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Effects of Prolonged Administration of Phthalate Ester on the Liver*

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Di(2-ethylhexyl)phthalate (DEHP) was administered to male rats in the diet at concentrations of 2.0, 0.2, and 0.02% for up to 102 weeks. Low doses resulted in moderate increases in certain hepatic enzymes during the initial phase of exposure and in a continuous increase in the activities of these same enzymes throughout the treatment period. An increased level of dolichol and decreased concentration of dolichyl-P were observed. Furthermore, the rate of protein glycosylation diminished. Liver biopsies from patients subjected to hemodialysis demonstrated an increased number of peroxisomes. Phthalate ester seems to interfere with protein turnover, so that the half-life of total mitochondrial and microsomal protein is considerably increased.

Introduction

Humans are exposed to low doses of plasticizers for long periods of time. Most people take up plasticizers from food, water, and air, but the individual level of exposure varies greatly, as was documented in this symposium. Those exposed to the highest levels of plasticizers are found among dialysis and hemophilia patients and in certain branches of industry. However, all of us encounter plasticizers in our environment, and we have to expect a certain risk from lifetime exposure (*1,2*).

The validity of the usual experimental model, involving administration of high doses of plasticizer for a short time and subsequent extrapolation of the data obtained to low dose administration for a long period, can be questioned on several counts. In addition, because plasticizers do not demonstrate acute toxicity, most test systems employed in present day toxicology are less effective in evaluating the risks associated with exposure to these compounds.

In the present study we approached this problem by treating one group of rats with 2% di(2-ethylhexyl)phthalate (DEHP) (the dose commonly employed) in the diet and treated other groups of animals with doses 10 and 100 times lower. The experiment was continued over a 2-year period in order to determine whether certain hepatic chemical or enzymatic parameters are influenced by such long-term exposure.

Materials and Methods

The materials, experimental procedures, and ultrastructural, chemical, and enzymatic measurements employed here are those currently used in our laboratory and are described in the literature (*3–5*).

Results

Electron Microscopy of Liver Tissue

DEHP, like other peroxisome-inducing agents, has prompt and extensive effects on the rat hepatocyte. The number of peroxisomes is increased dramatically. Other characteristic alterations include changes in the size and even disappearance of the core structure of these organelles. DEHP has an additional effect not shared by other peroxisome-inducing agents, namely, induction of mitochondria.

Dialysis patients have a particularly high level of exposure to DEHP, receiving 100 to 200 mg of this compound two to three times per week. If phthalate esters have effects on the structure of human hepatocytes, one would expect such changes to appear some time after the onset of the dialysis treatment. In Sweden it is not possible to systematically obtain liver biopsies from patients subjected to dialysis. In some cases, when biopsies were taken for diagnostic purposes, we were able to analyze these samples under the electron microscope.

The structure of human peroxisomes is less characteristic than in the rat, as this organelle in man is of

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moderate density and lacks a core. Figure 1 illustrates a liver biopsy from a patient dialyzed for 1 month. Peroxisomes are visible only occasionally and are greatly outnumbered by mitochondria. In another case, after 12 months of hemodialysis (Fig. 2), appreciable numbers of peroxisomes are visible. As these patients did not receive any drugs known to affect peroxisomes, phthalate esters may be responsible for this change.

Protein Synthesis and Breakdown

The appearance of new membranes in rats treated with DEHP is extensive and cannot occur without *de novo* synthesis. Previously, we found that in the initial phase of DEHP treatment, the rate of phospholipid synthesis in the endoplasmic reticulum increased by about 20%. Judging from the level of incorporation of radioactive precursors into total microsomal protein, the rate of protein synthesis is also increased (Fig. 3*B*). The high rate of incorporation in mitochondria is explained by increased mitochondrial protein synthesis together with import of components synthesized extramitochondrially (Fig. 3*A*).

