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ACCUMULATION OF HEPATIC PHOSPHOLIPIDS IN RATS FED DI-2-ETHYLHEXYL PHTHALATE

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Abstract—Feeding normal or low protein diets containing di-2-ethylhexyl phthalate (DEHP), a plasticizer commonly used, at a 0.5 per cent level to young rats for 10 days resulted in an enlargement of liver and a significant increase in hepatic phospholipids (PL) compared with those of control rats. The percentage of phosphatidylethanolamine (PE) in rats fed DEHP was increased significantly whereas that of phosphatidylcholine (PC) was decreased. Time–course studies with a low protein diet showed that the increase in hepatic PL was already detected 1 day after feeding DEHP. Hepatic triglycerides (TG) were decreased in rats fed DEHP. In hepatic PE, the percentage of stearic and arachidonic acids was increased at the expense of palmitic and docosahexaenoic acids. The percentage of major fatty acids of hepatic PC and TG was also altered significantly. The content of liver glycogen was decreased and that of liver protein was increased by feeding DEHP. In plasma of rats fed DEHP at a normal protein level for 10 days, the concentrations of PL, TG and cholesterol were decreased. In a low protein diet, however, there were no remarkable changes in the concentrations of these lipids. The concentration of plasma free fatty acids was increased by feeding DEHP.

Phthalic acid esters (PAE) are widely used as plasticizers, particularly in polyvinylchloride-type plastics, and may account for as much as 40 per cent of the weight of plastic products. Because these plasticizers are interspersed throughout the polymer matrix, they can migrate, under various conditions, from the plastics [1, 2]. Thus, PAE became one of the notable contaminants of environmental and biological systems. The existence of PAE in water [3, 4], air [1], milk [5], fat and oil [6] and animal tissues [7] has already been reported.

In general, PAE have a low order of acute toxicity [2]. The rate of excretion of PAE from the tissue or body varies with the type of PAE [1]. There are few reports of chronic toxicity studies or of PAE effects on the biological systems.

It seems likely that PAE influence lipid metabolism in animals because of their hydrophobic properties. Bell and Nazir [8] have shown that incorporation of [^{14}C]acetate into total lipids by liver slices from rats fed DEHP* at the 0.5 or 1.0 per cent level decreases to 50 per cent of the control values, and that the decreased incorporation is not attributable to any one lipid fraction. In contrast, Arikaki and Ariyoshi [9] were not able to demonstrate any changes in the synthesis of hepatic lipids of rats fed DBP, which is one of the representative PAE. Our preliminary experiments, however, revealed that, in female and male mice, hepatic PL were increased significantly by the administration of DEHP or DBP.

In the present paper, effects of DEHP, which is the most commonly used plasticizer, on liver and plasma lipid components of rats were investigated in detail.

EXPERIMENTAL

Animal experiments

Male rats of the Wistar strain were housed individually in metabolic cages in a room maintained at 22–24° and reared on a commercial pellet ration (type NMF, Oriental Yeast Co., Ltd, Tokyo, Japan).

Experiment 1. Rats weighing 92 g were fed a 20% casein diet *ad lib.* for 10 days. DEHP was added at the 0.5 per cent level.

Experiment 2. Rats weighing 80 g were fed a 10% casein diet *ad lib.* for 10 days. DEHP was added at the 0.5 or 1.0 per cent level.

Experiment 3. Rats maintained on a 10% casein diet for 5 days and weighing 95 g were fed the low protein diet *ad lib.* for 1, 3 and 6 days. DEHP was added at the 1.0 per cent level.

Basal (control) diets contained (in per cent): casein, 10 or 20; corn oil, 5; mineral mixture, 4; vitamin mixture, 1; cellulose powder, 2; choline chloride, 0.15; and sucrose to 100. Mineral and vitamin mixtures according to Harper [10] were purchased from Oriental Yeast Co., Ltd.

DEHP was the product of Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and the purity was checked by gas–liquid chromatography (g.l.c.) with an electron capture detector using a 2 m × 3 mm glass column (internal diameter) packed with 2% OV-1 on Chromosorb W, 80–100 mesh. The temperature of the column was 200° and that of the detector was 250°. The nitrogen flow-rate was 40 ml/min. Only one peak was detected.

* Abbreviations used in the text are as follows: DEHP, di-2-ethylhexyl phthalate; DBP, di-*n*-butyl phthalate; PL, phospholipids; TG, triglycerides; PC, phosphatidylcholine; PE, phosphatidylethanolamine, and PAE, phthalic acid esters.

