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# EFFECTS OF DI-(2-ETHYLHEXYL) PHTHALATE ON THE CONTENT AND COMPOSITION OF HEPATIC MITOCHONDRIAL AND MICROSOMAL PHOSPHOLIPIDS IN THE RAT

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**Abstract**—Feeding a 20% casein diet containing di-(2-ethylhexyl) phthalate (DEHP) at a 0.5% level to young male rats for 7 days resulted in a significant increase in hepatic phospholipids (PL), based either on per unit weight of the liver or on protein. Although the concentration of PL increased in both the mitochondrial and the microsomal fractions, the magnitude of the increase was much more marked in the former. The increase of PL in hepatic microsomal and mitochondrial fractions was attributed to increases in phosphatidylethanolamine (PE) and phosphatidylcholine (PC). In terms of percentage composition, PE increased significantly, whereas PC remained unchanged, leading to an elevation in the PE/PC ratio in both fractions. A similar response was observed in rats fed 0.1 to 0.5% DEHP for 30 days. Although hepatic PL accumulation was observed in rats fed a diet containing different dietary levels of casein and corn oil, the extent of the increase was much greater on a low protein diet. DEHP caused a decrease in the concentration of hepatic triglycerides (TG), and the magnitude of the reduction appeared to be greater in rats fed diets containing zero or low levels of essential fatty acids. The fatty acid profiles of PE and PC were modified differently by DEHP. Of interest was a significant increase in arachidonic acid in PE and a decrease in PC in two subcellular fractions examined. The rate of swelling of isolated mitochondria from the livers of rats fed DEHP was markedly slower than that of the controls. Some structural changes were also observed by electron microscopy.

In a previous study [1], we found that feeding di-(2-ethylhexyl) phthalate (DEHP), the most commonly used plasticizer in polyvinylchloride formulations, to rats results in an accumulation of phospholipids (PL) in the liver and in a change in the pattern of the PL components, characterized by an increase in the percentage of phosphatidylethanolamine (PE) and a decrease in that of phosphatidylcholine (PC). Analyses of the fatty acid compositions of hepatic glycerolipids showed that DEHP increased the percentage of oleic acid at the expense of linoleic acid. These data [1], together with those from the available literature [2, 3], suggest that DEHP is biologically active and capable of modifying lipid metabolism in experimental animals. Bell *et al.* [2, 4] reported that, in rats, dietary DEHP inhibited lipid biosynthesis from labeled acetate and mevalonate in liver minces and stimulated oxidation of fatty acids by isolated hepatic mitochondria. In contrast, Sakurai *et al.* [3] have reported recently that the synthesis of fatty acids and PL is enhanced with DEHP. DEHP is also toxic to a variety of cells in culture and may be a vascular toxin [5].

In the present study, we investigated the quantitative and qualitative changes in the PL of hepatic microsomal and mitochondrial fractions in rats fed DEHP. The effects of this plasticizer on the swelling pattern and electron microscopic appearance of isolated hepatic mitochondria were also studied. In addition, the effects of dietary manipulations on the responses of hepatic lipids to feeding DEHP were examined.

## EXPERIMENTAL

### *Animals and diets*

Male rats of the Wistar strain, with initial weights of 68–73 g, were individually housed in wire-bottom cages maintained in an air-conditioned room at a temperature of approximately 23°. Experimental diets and water were given *ad lib.* throughout the experiments. Body weight and food intake were recorded every day during the experimental periods.

*Experiment* 1. Rats were fed a 20% casein diet for 7 days. DEHP was added at a 0.5% level.

*Experiment* 2. Rats were fed a 20% casein diet for 30 days. DEHP was added at 0.1, 0.25 or 0.5% levels.

*Experiment* 3. Rats were fed 10, 20, or 42% casein diets for 10 days. DEHP was added at a 0.5% level.

*Experiment* 4. Rats were fed 0, 5, or 15% corn oil diets for 10 days. Diets containing 5% lard or coconut oil were also used. DEHP was added at a 0.25% level.

In each experiment, rats fed diets free of DEHP served as the controls.

The percentage composition of the basal diet was casein, 20; corn oil, 5; mineral mixture, 4; vitamin mixture, 1; choline chloride, 0.15; cellulose powder, 2; and sucrose to 100. The amount of protein (experiment 3) or fat (experiment 4) was adjusted at the expense of the sucrose. Vitamins A, D and E (α-tochopherol) were added (2000 I.U., 200 I.U. and 10 mg/100 g of diet respectively). Mineral and vitamin mixtures, according

to Harper [6], were purchased for the Oriental Yeast Co., Ltd., Tokyo.