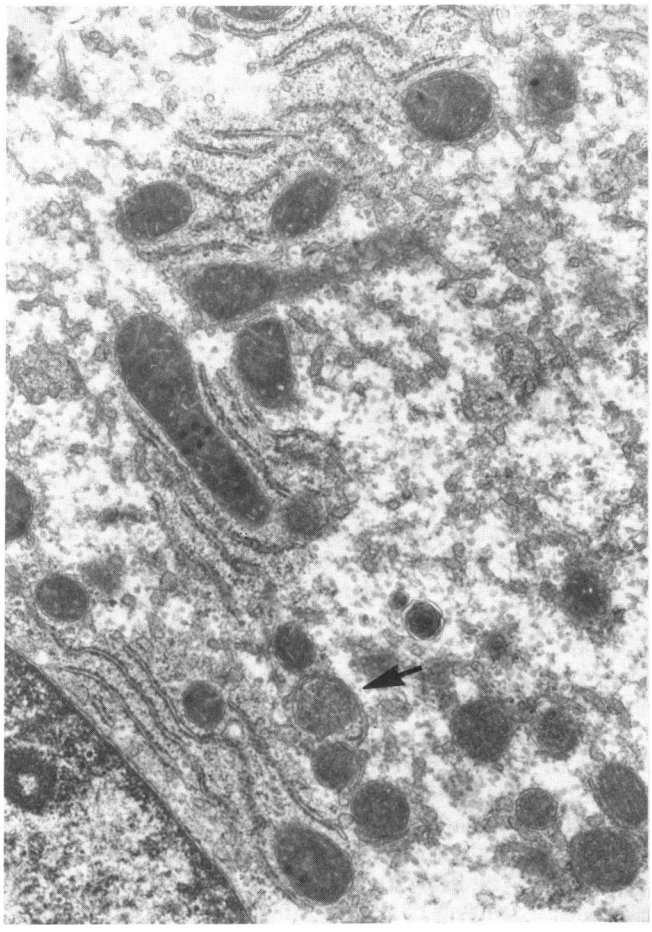

FIGURE 1. Electron micrograph of the liver of a patient after 1 month of hemodialysis. Human liver biopsy specimens were fixed in glutaraldehyde immediately after removal. The arrow points to a peroxisome. × 14,500.

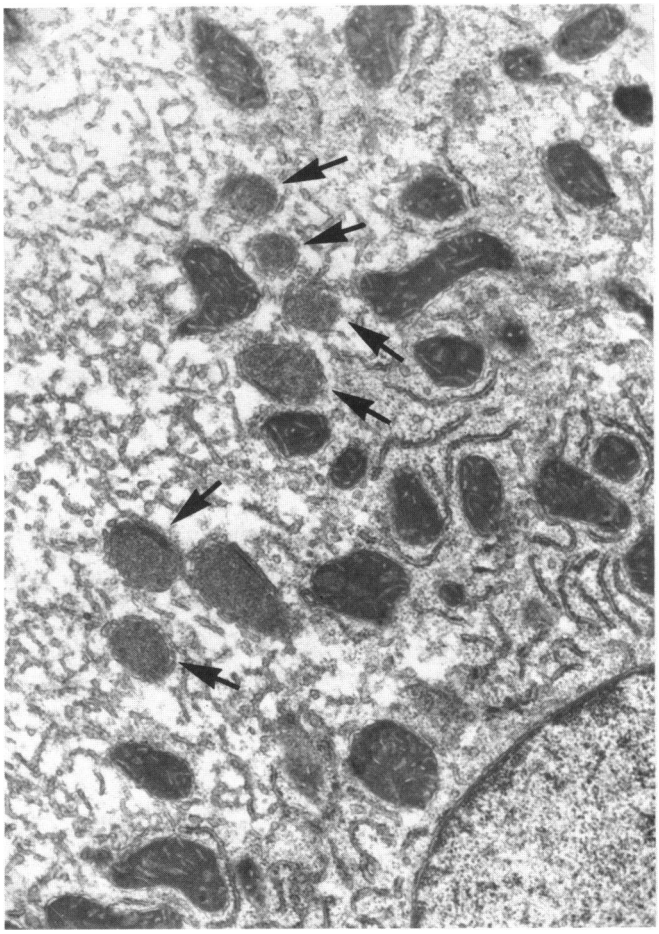

FIGURE 2. Electron micrograph of the liver of a patient following 12 months of hemodialysis. The arrows point to peroxisomes. × 14,500.