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

Groups†		Body wt		Food intake	Liver wt	Kidney wt
		Initial (g)	Gain (g/10 days)	(g/day)	(g/100 g body wt)	
Experiment 1						
Normal protein	Control	92 ± 2	66 ± 3	15.2 ± 0.4	5.6 ± 0.1	1.24 ± 0.05
	DEHP 0.5%	92 ± 2	64 ± 3	14.6 ± 0.2	8.4 ± 0.2‡	1.35 ± 0.06
Experiment 2						
Low protein	Control	80 ± 1	22 ± 2	11.3 ± 0.9	5.8 ± 0.1	1.17 ± 0.04
	DEHP 0.5%	79 ± 2	17 ± 3	10.2 ± 0.8	6.6 ± 0.1‡	1.26 ± 0.02
	DEHP 1.0%	80 ± 1	13 ± 3‡	9.4 ± 0.8§	7.2 ± 0.1‡	1.36 ± 0.04

* Values are the means ± S. E. of six rats.
† Rats were fed normal protein (20% casein) diet in experiment 1 or low protein (10% casein) diet in experiment 2 with or without (control) addition of DEHP for 10 days.
‡ Difference from the control is significant at $P < 0.01$.
§ Difference from the control is significant at $P < 0.05$.

Analytical procedure

The animals had free access to food until sacrifice by decapitation at 10.00 to 10.30 a.m. Liver and plasma lipids were extracted and purified by the procedure of Folch *et al.* [11]. Lipid phosphorus, cholesterol, triglycerides and free fatty acids were determined by the methods of Gomori [12], Sperry and Webb [13], Fletcher [14], and Noma *et al.* [15] respectively. Fractionation of lipids by thin-layer chromatography (t.l.c.) and determination of fatty acid composition by g.l.c. were performed as described elsewhere [16, 17]. PL were fractionated by t.l.c. according to the method of Mangold [18] and lipid phosphorus was determined by the method of Rouser *et al.* [19]. Liver glycogen and protein were determined by the methods of Seifer *et al.* [20] and Gornall *et al.* [21] respectively. Plasma glucose was determined by the Somogyi–Nelson procedure [22].

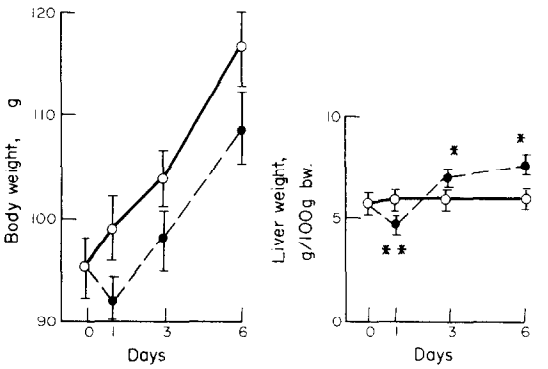

Fig. 1. Effects of DEHP on body weight and liver weight (experiment 3). Rats were fed a low protein (10% casein) diet with or without (control) addition of 1.0% DEHP. Key: control rat, ○——○; DEHP-fed rat, ●- - -●. Values are the means ± S.E. of five rats. Difference from the control is significant at $P < 0.01$ (single asterisk) and < 0.05 (double asterisk) respectively.

RESULTS

Weight gain, food intake and liver and kidney weight

Experiments 1 and 2. The results are summarized in Table 1. In rats fed normal or low protein diets containing 0.5% DEHP for 10 days, there were no significant differences in weight gain, food intake and kidney weight compared with those of the controls, whereas liver weight was increased significantly. Based on an average food intake, the daily consumption of DEHP was estimated to be one-fiftieth the amount of acute oral LD_{50}, which is ca. 30 g/kg.

At the low protein level, feeding the animals 1.0% DEHP resulted in decreases in weight gain and food intake, and both liver and kidney weights were increased significantly.

Experiment 3. As shown in Fig. 1, 1 day after the rats were fed a low protein diet containing 1.0%

Table 2. Effects of DEHP on liver lipids, protein and glycogen*

Groups†		Phospholipids	Triglycerides	Cholesterol (mg/g liver)	Protein	Glycogen
Experiment 1						
Normal protein	Control	25.9 ± 0.7	12.4 ± 1.6		156 ± 6	56 ± 2
	DEHP 0.5%	34.0 ± 1.0†	9.9 ± 0.7		171 ± 6	33 ± 4‡
Experiment 2						
Low protein	Control	19.2 ± 0.5	14.5 ± 0.2	2.3 ± 0.03	133 ± 6	104 ± 7
	DEHP 0.5%	28.6 ± 0.7‡	9.4 ± 0.4‡	2.3 ± 0.06	169 ± 4‡	48 ± 4‡
	DEHP 1.0%	31.2 ± 0.4‡	9.4 ± 0.9‡	2.4 ± 0.05	180 ± 10‡	36 ± 3‡