DEHP was the product of the Tokyo Kasei Co., Ltd., Tokyo, and the purity was checked by gas–liquid chromatography (g.l.c.) with an electron capture detector using a OV-1 column, as reported previously [1]. Only one peak was detected.

*Analytical procedure*

The animals had free access to diets until decapitation at 10:00 to 10:30 a.m. Liver and plasma lipids were extracted with chloroform–methanol (2:1, w/w) [7]. For isolation of subcellular fractions, livers were homogenized with 5 vol. of 0.25 M sucrose containing 1 mM EDTA and the homogenates were centrifuged at 700 *g* for 10 min. The supernatant fraction was centrifuged at 7,000 *g* for 10 min to sediment mitochondria. The pellets were washed with 1.15% KCl–0.025 M Tris HCl, pH 7.4, and recentrifuged at 6,000 *g* for 10 min. After centrifuging the post-mitochondrial supernatant fraction at 24,000 *g* for 10 min, the microsomal fraction was sedimented from this supernatant fraction by centrifugation at 54,000 *g* for 60 min. The microsomal fraction contains endoplasmic reticulum membranes and ribosomes, and also contains three types of protein (membrane protein, ribosomal protein and secretory protein) [8]. The mitochondrial and microsomal pellets were suspended in the KCl–Tris, and lipids were extracted and purified by the procedure of Folch *et al.* [7]. PL were fractionated by thin-layer chromatography (t.l.c.) according to the methods of Skipski *et al.* [9], and lipid phosphorus was determined by the methods of Rouser *et al.* [10]. Determination of fatty acid composition by g.l.c. was performed as reported elsewhere [11]. Protein was determined by the procedure of Hartree [12].

Samples of the isolated mitochondria were also examined by electron microscopy.

*Assay of mitochondrial swelling*

Swelling of mitochondria was measured according to the method of Johnson [13]. Liver was homogenized with 5 vol. of 0.25 M sucrose with a loose fitting Teflon homogenizer and the homogenate was centrifuged at 600 *g* for 10 min. The supernatant fraction was centrifuged at 15,000 *g* for 5 min to sediment mitochondria. The mitochondrial fraction was suspended in 0.25 M sucrose at 4° at the concentration required to give an initial optical density of approximately 0.7 to 0.8 when 0.1 ml of the suspension was transferred to 2.9 ml of the swelling medium containing 0.25 M sucrose–0.02 M Tris–HCl buffer (pH 7.4). Mitochondrial swelling was determined by measuring the decrease in the optical density at 520 nm at 25°.

*Preparation for electron microscopy*

Tissues were prefixed with a paraformaldehyde–glutaraldehyde mixture in cacodylate buffer at pH 7.4 and postfixed with 2% osmium tetroxide in the same buffer solution. After dehydration in ascending concen-