Under steady-state conditions, the rate of membrane synthesis is about the same as the rate of membrane degradation. It is now well established that a number of drugs that interfere with membrane biosynthesis also alter the rate of breakdown. In order to examine this possibility, we followed the disappearance of [^{35}S]-methionine from the total protein of mitochondria, microsomes, and supernatant (Fig. 4). DEHP treatment resulted in an increase in the average half-life of mitochondrial proteins from ~6 days to ~25 days. Obviously, this decreased breakdown is an important factor in mitochondrial proliferation. The increase in average protein half-life for microsomes and supernatant is more moderate, i.e., from ~3.5 to ~5.5 days and from ~2.5 to ~5 days, respectively.

Effects on Dolichol and Dolichyl-P

All tissues and virtually all biological membranes contain the polyisoprenoid compound dolichol (*6*). This lipid is present in milligram quantities in certain human tis-

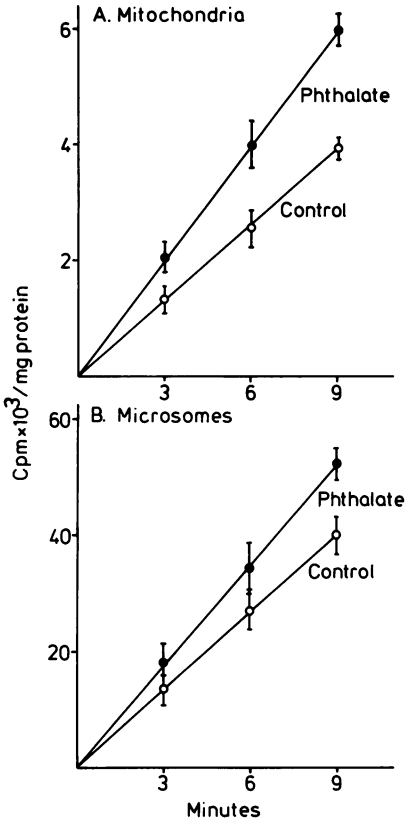

FIGURE 3. Effect of DEHP treatment on the incorporation of radioactive protein precursors. Rats were fed a diet containing 2% DEHP for two days. 200 μCi [^{3}H]leucine was then injected into the portal vein at specified time points before sacrifice. Mitochondria (*A*) and microsomes (*B*) were isolated and the radioactivity bound to protein precipitated by trichloroacetic acid was determined.

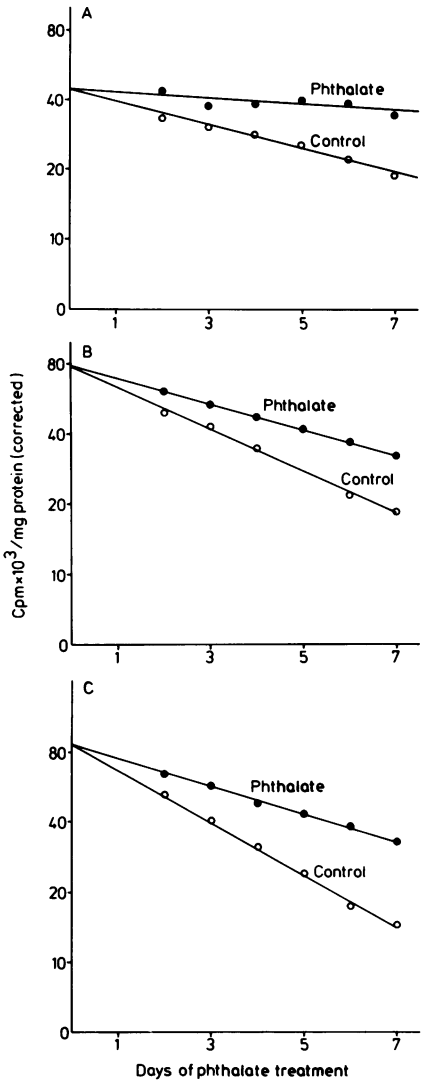

FIGURE 4. Changes in the apparent half-lives of intracellular proteins caused by DEHP treatment. Rats fed a diet containing 2% DEHP for 2 days were subsequently injected with 0.5 mCi [^{35}S]methionine IP. The animals were then sacrificed at specified time points after this injection. Mitochondria (*A*), microsomes (*B*), and particle-free supernatant (*C*) were prepared. The TCA-precipitable radioactivity in these individual fractions was determined.