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

Table 3. Effects of DEHP on hepatic phospholipid composition*

Groups†		Lysolecithin	Sphingomyelin	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidic acid	PE‡/PC (%)
		(per cent of total phospholipids)					
Normal protein	Control	4.6 ± 0.4	10.2 ± 1.3	58.6 ± 1.1	20.6 ± 0.4	6.1 ± 0.2	35.2
	DEHP	4.0 ± 0.3	12.3 ± 0.3	49.6 ± 0.6§	26.1 ± 0.8§	8.0 ± 0.3§	52.6

* Liver phospholipids were separated by thin-layer chromatography and the content of phospholipid in each fraction was determined. Values are the means ± S. E. of six rats.
† See Table 1.
‡ PE = phosphatidylethanolamine; PC = phosphatidylcholine.
§ Difference from the control is significant at $P < 0.01$.

DEHP, there were decreases in body and liver weights. However, after 3 and 6 days, liver weight was significantly increased.

Liver lipid components, protein and glycogen

Experiments 1 and 2. By feeding the rats normal or low protein diets containing DEHP, the concentration of hepatic PL was significantly increased and that of TG decreased (Table 2). The content of hepatic protein was increased and that of glycogen decreased.

In rats fed a normal protein diet containing 0.5% DEHP, the percentage of PE and phosphatidic acid fraction was increased by approximately 30 per cent, respectively, whereas that of PC was decreased by 15 per cent. Thus, the ratio of PE/PC was elevated after feeding DEHP (Table 3).

Experiment 3. Significant increases in hepatic PL were already observed on days 1 and 3 after feeding DEHP (Fig. 2). The concentration of TG was decreased 1 day after feeding DEHP and the difference between two groups became more apparent after 3 days. There was no difference in the concentration of cholesterol between control and DEHP groups.

The percentage of PC was decreased significantly 1 and 3 days after feeding DEHP, compared with control values [48.3 ± 1.00% vs 51.4 ± 0.47 per cent, ($P < 0.05$) and 47.9 ± 1.21 vs 51.4 ± 0.08 per cent, ($P < 0.01$), respectively], but the difference was not apparent on day 6. The percentage of PE was increased significantly 6 days after feeding DEHP [34.3 ± 0.10 vs 27.2 ± 0.47 per cent ($P < 0.01$)].

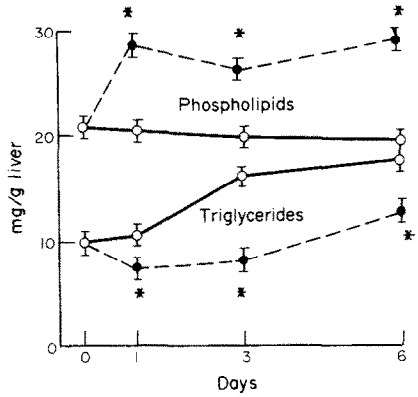

Fig. 2. Effects of DEHP on hepatic phospholipids and triglycerides (experiment 3). Key: control rat, O——O; DEHP-fed rat, ●- - -●. See also Fig. 1. Values are the means ± S. E. of five rats. An asterisk indicates that difference from the control is significant at $P < 0.01$.

Plasma lipids and glucose

Experiments 1 and 2. In plasma of rats fed a normal protein diet containing DEHP, the concentrations of PL, TG and cholesterol were decreased significantly compared with that of the controls, whereas that of plasma free fatty acids tended to increase (Table 4).

Table 4. Effects of DEHP on plasma lipids and glucose*

Groups†		Phospholipids	Triglycerides (mg/dl)	Cholesterol	Free fatty acids (μEquiv/dl)	Glucose (mg/dl)
Experiment 1						
Normal protein	Control	223 ± 9	129 ± 9	106 ± 5	44 ± 3	
	DEHP 0.5%	184 ± 9‡	93 ± 5§	92 ± 4‡	66 ± 8‡	
Experiment 2						
Low protein	Control	182 ± 18	140 ± 15	87 ± 10	71 ± 5	128 ± 10
	DEHP 0.5%	212 ± 20	129 ± 5	87 ± 5	84 ± 5	101 ± 7
	DEHP 1.0%	207 ± 20	119 ± 17	86 ± 9	100 ± 3§	87 ± 3§

* 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 $P < 0.01$.