Table 1. Effects of DEHP on body weight gain, food intake and liver weight*

| Expt. | Groups† | | Body weight (g) Initial | Body weight (g) Gain | Food intake (g/day) | Liver weight (g/100 g body wt) |
|---|---|---|---|---|---|---|
| 1 | 20% Casein | Control | 76 ± 2 | 48 ± 2 | 12.9 ± 0.4 | 5.4 ± 0.2 |
| | | DEHP, 0.5% | 75 ± 2 | 43 ± 3 | 11.7 ± 0.7 | 7.5 ± 0.2‡ |
| 2 | 20% Casein | Control | 73 ± 2 | 172 ± 2 | 15.9 ± 0.2 | 3.9 ± 0.1 |
| | | DEHP, 0.1% | 73 ± 2 | 174 ± 5 | 15.7 ± 0.3 | 4.7 ± 0.2‡ |
| | | DEHP, 0.25% | 73 ± 2 | 176 ± 5 | 16.2 ± 0.4 | 6.0 ± 0.1‡,§ |
| | | DEHP, 0.5% | 74 ± 2 | 156 ± 5‖ | 14.6 ± 0.6 | 6.9 ± 0.2‡,§,¶ |
| 3 | 10% Casein | Control | 92 ± 2 | 23 ± 2 | 11.3 ± 0.9 | 5.8 ± 0.1 |
| | | DEHP, 0.5% | 92 ± 2 | 18 ± 3 | 10.2 ± 0.8 | 6.6 ± 0.1‡ |
| | 20% Casein | Control | 92 ± 2 | 66 ± 3 | 15.2 ± 0.4 | 5.6 ± 0.1 |
| | | DEHP, 0.5% | 92 ± 2 | 64 ± 3 | 14.6 ± 0.2 | 8.4 ± 0.2‡ |
| | 42% Casein | Control | 92 ± 2 | 65 ± 3 | 14.8 ± 0.4 | 5.5 ± 0.2 |
| | | DEHP, 0.5% | 92 ± 2 | 64 ± 2 | 14.2 ± 0.2 | 7.7 ± 0.2‡ |
| 4 | Fat free | Control | 72 ± 1 | 60 ± 2 | 15.2 ± 0.3 | 5.3 ± 0.2 |
| | | DEHP, 0.25% | 72 ± 2 | 63 ± 2 | 15.0 ± 0.5 | 7.3 ± 0.2‡ |
| | Corn oil, 5% | Control | 73 ± 2 | 69 ± 3 | 14.9 ± 0.5 | 5.5 ± 0.1 |
| | | DEHP, 0.25% | 74 ± 2 | 64 ± 3 | 14.2 ± 0.4 | 7.0 ± 0.1‡ |
| | Corn oil, 15% | Control | 74 ± 2 | 66 ± 4 | 13.5 ± 0.6 | 4.9 ± 0.2 |
| | | DEHP, 0.25% | 74 ± 2 | 63 ± 4 | 13.1 ± 0.6 | 6.4 ± 0.1‡ |
| | Lard, 5% | Control | 91 ± 2 | 65 ± 3 | 16.2 ± 0.6 | 4.8 ± 0.2 |
| | | DEHP, 0.25% | 91 ± 1 | 66 ± 2 | 15.3 ± 0.2 | 7.1 ± 0.2‡ |
| | Coconut oil, 5% | Control | 91 ± 1 | 73 ± 2 | 15.8 ± 0.4 | 5.4 ± 0.2 |
| | | DEHP, 0.25% | 91 ± 1 | 69 ± 2 | 15.9 ± 0.3 | 7.3 ± 0.3‡ |

* Values are the means ± S.E. of six rats.

† Rats were fed diets with or without (control) addition of DEHP for 7 days in Expt. 1, for 30 days in Expt. 2 and for 10 days in Expt. 3 and 4.

‡ Difference from the corresponding control is significant at $P < 0.01$.

§ Difference from the 0.1% DEHP group is significant at $P < 0.01$.

‖ Difference from the corresponding control is significant at $P < 0.05$.

¶ Difference from the 0.25% DEHP group is significant at $P < 0.01$.

Table 2. Effects of DEHP on hepatic mitochondrial and microsomal phospholipids and protein (Expt. 1)*

| | Groups† | |
|---|---|---|
| | Control | DEHP |
| Liver phospholipid phosphorus (μg/g liver) | 1,024 ± 20 | 1,336 ± 12‡ |
| Mitochondria | | |
| Phospholipid phosphorus (μg/g liver) | 119 ± 5.0 | 216 ± 15.0‡ |
| Protein (mg/g liver) | 14.1 ± 0.7 | 22.6 ± 1.4‡ |
| Phosphorus/protein (μg/mg) | 8.4 ± 0.2 | 9.5 ± 0.1‡ |
| Microsomes | | |
| Phospholipid phosphorus (μg/g liver) | 247 ± 5.5 | 305 ± 19‡ |
| Protein (mg/g liver) | 20.0 ± 0.8 | 23.1 ± 1.0§ |
| Phophorus/protein (μg/mg) | 12.4 ± 0.5 | 13.7 ± 0.3 |
| Plasma phospholipid phosphorus (mg/dl) | 9.28 ± 0.28 | 8.20 ± 0.44 |

* Values are the means ± S.E. of six rats.
† See Table 1.
‡ Difference from the control is significant at $P < 0.01$.
§ Difference from the control is significant at $P < 0.05$.

trations of acetone, they were embedded in Epon 812. Thin sections stained with uranyl acetate and lead citrate were examined in a Hitachi H-500 electron microscope.

## RESULTS

### *Weight gain, food intake and liver weight*

The results are summarized in Table 1.

*Experiments 1 and 2.* In rats fed a diet containing 0.5% DEHP for 7 days (experiment 1), there were no significant differences in food intake and weight gain compared with the controls, whereas liver weight increased significantly. Feeding lower levels of DEHP, 0.1 and 0.25%, for 30 days (experiment 2), did not influence weight gain and food intake, but at the 0.5% level growth was inhibited significantly. There were gradual increases in liver weights with increasing dietary levels of DEHP; a high correlation between these parameters was demonstrated ($r = 0.987$).