sues, but much lower levels are seen in rat tissues. Only a small portion of the total dolichol is phosphorylated and participates as an obligatory intermediate in the biosynthesis of *N*-glycosidically linked oligosaccharides. The exact function(s) of dolichol is not yet known, but recent observations indicate that this lipid destabilizes model membranes and increases fatty acid fluidity (*7*).

In control rats, hepatic mitochondrial membranes contain low levels of dolichol. The microsomes demonstrate a relatively low content of this lipid as well, even though the endoplasmic reticulum is the site of dolichol biosynthesis (Table 1). On the other hand, lysosomes have 15 times more dolichol than is found in microsomes.

Treatment of rats with 2% DEHP for 5 weeks affected only the dolichol content of lysosomes, where this value was tripled. In microsomes about 20% of the total dolichol is phosphorylated. Almost all of the initial phase of protein glycosylation occurs in the endoplasmic reticulum and the evidence indicates that the phosphorylated intermediate is under certain conditions rate limiting for sugar transfer from the nucleotide-sugar to protein. In contrast to the free alcohol, the level of dolichyl-P decreases substantially as a result of DEHP

Table 1. Distribution of dolichol and dolichyl-P in liver subfractions after treatment of rats with 2% DEHP for 5 weeks.

	μg/mg Protein			
	Dolichol		Dolichyl-P	
Fraction	Control	Treated	Control	Treated
Homogenate	0.192	0.289	0.101	0.070
Heavy mitochondria	0.094	0.117	0.010	0.013
Light mitochondria	0.072	0.100	0.008	0.008
Heavy lysosomes	3.85	9.88	0.204	0.194
Light lysosomes	3.73	9.51	0.181	0.165
Microsomes	0.215	0.238	0.159	0.083

treatment and this decrease is localized to the key subfraction, the microsomes.

Lysosomal dolichol is changed not only in amount, but also in composition during DEHP treatment (Table 2). Dolichols containing 17 and 18 isoprene residues decrease in amount during 57 weeks of treatment. The amounts of longer dolichols, D19 and D20, gradually increase during this period. This pattern of change has not been observed previously (*8*).

The amount of dolichol present in lysosomes increases with age, and phthalate esters cause an additional increase (Fig. 5). After 8 months of dietary administration of 2% DEHP, a fourfold increase is observed, which is close to maximal. Both the 0.2% and the 0.02% dose of dietary DEHP also resulted in significant elevations of the polyisoprenoid content in lysosomes. With these low doses the effect is observed only after dietary treatment for a long period of time.

Many intracellular and secretory proteins are glycosylated, a process that is important from a functional point of view (*9*). Protein-bound oligosaccharide chains may be involved in protein association with various membranes or protein transport within the endoplasmic reticulum-Golgi system. The decrease in dolichyl-P observed here during treatment of rats with plasticizer raises the question as to whether various glycosyltransferase systems are also affected. Three sugars present in the oligosaccharide core of glycoproteins, i.e., *N*-acetyl-glucosamine (GlcNAc), mannose, and glucose, are transported and collected in the membrane of the endoplasmic reticulum with the help of dolichyl-P or dolichyl-PP. For this reason, the corresponding glycosyltransferases were assayed (Table 3). Transfer of sugar from UDP-GlcNAc and GDP-mannose to dolichyl-P, and also to protein, is considerably decreased. In agreement with findings in other systems, the UDP-glucose transferase system is less sensitive and not affected to any great extent.

Table 2. Distribution of individual dolichols in the mitochondrial-lysosomal fraction from rat liver after DEHP treatment.