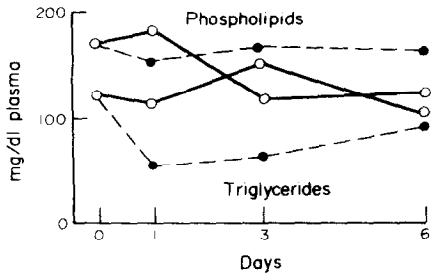

Fig. 3. Effects of DEHP on plasma phospholipids and triglycerides (experiment 3). Key: control rat, O——O; DEHP-fed rat, ●- - -●. See also Fig. 1. Values are from pooled plasma of five rats/group.

On a low protein diet, DEHP caused a response similar to that in the rats on a normal protein diet, but the change was not statistically significant. The level of cholesterol was unaltered. In contrast, feeding DEHP caused an increase in plasma free fatty acids and a decrease in glucose.

Experiment 3. As shown in Fig. 3, plasma PL in rats fed DEHP were initially decreased slightly, but increased markedly after 3 and 6 days. Plasma TG appeared to decrease immediately after feeding DEHP. There was no effect of DEHP on the cholesterol content.

Fatty acid composition of liver and plasma lipids (experiment 1)

Table 5 shows the fatty acid composition of hepatic PE, PC and TG in rats fed DEHP or control diets.

In hepatic PC, feeding DEHP at a 0.5 per cent level induced an increase in the percentage of stearic and arachidonic acids whereas the percentage of palmitic and docosahexaenoic acids decreased. In hepatic PC, an increase in the percentage of oleic acid was demonstrated after feeding 0.5% DEHP. In hepatic TG, the percentage of oleic acid increased and this was balanced by a decrease in linoleic acid.

In plasma PL and TG, the percentage of oleic acid was increased whereas that of linoleic acid was decreased (Table 6).

DISCUSSION

The present study indicates that addition of DEHP to the normal or low protein diet induced a marked enlargement of liver and significant changes in hepatic components of rats. In liver, significant accumulation of PL—hepatic phospholipidosis—was apparent (Table 2), and this phenomenon was already detectable 1 day after feeding DEHP (Fig. 1). The increase in liver weight in experiments 1 and 2

Table 5. Effects of DEHP on the fatty acid composition of hepatic lipids (Experiment 1)*

	Fatty acids (%)						
Groups†	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	20 : 4	22 : 6
Phosphatidylethanolamine							
Control	17.5 ± 0.4	1.0 ± 0.1	22.4 ± 0.4	6.1 ± 0.3	3.6 ± 0.3	30.0 ± 0.6	17.2 ± 1.6
DEHP	13.1 ± 0.7‡	0.9 ± 0.2	27.9 ± 0.5‡	6.1 ± 0.3	3.1 ± 0.2	39.3 ± 0.8‡	8.9 ± 0.4‡
Phosphatidylcholine							
Control	26.6 ± 1.6	1.6 ± 0.2	21.4 ± 1.0	7.2 ± 0.3	7.9 ± 0.4	29.4 ± 1.3	4.4 ± 0.5
DEHP	24.1 ± 0.8	1.1 ± 0.1	19.8 ± 0.8	11.1 ± 0.8‡	7.9 ± 0.6	32.0 ± 0.9	3.4 ± 0.1
Triglycerides							
Control	30.5 ± 1.6	8.0 ± 0.8	2.9 ± 0.3	40.1 ± 2.0	14.9 ± 2.7	1.7 ± 0.7	0.6 ± 0.1
DEHP	30.0 ± 0.5	4.3 ± 0.2‡	3.2 ± 0.2	52.6 ± 1.5‡	4.9 ± 0.5‡	3.3 ± 1.1	0.3 ± 0.1

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

Table 6. Effects of DEHP on the fatty acid composition of plasma lipids (Experiment 1)*

	Fatty acids (%)						
Groups†	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	20 : 4	22 : 6
Phospholipids							
Control	29.2 ± 3.4	2.3 ± 0.6	23.6 ± 1.6	10.6 ± 0.6	18.1 ± 1.1	13.3 ± 1.7	2.2 ± 0.6
DEHP	33.2 ± 1.5	2.2 ± 0.2	20.4 ± 0.5	15.6 ± 1.1‡	15.7 ± 1.2	11.7 ± 2.8	1.0 ± 0.2
Triglycerides							
Control	25.2 ± 2.2	7.0 ± 1.0	3.1 ± 0.3	32.8 ± 1.1	27.3 ± 2.6	2.0 ± 0.5	
DEHP	27.3 ± 1.0	5.2 ± 0.4	3.7 ± 0.2	45.1 ± 2.3‡	15.7 ± 3.4§	1.5 ± 0.3	

* 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 $P < 0.05$.

was in agreement with toxicological studies of DEHP in rats. [23, 24].