*Experiments 3 and 4.* There were no significant differences in weight gain and food intake between the control and the DEHP-fed rats, but liver weight increased significantly in all cases. Although the extent of the increase in liver weight appeared greater in the animals fed 20 or 42% protein diets (experiment 3), the amount and the type of dietary fat showed no effect (experiment 4).

### *Concentrations of PL and protein in hepatic microsomal and mitochondrial fractions*

*Experiment 1.* Feeding of DEHP caused a significant increase in the concentration of hepatic PL. The extent of the increase was more remarkable in the mitochondrial fraction than in the microsomal fraction (Table 2), the increase being 85 and 25 per cent for mitochondrial

Table 3. Effects of DEHP on the phospholipid composition of hepatic mitochondria and microsomes (Expt. 1)*

| Groups† | LPC | Sph | PC | PI + PS | PE | PA¶ etc. | PE/PC |
|---|---|---|---|---|---|---|---|
| | | | Mitochondrial PL–phosphorus (μg/g liver) | | | | |
| Control | 5.1 ± 0.2 | 7.9 ± 0.5 | 60.1 ± 5.4 | 14.2 ± 1.1 | 24.4 ± 3.4 | 8.3 ± 0.4 | |
| DEHP | 3.0 ± 0.9‡ | 4.5 ± 1-2‡ | 116.0 ± 4.6§ | 22.5 ± 2.6§ | 63.1 ± 7.2§ | 16.4 ± 2.7§ | |
| | | | Percent of total mitochondrial PL | | | | |
| Control | 4.3 ± 0.4 | 6.7 ± 0.8 | 50.5 ± 1.9 | 11.9 ± 0.7 | 20.5 ± 1.7 | 7.0 ± 0.5 | 0.41 ± 0.02 |
| DEHP | 1.4 ± 0.5§ | 2.1 ± 0.7§ | 49.4 ± 1.2 | 10.4 ± 0.7 | 29.2 ± 1.8§ | 7.6 ± 0.9 | 0.59 ± 0.03§ |
| | | | Microsomal PL–phosphorus (μg/g liver) | | | | |
| Control | 7.4 ± 0.8 | 12.4 ± 1.7 | 155.0 ± 5.7 | 38.4 ± 1.0 | 32.9 ± 2.2 | 1.5 ± 0.6 | |
| DEHP | 5.2 ± 0.7 | 4.9 ± 0.5§ | 181.8 ± 16.8§ | 41.2 ± 2.5 | 70.2 ± 6.1§ | 0.6 ± 0.2 | |
| | | | Percent of total microsomal PL | | | | |
| Control | 3.0 ± 0.3 | 5.0 ± 0.6 | 62.2 ± 0.9 | 15.5 ± 0.2 | 13.3 ± 0.6 | 0.6 ± 0.2 | 0.21 ± 0.01 |
| DEHP | 1.7 ± 0.3‡ | 1.6 ± 0.4§ | 59.9 ± 0.2 | 13.5 ± 0.7 | 23.0 ± 1.5§ | 0.2 ± 0.1 | 0.39 ± 0.03§ |

* Values are the means ± S.E. of six rats.
† See Table 1.
‡ Difference from the control is significant at $P < 0.05$.
§ Difference from the control is significant at $P < 0.01$.
¶ PA: Phosphatidic acid.

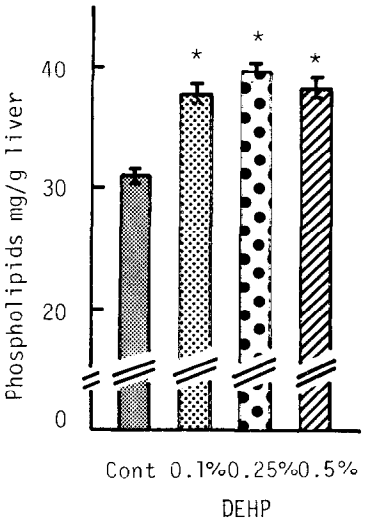


Fig. 1. Effects of DEHP on the concentration of hepatic phospholipids in rats (Expt. 2). Values are the means ± S.E. of six rats. See Table 1. The asterisk (*) indicates that the difference from the control is significant at $P < 0.01$.

and microsomal fractions respectively. In both fractions, the concentration of protein also increased, but again the increase was much greater in mitochondria. As shown in Table 3, in the mitochondrial fraction, the concentrations of PE, PC and PS + PI (phosphatidylserine + phosphatidylinositol) increased significantly after feeding DEHP. However, in terms of the percentage composition, the increase was found only in PE. In the microsomal fraction, the concentrations of PE and PC increased, whereas that of sphingomyelin decreased. However, in terms of the percentage composition, only PE was again increased markedly.