Weeks of DEHP treatment	Composition, % of total				
	D17	D18	D19	D20	D21
0	12	38	34	11	5
18	8	39	39	13	6
33	5	30	41	17	7
57	4	28	42	19	7

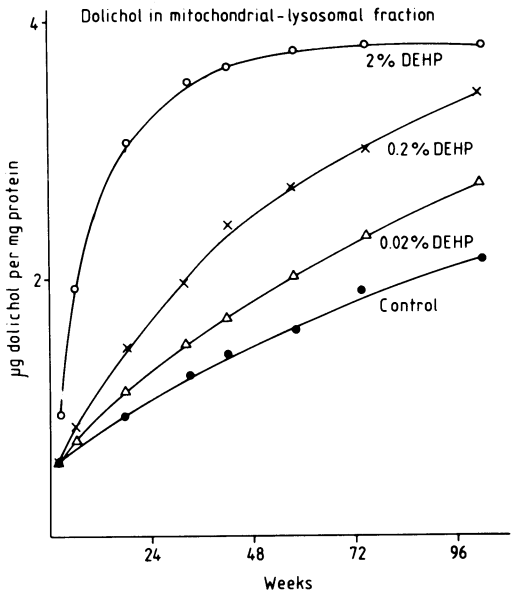

FIGURE 5. Levels of dolichol in the mitochondrial-lysosomal fraction isolated from rat liver. Rats were kept on a diet containing 2.0, 0.2, or 0.02% DEHP for 3–102 weeks.

Enzymic Pattern

Induced changes of the metabolism were followed by analyses of the activities or amounts of some key enzymes. During the entire 102-week exposure to the three levels of DEHP, we monitored a number of enzymes. Cyanide-insensitive palmitoyl-CoA dehydrogenase is one of the enzymes involved in peroxisomal β-oxidation of fatty acids. This enzyme is rapidly induced (15–20 times) even during the early phase of treatment with the highest dose of DEHP (Fig. 6). Lowering the dose 10-fold results in a slower, but continuous increase in this dehydrogenase activity. After 2 years, the activity is still increasing in this case, but has not yet reached its maximal level. A continuous elevation of this activity was also observed with 0.02% DEHP.

One of the major effects of DEHP is induction of carnitine-mediated fatty acid transport in mitochondrial inner membranes. Carnitine acetyltransferase (CAT) is the enzyme most rapidly induced, and Figure 7*A* illustrates the changes in this activity during the experimental period. Carnitine acetyltransferase is induced very rapidly 30-fold by the diet containing 2% DEHP; within the first couple of weeks a striking increase is observed. Similar to the situation with other membrane components, no further induction is possible for sterical or chemical reasons. As is the case for cyanide-insensitive palmitoyl-CoA dehydrogenase, at the two lower doses of DEHP, carnitine acetyltransferase is induced much more slowly, but there is a continuous increase in activity.

With regard to components of the microsomal fraction, no dramatic effects of exposure to phthalate esters

Table 3. Glycosyltransferase activities in liver microsomes after dietary administration of 2% DEHP to rats for 6 weeks.

Sugar-nucleotide substrate	cpm/mg Protein			
	Dol-P monosaccharide		Protein	
	Control	Treated	Control	Treated
UDP-GlcNAc	1559	826	589	308
GDP-Mannose	2385	1486	606	327
UDP-Glucose	1735	1462	451	445

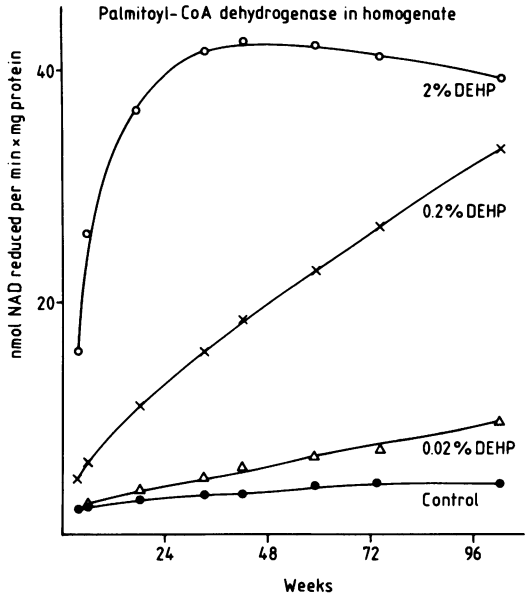

FIGURE 6. Palmitoyl-CoA dehydrogenase activity of rat liver homogenate. The enzyme activity was measured in the presence of KCN.

