver	Pig	0.8 mg/kg per os, b.i.d.	2	170	2		
	Rabbit	1.0% in diet	12	236 ± 77	4		
	Rat	0.1% in diet	2	284	1		
			3	191 ± 1	3		
			4	299	1		
	Rat	0.5% in diet	4	548	2		
			8	272 ± 16	3		
			10	199 ± 21	3		
			16	329	2		
			Rat	1.0% in diet	16	227 ± 15	3
					35	278	2

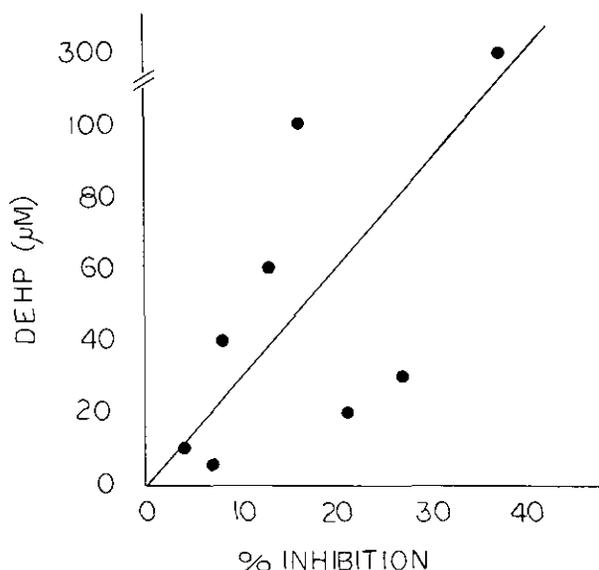
<sup>a</sup>Mitochondria were isolated as previously described (72) and incubated for 3 hr at 37°C in the presence of 1- $^{14}\text{C}$ -palmitoyl CoA (SA 56 Ci/mole). Oxidation of  $^{14}\text{C}$ -palmitoyl CoA was assessed by measuring  $^{14}\text{CO}_2$  production (72).

**Table 12. Stimulation of  $^{14}\text{C}$ -palmitoyl CoA oxidation by carnitine and cofactors in heart mitochondria from DEHP-fed rats.<sup>a</sup>**

Additions to incubation medium	$^{14}\text{CO}_2$ production, dpm/mg mitochondrial protein
None	2100
DL-Carnitine	2740
ATP, CoA-SH	4660
ATP, CoA-SH, DL-carnitine	5710

<sup>a</sup>Male Sprague-Dawley rats (225-250 g) were fed Purina Laboratory Chow containing 1.0% DEHP (w/w) for 6 days. Mitochondria were isolated from pools of two hearts, and all assays were performed in triplicate (see footnote to Table 11) in a total volume of 7.0 ml (72). When present, the levels of the various cofactors in the incubation mixtures were: ATP, 15 mg; CoA-SH, 4 mg; NaF, 0.8; MgCl $\cdot$ 6H $_2$ O, 3 mg. DL-Carnitine  $\cdot$ HCl was added to a final concentration of 2.6 mM.

also be related to the decrease in spontaneous heart rate that is observed in rat hearts perfused with DEHP-containing medium (75, 76). Despite the fact that DEHP accumulates in the hearts of DEHP-fed rats (50), we did not observe a change in adenine nucleotide translocase activity in mitochondria isolated from hearts of rats fed 0.5% DEHP for up to 10 days (73). The fact that DEHP inhibited adenine nucleotide translocase when added directly to mitochondria, but not when fed to the rats, may indicate that the level of DEHP accumulating in the hearts during the 10-day feeding trials was insufficient to significantly affect the enzyme.



**FIGURE 2.** Inhibition of mitochondrial adenine nucleotide translocase. Mitochondria were isolated from normal male rat heart and assayed (73) in the presence of various concentrations of DEHP. Values are means of 15 animals at 20, 40 and 60 M DEHP and means of three to four animals at all other levels.

### Effect of DEHP Feeding on Platelet Lipid Synthesis and Function in Rats and Rabbits

The well-known fact that blood and blood products become contaminated with DEHP during stor-

Table 13. Effect of DEHP feeding on platelet function and prothrombin time.<sup>a</sup>

Species	Group	Platelet number × 10 <sup>6</sup> /mm <sup>3</sup>	Aggregation		Prothrombin time, sec
			Collagen-induced	ADP-induced	
Rat	Control	1.57 ± 0.03	30.7 ± 6.1	27.8 ± 1.9	12.7 ± 0.3
	DEHP-fed	1.66 ± 0.09	35.7 ± 4.9	30.2 ± 2.6	11.6 ± 0.3
Rabbit	Control	0.61 ± 0.05	18.0 ± 3.6	—	5.5 ± 0.1
	DEHP-fed	0.60 ± 0.04	17.9 ± 1.9	—	5.6 ± 0.1