In addition to these alterations, administration of DEHP caused a change in the pattern of hepatic PL components, characterized by an increase in the percentage of PE and the phosphatidic acid fraction, and by a decrease in the percentage of PC (Table 3). The concentration of hepatic TG was decreased by feeding DEHP. Again, a decrease in TG was detected 1 day after feeding DEHP. One can consider effects of fasting on this phenomenon when rats were fed a low protein diet containing DEHP. However, the hepatic phospholipidosis caused by administration of DEHP was also detected with a normal protein diet, in spite of the unaltered food intake compared with the control group.

Only limited information is available about drug-induced accumulation of hepatic PL. Administration of phenobarbital to rats causes an increase in hepatic PL, without influencing the percentage composition of each PL component [25]. Administration of ethionine does not change the content of hepatic PL [25], but the percentage of PE increases whereas that of PC decreases. Similar trends have been demonstrated in rats dosed with carbon tetrachloride [26].

Yamamoto *et al.* [27] have reported that 4,4′-diethylaminoethoxyhexestrol (4,4′-DH) induces a hepatic phospholipidosis which resembles the Niemann–Pick disease and increases lysobisphosphatidic acid and phosphatidylinositol. They have further shown [28] that administration of chloroquine to rats also produces a phenomenon similar to that observed with 4,4′-DH. However, 4,4′-DH or chloroquine causes an increase in hepatic cholesterol content and plasma lipid components simultaneously, so that this type of phospholipidosis appears to differ from that shown after administration of DEHP.

Administration of DEHP caused alteration in the composition of major fatty acids of hepatic glycerolipids (Table 5). As a consequence, the amount of linoleic acid in hepatic and plasma TG was decreased whereas that in hepatic PL was increased.

In plasma, the concentration of PL was altered by feeding DEHP. At the normal protein level, the change in the concentration of plasma PL did not reflect that of hepatic PL. At the low protein level, the change in plasma appeared to reflect that in liver. However, a time–course study showed that the concentration of plasma PL was lower than that of the control 1 day after feeding DEHP in spite of considerable accumulation of hepatic PL at that time. It seems likely that transport of PL from liver to the blood stream is suppressed by feeding DEHP when rats are fed a diet containing a normal level of protein, but not when fed a low protein diet. Differences in responses in plasma PL due to differences in feeding periods or dietary protein levels are obscure.

Bell and Nazir [8] have reported that incorporation of [^{14}C]acetate into total lipids by liver slices from adult rats fed a commercial chow containing 0.5 or 1.0% DEHP for 10 or 18 days decreases to 50 per cent of the control values and this is not attributable to any one lipid fraction. However, in that experiment liver slices in which PL might have already accumulated were used, so that it seems necessary to consider the possibility of a negative feedback control, if any, by pre-existing PL. A similar examination at an earlier stage of DEHP feeding would be required in order to confirm the reported data.

The possibility of a modification of lipid catabolism might also be feasible by DEHP (or a metabolite of DEHP) [24]. From our study, the mechanism by which DEHP alters liver lipid metabolism and the physiological importance of such alterations are not apparent.

Other observed changes were the increase in hepatic protein and the decrease in hepatic glycogen (Table 2). The increase in protein content may partly be attributable to an induction of mixed-function oxidase systems, mainly cytochrome p-450, by feeding DEHP. In fact, Arikaki and Ariyoshi [9] have reported an increase in drug-metabolizing enzyme activities by the administration of PAE. Plasma glucose was decreased, while plasma free fatty acids were increased after feeding DEHP. These modifications in circulating fuels may represent changes in the sources of energy supply.

The present study demonstrates for the first time that feeding DEHP to rats modifies the hepatic lipid components, characterized by an accumulation of PL. The relationship between the levels given in the present study and those encountered environmentally is not clear. However, the possible cumulative nature of the phthalate esters must be considered, since DEHP has been found to be as high as 27 mg/100 g dry wt in human tissue [29]. A series of experiments with phthalate has shown that rats fed DEHP at a 0.1 per cent level or rats fed DBP at a 0.5 per cent level also accumulate PL in the liver (T. Yamagita and N. Enomoto, unpublished observations). These studies emphasize the need for additional studies to evaluate the biological effect of phthalate esters.

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