have been found. One of few changes observed in this case is induction of the cytochrome P-450 system (Fig. 7*B*), possibly because of its involvement in phthalate ester metabolism. Initially, the microsomal content of cytochrome P-450 increases in a manner similar to the increase in NADPH-cytochrome *c* reductase activity. However, unlike the other enzyme activities described above, the level of cytochrome P-450 decreases again upon prolonged exposure, although it is still higher than the control value. As expected, lower doses of DEHP have little or no effect on the total amount of cytochrome P-450.

Effects of Metabolites

The metabolism of DEHP has been studied in detail (*10*). It is well established that various modifications of the side chain take place *in vivo* and that hydrolytic cleavage also occurs. It is possible that the various effects observed after dietary administration of this plasticizer are caused not by one, but by several different metabolites. We have compared the effects of DEHP with those of the monoester, the benzoate derivative, phthalic acid, and the isolated side chain (Fig. 8). Both DEHP and monoethylhexylphthalate (MEHP) effectively induce peroxisomal β-oxidation, while this effect is completely absent when 2-ethylhexylbenzoate (EHB) is administered (Table 4). Similarly, both phthalic acid (PA) and 2-ethylhexanol (EH) lack the capacity for induction. The specific activity of catalase is decreased upon exposure to DEHP and increased by MEHP. Microsomal NADPH-cytochrome *c* reductase activity is increased by DEHP and MEHP, but not by the other metabolites.

Discussion

Possible chronic toxicity resulting from phthalate esters is a difficult problem to investigate from several points of view. The levels that occur in our environment are low, but on the other hand, phthalate esters are more widely distributed than any other chemicals (*11*). Consequently, man is continuously exposed.

At present, we do not know whether phthalate esters are metabolized and the products are excreted completely or if accumulation in certain tissues occurs. Elucidation of these problems obviously requires extensive data.

It is virtually impossible to definitively establish deleterious effects of low-dose exposure to plasticizers on human health due to the time element involved. If we

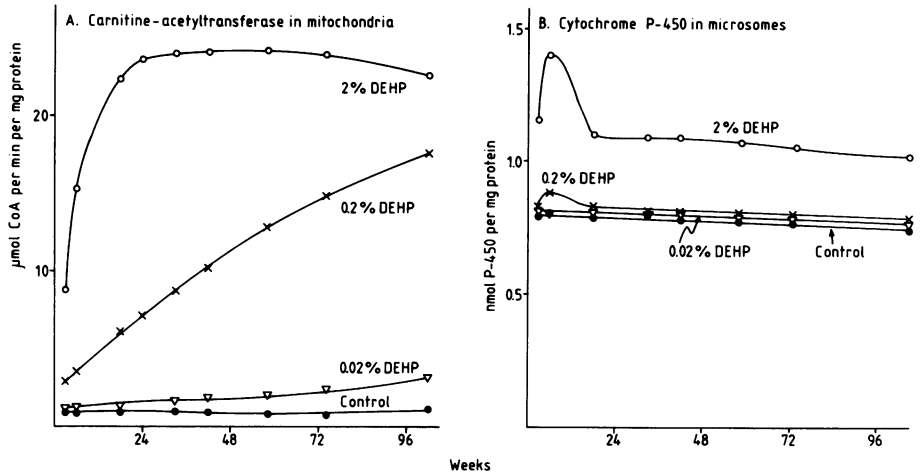

FIGURE 7. Effect of prolonged phthalate ester treatment on mitochondrial carnitine acetyltransferase and microsomal cytochrome P-450. (*A*) Carnitine acetyltransferase activities in isolated mitochondria. (*B*) Cytochrome P-450 levels in isolated microsomes.