<sup>a</sup>Male New Zealand rabbits (2.5-3 kg) and male Sprague-Dawley rats received Purina Chow or the chow supplemented with DEHP. Rats (150-160 g at beginning of study) received 0.5% DEHP for 32 days and rabbits received 1.0% DEHP for 28 days. All methods used are detailed elsewhere (69). Values are means ± SEM of six animals in each control group, and seven animals in each treated (DEHP) group.

age in PVC plastic storage bags has caused great concern because of the uncertainty as to what DEHP may do to blood cell physiology (77). For example, it has been reported that DEHP may cause increased platelet aggregation in blood stored in PVC storage bags (45), increased platelet adhesion to artificial surfaces (78) and decreased survival of platelets stored in the frozen state in PVC bags (79). In view of the above, we studied the function of platelets exposed to DEHP *in vivo* through a regimen of DEHP feeding. DEHP was fed in the diets of rats (0.5%) and rabbits (1.0%) for 32 and 28 days, respectively. At the end of the feeding period, platelet function was studied. Synthesis of phospholipids and sterols by the platelets was also studied because of the importance of these two lipid classes to membrane structure and stability (80), particularly since platelet aggregation is a membrane-dependent phenomenon. DEHP feeding did not affect phospholipid synthesis from acetate-1-<sup>14</sup>C, or sterol synthesis from mevalonic-5-<sup>3</sup>H acid, in platelets from young (6-week-old) or old (5-month-old) rats (69). Additionally, the feeding of DEHP did not significantly ( $p > 0.05$ ) alter platelet number, collagen-induced platelet aggregation or ADP-induced aggregation in either the rabbit or rat (Table 13). Prothrombin time was also essentially unchanged (Table 13). The results suggest that the increased sensitivity of platelets exposed to DEHP *in vitro* (45, 78, 79) does not accurately reflect their response to DEHP exposure *in vivo*.

## Conclusions and Suggestions

The data presented here make it abundantly clear that the exposure of animals to phthalate esters can result in a significant perturbation of normal metabolic patterns in liver, heart, testes, adrenal gland and brain and can affect blood lipids (51-53, 55, 59-61, 68, 69, 72, 73, 77). The ability of phthalates to alter lipid metabolism in the developing fetus and in suckling animals is also apparent

and becomes a point of particular concern. At the time of this writing, no published biochemical studies similar to those reported here have been conducted in tissues from subhuman primates or man; such studies are urgently needed in order to assess more accurately the potential hazard of the phthalates to human health. At the present time, the concern of the medical profession over the exposure of patients to phthalates while undergoing medical treatment has attracted widespread attention in government and industry. Hopefully, the concern over the use of phthalate-containing devices in the field of medicine will attract greater attention to the more important issue of what potential health hazards mankind is exposed to as a result of involuntary chronic exposure to phthalates in the environment. Evidence that chronic exposure of man to DEHP may result in neurological disorders has been reported (81). A further point to be made is a suggestion that pharmacokinetic studies with phthalates should be less preoccupied with tissue distribution and more concerned with subcellular distribution; the reasoning follows. Much of the work described here has been concerned with biochemical systems dependent upon the activity of membrane-bound (particulate) enzymes—e.g., sterol synthesis (HMG CoA reductase), adenine nucleotide translocase, and fatty acid oxidation. Membrane-bound enzymes, in general, are sensitive to changes in membrane fluidity which reflects alterations in the physical state of the membrane-lipids (82, 83). The possibility exists that the effects of phthalates (inhibitory or stimulatory) on various membrane-bound enzymes is an indirect effect of their ability to modify membrane fluidity. Being lipophilic, the phthalates can partition across organellar membranes and perturb the composition and physical state of the lipid microenvironment (domain) surrounding certain particulate enzymes. Since the response of specific membrane-bound enzymes to changes in membrane fluidity cannot be determined *a priori*, it is not inconsistent that phthalate exposure should inhibit some enzymes while stimulating others. Con-

sequently, pharmacokinetic studies would yield information of greater potential value if distribution data were obtained for specific cell fractions (endoplasmic reticulum, mitochondria, cytosol, etc.) rather than for total tissue. The growing knowledge of factors regulating particulate enzymes makes it clear that relatively low levels of a chemical agent in tissue may be misleading in terms of toxicity potential, since levels considered to be inconsequential on a tissue or organ basis may be disastrously high on an organellar basis.

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