*Experiment* 2. The concentration of hepatic PL was increased by the feeding of DEHP (Fig. 1). Although there was no correlation between the concentrations of PL and DEHP consumed, a gradual increase in PL with

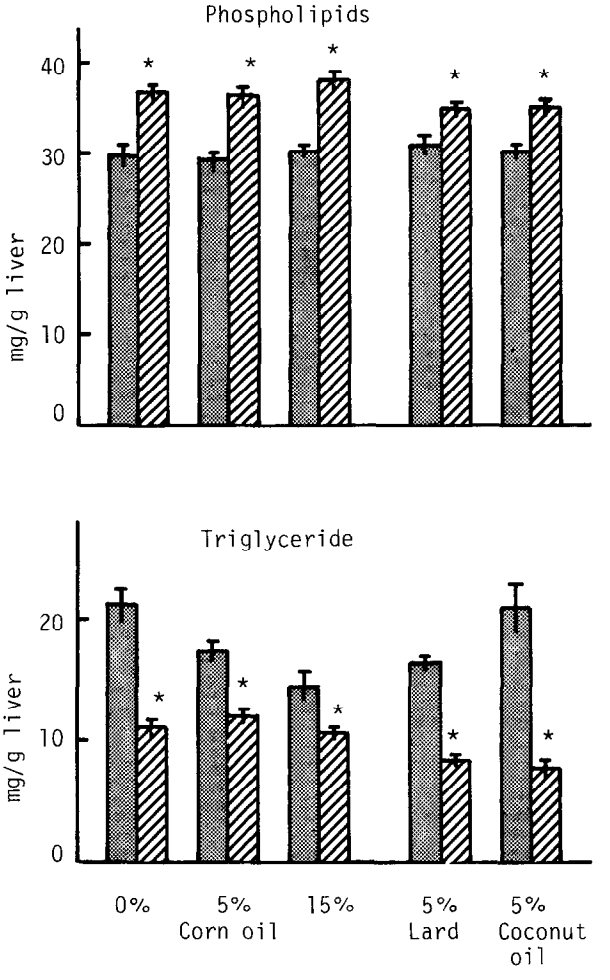


Fig. 3. Effects of DEHP on the concentration of hepatic phospholipids and triglyceride (Expt. 4). Values are the means + S.E. of six rats. Key:( ▨ ), control rats; and ( ▨ ). DEHP-fed rats. See Table 1. The asterisk (*) indicates that the difference from the control is significant at $P < 0.01$.

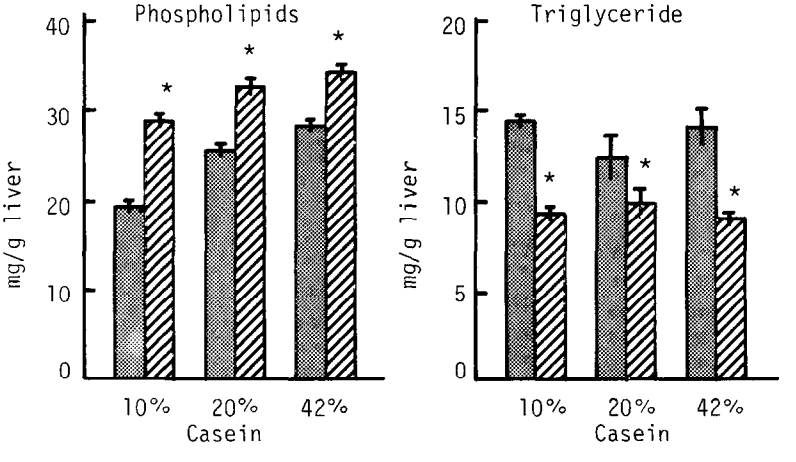


Fig. 2. Effects of DEHP on the concentration of hepatic phospholipids and triglyceride (Expt. 3). Values are the means ± S.E. of six rats. Key: ( ▨ ), control rats; and ( ▨ ), DEHP-fed rats. See Table 1. The asterisk (*) indicates that the difference from the control is significant at $P < 0.01$.