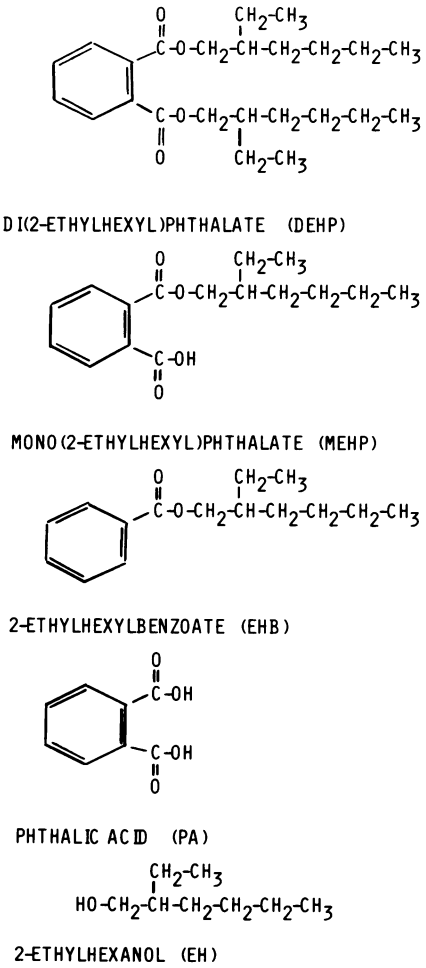

FIGURE 8. Chemical formulae of the metabolites tested in the experiment documented in Table 4.

Table 4. Effects of DEHP and certain of its metabolites on some enzymes in homogenates and microsomes prepared from rat liver.[a]

Treatment[b]	Palmitoyl-CoA oxidation[c]	Catalase[d]	NADPH-cytochrome *c* reductase[e]
Control	4.8	81	0.089
DEHP	28.8	49	0.142
MEHP	16.7	113	0.154
EHB	3.5	65	0.092
PA	4.2	70	0.095
EH	3.8	81	0.101

[a]The compounds tested were administered *ad libitum* in the diet at a level of 2% for 2 weeks. Palmitoyl-CoA oxidation (in the presence of KCN) and catalase were measured in the homogenate; NADPH-cytochrome *c* reductase activity was determined in isolated microsomes.

[b]DEHP = di(2-ethylhexyl)phthalate; MEHP = mono(2-ethylhexyl) phthlate; EHB = 2-ethylhexylbenzoate; PA = phthalic acid; EH = 2-ethylhexanol.

[c]nmole NAD reduced/min/mg protein.

[d]μmole H_2O_2 decomposed/min/mg protein.

[e]μmole cytochrome c reduced/min/mg protein.

assume, in analogy to certain other environmental pollutants, that some bioaccumulation of phthalate esters does occur with time, it may take 30 to 40 years to reach a toxic level. Extensive use of PVC materials was introduced in the 1960s. This means that evaluation of a possible human toxicity would have to wait until the year 2000.

There are some situations where humans are exposed to relatively large amounts of plasticizers (*12,13*). The tubing used for hemodialysis is made of PVC material and patients may thus receive as much as 500 to 600 mg DEHP/week. Other well-established medical procedures that result in administration of phthalates include systemic blood transfusion and injection of blood products, e.g., to hemophilic patients.

For the past couple of years we have been able to investigate liver biopsies from patients undergoing hemodialysis for various periods of time. These biopsies were taken in order to diagnose possible hepatitis. Electron micrographs revealed that, as in the rat, peroxisome proliferation in human liver does occur. These patients had not received any drugs known to have peroxisome-proliferating effects. On the other hand, they were systematically exposed to phthalate esters in connection with their two to three dialysis treatments per week.

We are well aware of two problems in interpreting these data. One is the general health condition of patients subjected to dialysis. Renal insufficiency and a continuous suburemic state have significant effects on general metabolism and on the function of various organs. It will be a difficult task to distinguish the specific effect of a foreign chemical compound from these other effects. Another difficulty is in obtaining a sufficient number of biopsies to allow statistical analysis of the effect on peroxisomes. This is not possible in Sweden, as biopsies may be taken only for diagnostic purposes when diagnosis by other means is unreliable.

Phthalate esters have been tested in various *in vitro* systems, but insufficient information about the toxicity of these plasticizers has been obtained. At present, *in vivo* experiments are also required and all such studies to date have utilized high doses and short-time administration. The usual dietary dose of DEHP is 2%, a dose which gives considerable effects within a few weeks. Here we have also chosen doses that are 10- and 100-fold less; the latter dose approaches the level of exposure in dialysis patients. With these doses we followed the response during a 2-year period, i.e., most of the rat's lifespan.

Although these studies with very low doses of phthalate esters may be more adequate models from the point of view of human toxicology, they also involve problems. Aging itself has effects on cellular structure and metabolism, e.g., a significant increase in lysosomal dolichol. Furthermore, even a well-kept animal room is far from being free of chemicals, and the result is a sizeable background exposure to xenobiotics, particularly in experiments lasting years (*14*). Finally, various chemicals, including plasticizers, have different effects on young

and adult animals. For this reason, long-term exposures to phthalate esters were begun with adult rats, but this procedure does not accurately mirror the pattern of human exposure.

The most apparent results of long-term, low-dose exposure to phthalate esters are effects which are initially limited, but increase in a continuous, almost linear manner with prolonged exposure. Consequently, after sufficiently long exposure, even a very low dose of a plasticizer could give the same effects as a high dose for a short period of time. According to this argumentation, no threshold values for phthalates exist, i.e., any level of intake continued over a sufficiently long period of time can have deleterious effects. The major questions, however, remain: What is the precise period of exposure required for toxic effects in humans with environmental levels of phthalate esters? Will this time period be completed during a life-span?

The pattern of changes in membranes and enzymes which results from DEHP treatment is not very common (*14*), as it involves several intracellular compartments. The complexity of these changes is also revealed by studies of the biosynthesis and breakdown of individual components. The considerable decrease in $t_{1/2}$ for total mitochondrial protein and for proteins in other compartments as well indicates that the changes are mediated not only by an increased synthesis, but also by decreased breakdown. We have previously shown that the rate of phospholipid synthesis is increased during the initial phase of phthalate treatment (*3*). The rate of phospholipid breakdown under these same conditions has not yet been investigated, but this rate may also decrease.

Studies of the effect of DEHP treatment on the pattern of individual dolichols explain certain functional changes. The phosphorylated form of this lipid is an obligatory intermediate in glycoprotein synthesis and is present chiefly in the endoplasmic reticulum. A number of observations indicate that the level of this lipid may be rate-limiting in certain glycosyltransferase reactions (*15,16*). The extensive reduction of microsomal dolichyl-P content, together with modifications of the glycosyl transferases, may be the explanation for the decrease in protein glycosylation caused by DEHP. Since many of the proteins that are synthesized in the endoplasmic reticulum are glycoproteins with important enzymatic or receptor functions, the possible functional consequences of such changes are obvious. It will be a future task to identify the proteins whose states of glycosylation are altered by DEHP treatment.

The presence of dolichol in different cellular organelles is well established, but all the functions of this lipid are not yet clear. It is now known that a large portion of the dolichol is associated with the membrane itself, and that a portion is probably distributed as lipid particles in the lumen. Recent investigations have shown that dolichol affects the structure of model membranes (*7*), which is likely to be true also for biological membranes. The principal effects observed are membrane destabilization and an increase in phospholipid fatty acid fluidity, properties which are of essential importance to membrane function. Phthalates increase the level of dolichol in lysosomal membranes, leading to destabilization and a possible increase in permeability. If some of the lysosomal contents, such as hydrolytic enzymes, are released, the possible severity of the effects on the cell are obvious.

An important task in future research on phthalate ester toxicology will be to analyze the effects of individual metabolites. It seems unlikely that so many diverse effects are caused by a single agent. The levels of different metabolites, their tissue and membrane distributions, and the extent of their binding to various cellular macromolecules may explain on a molecular level the effects in cellular function caused by DEHP. Removal of one alkyl chain does not alter the pattern of induction very greatly. Removal of one alkyl chain plus its carboxyl group, however, abolishes all the biological effects caused by DEHP administration. It is quite possible that minor modifications of the structure of this compound reduce, for example, its effects on one organelle, but not on another.

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