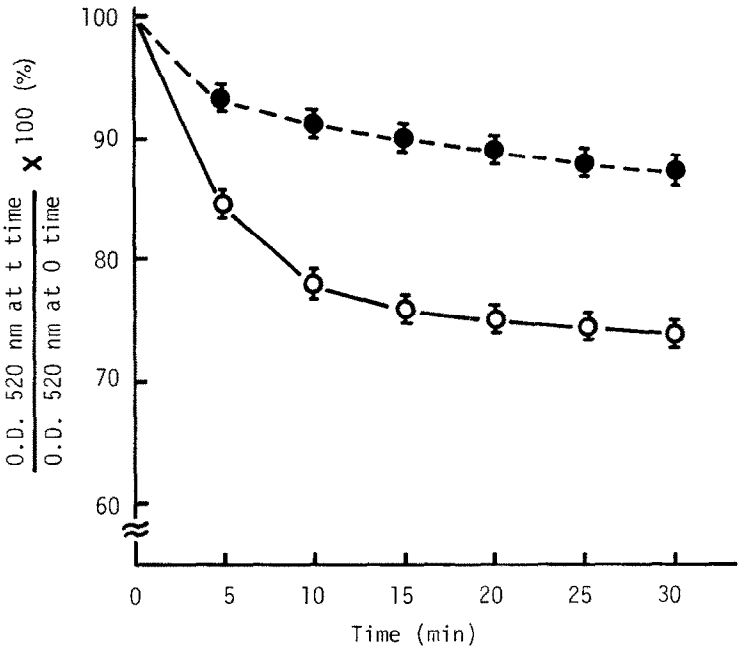


Fig. 4. Effects of DEHP on hepatic mitochondrial swelling (Expt. 1). Values are the means ± S.E. of four rats. Key: (○——○), control rats, and (●---●), DEHP-fed rats. At any time after incubation, difference from the control is significant at $P < 0.01$.

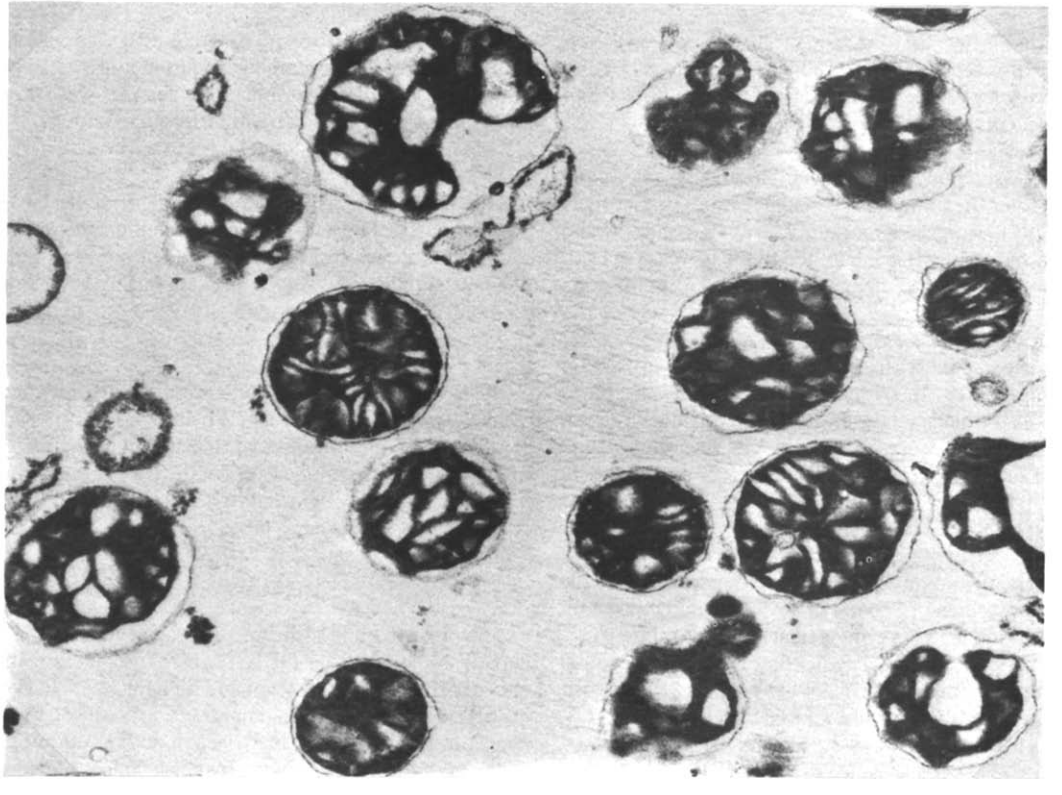

Fig. 5. Electron microphotograph of mitochondria isolated from a rat fed the 20% casein diet containing DEHP at a level of 0.5% for 7 days (× 20,000).

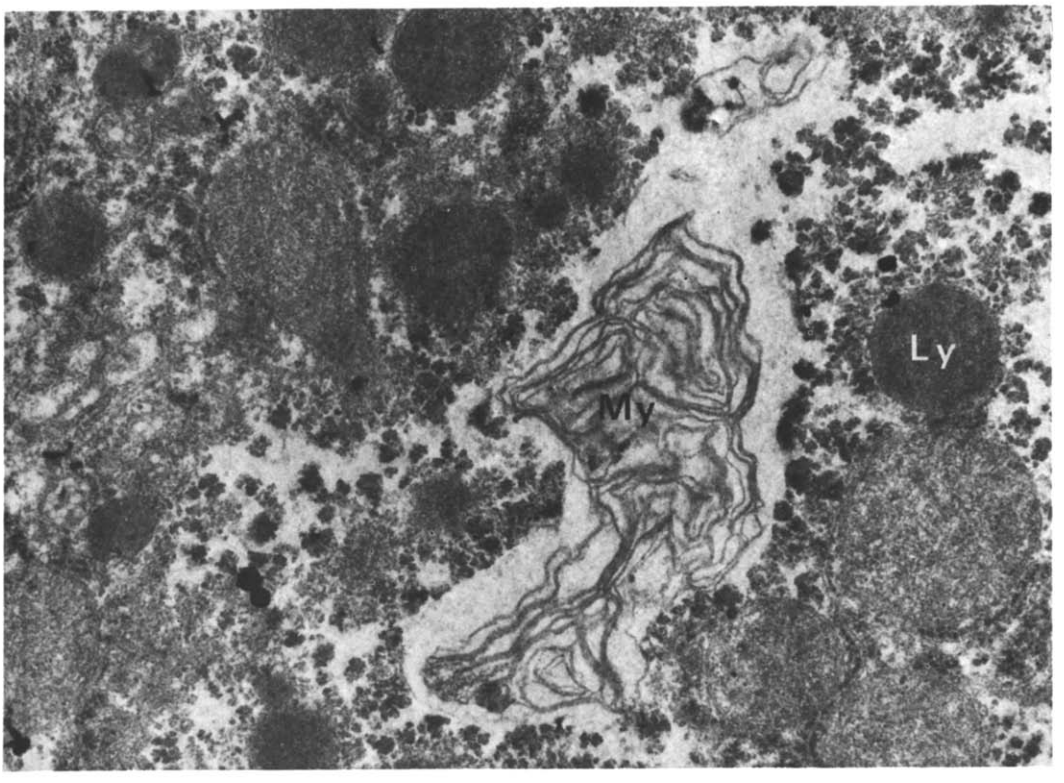


Fig. 6. Electron microphotograph of liver from a rat fed 20% casein diet containing DEHP at a level of 0.5% for 7 days (× 24,000).

increasing levels, up to 0.25% of DEHP, was observed when expressed in terms of mg/whole liver.

*Experiment* 3. Feeding DEHP caused an increase in the concentration of hepatic PL irrespective of the dietary levels of casein, as shown in Fig. 2. The extent of the increase, however, was much greater on a low protein diet. In contrast, the concentration of triglycerides (TG) decreased markedly. The increase in the concentration of PL is attributed to the increase in both PE and PC, in agreement with previous observations [14].

*Experiment* 4. Feeding DEHP at the 0.25% level also caused an increase in the concentration of hepatic PL (Fig. 3), and this was not influenced by the types and levels of dietary fats. Hepatic TG again decreased, the extent of the decrease being much greater in rats fed fat-free or 5% coconut oil diets.

*Fatty acid composition of hepatic microsomal and mitochondrial PE and PC*

Effects of feeding DEHP on the composition of the major fatty acids of hepatic microsomal and mitochondrial PE and PC were investigated in experiment 1.

In rats fed DEHP, PE in the hepatic microsomal fraction contained, in terms of the percentage composition, significantly more arachidonic acid (15.9 vs 32.5) and stearic acid (23.8 vs 30.5) and less palmitic acid (30.3 vs 17.9), oleic acid (11.3 vs 6.1) and linoleic acid (5.4 vs 1.5) in comparison with the controls. On the other hand, PC contained more palmitic acid (23.3 vs 30.4) and oleic acid (8.9 vs 15.7) and less stearic acid (21.5 vs 14.1), arachidonic acid (27.8 vs 23.9) and docosahexaenoic acid (6.4 vs 4.0). Similar results were obtained in mitochondrial PE and PC in rats fed DEHP, although the changes were more moderate than those in the microsomal fraction.

*Effects of DEHP on hepatic mitochondrial swelling*

This was studied using mitochondria from the rats in experiment 1. The swelling pattern of hepatic mitochondria is shown in Fig. 4. The rate of the swelling at 25° was significantly slower in rats fed DEHP than in the controls.

*Electron microscopic examination*

Electron microscopic examination of rats in experiment 1 showed the existence of mitochondria with a condensed configuration and intracellular inclusions (myelin-like figure) in the livers of the treated rats (Figs. 5 and 6). No such abnormalities were observed in the control rats (figures not shown).

## DISCUSSION

The present study indicates that the accumulation of hepatic PL by feeding DEHP can be attributed to the increase in the concentration and content of PL in both mitochondrial and microsomal fractions. Although the concentration of protein increased in both fractions, the ratio of PL-phosphorus/protein was also elevated. These responses were much more marked with mitochondria.

The increase in the concentrations of PL in the mitochondrial and the microsomal fractions is attrib-

uted to the increases in PE and PC. On the other hand, the percentage of PE was increased significantly, whereas the percentage of PC remained unchanged or decreased slightly after feeding DEHP, resulting in a significant elevation of the PE/PC ratio in both fractions.

The properties of microsomal membranes may be regulated in part by PL components, as suggested by the role of PL in the functioning of hepatic microsomal drug-metabolizing systems [15]. However, only limited information is available as to drug-induced accumulation of hepatic PL. Yamamoto *et al.* [16] have reported that 4,4′-diethylaminoethoxyhexestrol and chloroquine induce hepatic phospholipidosis accompanied by an increase in lysobisphosphatidic acid and PI. Administration of phenobarbital or butyl-hydroxyl-toluene to rats causes an increase in hepatic microsomal PL, without influencing the percentage composition of each PL component, and secondarily changes the activities of drug-metabolizing enzymes [17]. The increase in hepatic microsomal PL by feeding DEHP might be an adaptive response in order to metabolize the DEHP ingested. Lake *et al.* [18] have reported an increase in drug-metabolizing enzyme activities in rats following administration of DEHP.

Although the exact mechanism leading to the accumulation of PL in the hepatic mitochondrial fraction, due to the feeding of DEHP, is not clear, the quantitative and qualitative changes of mitochondrial PL may be relevant to the function of this organelle, as can be presumed by the regression of the swelling properties.

By feeding DEHP, characteristic alterations of fatty acid composition in hepatic microsomal and mitochondrial PC and PE were observed. Since PL are integral components of the mitochondrial membrane, alteration in concentration and fatty acid composition of PL may influence the biological behavior of mitochondria, although our data do not necessarily represent changes in membrane composition alone. The swelling phenomenon has been known to be a measure for analyzing integrity of the mitochondrial membrane [19]. DEHP appears to depress the swelling markedly.

Since the concentration of plasma PL in rats fed DEHP was slightly lower than that of the controls (232 vs 205 mg/dl, experiment 1), in spite of the considerable accumulation of hepatic PL, the suppressed transport of PL from liver to blood stream may have been partly responsible for the accumulation of hepatic PL. An increase [3] or decrease [2] in hepatic lipid synthesis by feeding of DEHP has been reported. Because of these controversies, the mechanisms causing PL accumulation awaits further work.

The increase in hepatic PL by feeding DEHP was observed independently of the levels of dietary protein and dietary fat. However, the extent of the increase in the concentration of hepatic PL was much greater on a low protein diet. The extent of the decrease in the concentration of hepatic TG was much greater in rats fed diets containing no fat or 5% coconut oil. Thus, the lower the dietary supply of essential fatty acids, the greater the decrease in hepatic TG.

The present study is an extension of previous work [1], and confirms that feeding of DEHP affects, quantitatively and qualitatively, hepatic mitochondrial and microsomal PL in rats. The data emphasize not only the need for additional study concerning the function of hepatic subcellular fractions in rats fed other types of phthalate esters, but also the possibility that the nutritional status of the animals may influence the response to DEHP.

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