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Effects of Phthalic Acid Esters on the Liver and Thyroid*

by Richard H. Hinton,† Fiona E. Mitchell,† Alan Mann,† Dawn Chescoe,† Shirley C. Price,† Alistair Nunn,† Paul Grasso,† and James W. Bridges†

The effects, over periods from 3 days to 9 months of administration, of diets containing di-2-ethylhexyl phthalate are very similar to those observed in rats administered diets containing hypolipidemic drugs such as clofibrate. Changes occur in a characteristic order commencing with alterations in the distribution of lipid within the liver, quickly followed by proliferation of hepatic peroxisomes and induction of the specialized P-450 isoenzyme(s) catalyzing ω oxidation of fatty acids. There follows a phase of mild liver damage indicated by induction of glucose-6-phosphatase activity and a loss of glycogen, eventually leading to the formation of enlarged lysosomes through autophagy and the accumulation of lipofuscin. Associated changes are found in the kidney and thyroid. The renal changes are limited to the proximal convoluted tubules and are generally similar to changes found in the liver. The effects on the thyroid are more marked. Although the levels of thyroxine in plasma fall to about half normal values, serum triiodothyronine remains close to normal values while the appearance of the thyroid varies, very marked hyperactivity being noted 7 days after commencement of treatment, this is less marked at 14 days, but even after 9 months treatment there is clear cut evidence for hyperactivity with colloid changes which indicate this has persisted for some time. Straight chain analogs of di-2-ethylhexyl phthalate, di-*n*-hexyl phthalate and di-*n*-oxtyl phthalate differ entirely in their short-term effects on the liver and kidney but have similar effects on the thyroid.

The short-term *in vivo* hepatic effects of the three phthalate esters can be reproduced in hepatocytes in tissue culture. All three phthalate esters, as well as clofibrate, have early marked effects on the metabolism of fatty acids in isolated hepatocytes. The nature of these changes is such as to increase storage of lipid in the liver. A hypothesis is presented to explain the progress from these initial metabolic effects to the final formation of liver tumors.

Introduction

The demonstration that a significant increase in liver cancer in rats and mice occurs after administration of diets containing excessive amounts of di(2-ethylhexyl) phthalate (*1*) has raised concern regarding its effects on human health. This result was not totally unexpected, as the short-term hepatic effects of DEHP which include hepatomegaly and proliferation of peroxisomes had already been reported to occur with certain hypolipidemic drugs (*2*) exemplified by clofibrate, which were known to be hepatocarcinogenic in rats (*2–4*).

Several surveys indicate that clofibrate, although administered to humans at up to 10% of the rat carcinogenic dose for up to 15 years, does not increase the incidence of liver cancer in humans (*5,6*) and that the short-term effects on human liver are markedly different from the effects on rats (*7,8*). Accordingly it has been argued that the group of nonmutagenic hepatocarcinogens, which cause proliferation of liver peroxisomes in rodents typified by clofibrate and including DEHP, does not pose any risk to humans.

As it is not possible to study the livers of humans deliberately exposed to various doses of environmental pollutants such as DEHP, it is necessary to use some indirect methods to test this assertion of safety to humans, for example by: determining which short-term changes in the liver of rats treated with DEHP are directly connected with the development of liver cancer; developing conditions for culturing rat liver hepatocytes in which these *in vivo* changes could be reproduced and; testing the response of human hepatocytes under similar conditions. A further independent question is whether the response of rats to DEHP is typical of their response to phthalate esters as a class.

Carpenter and his colleagues (*9*) reported in 1953 that hepatomegaly occurred in rats but not guinea pigs or dogs administered diets containing DEHP. There is, however, little change in the results of standard liver function tests in any species (*10*). Apart from the increase in size, the most noticeable change in the liver

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†Robens Institute of Industrial and Environmental Health and Safety, University of Surrey, Guildford, Surrey GU2 6NH, UK.

is a very marked increase in the number of peroxisomes in the hepatocytes of rats (*11–14*), although not of guinea pigs (*15*) or ferrets (*16*). Both rats (*11*) and ferrets *16*) show some dilation of the endoplasmic reticulum after treatment with DEHP but the main specific endoplasmic reticulum change in rats treated with DEHP, as in rats treated with clofibrate (*17*) is a very marked induction of the specific P-450 isoenzyme(s) catalyzing the ω-oxidation of fatty acids. It has been reported that in rat hepatocytes mitochondria are increased in number by DEHP but that there is no effect *in vivo* on mitochondrial respiratory activity. Lake et al. (*11*) also reported a reduction in succinate dehydrogenase activity; this would appear to be variable between strains because in our experiments the decrease was less pronounced and was not statistically significant (*14*). We did, however, observe a change in the appearance of mitochondria under the electron microscope. Various studies have been made of the effect of phthalate esters on mitochondria *in vitro*. These have not provided much useful toxicological information to date partly because the authors have often failed to consider that in the living animal the liver is exposed to the monoester and its metabolites rather than DEHP itself and partly because nonspecific damage can arise due to the fact that long-chain alkyl monoesters of phthalic acid are surface-active.

As well as producing morphological change in the liver, peroxisome proliferating agents produce changes in other organs and in plasma components. For example, in the kidney of rats treated with hypolipidemic drugs there are alterations in the cells of the proximal tubule similar to those observed in hepatocytes (*18*). Changes are also evident in the testes. DEHP also produces alterations in plasma components; in particular, there is a marked reduction in plasma triglyceride with a smaller change in plasma cholesterol (*19*). Additionally there may be slight changes in carbohydrate metabolism as indicated by alteration in plasma glucose (*19,20*).

In regard to the question of the comparability of the effects of DEHP and other phthalates much more data are available on the effects of DEHP on rats than on other phthalate esters. Several other phthalic acid esters have been reported to cause hepatomegaly *in vivo* (*20*). Gray et al. (*21*) have shown that a variety of phthalate esters other than DEHP can induce a small degree of peroxisome proliferation in hepatocytes in tissue culture. Diethyl and dibutyl phthalate also modify the action of drug-metabolizing enzymes (*22–24*) but not glucose-6-phosphatase activity (*24*). Dibutyl phthalate, like DEHP, is reported to lower plasma cholesterol and inhibit cholesterogenesis (*25,26*), while diethyl phthalate, like DEHP, lowers plasma triglyceride but unlike DEHP does not affect hepatic peroxisomes (*12*).

From the limited data available it can be deduced that there are marked differences in the various effects of phthalate esters on rat livers, but the literature does not provide sufficient evidence to deduce whether these were qualitative or merely quantitative differences.

Materials and Methods

Di(2-ethylhexyl) phthalate (DEHP), mono(2-ethylhexyl) phthalate (MEHP), di(*n*-octyl) phthalate (DnOP), mono(*n*-octyl) phthalate (MnOP), di(*n*-hexyl) phthalate (DnHP), and mono(*n*-hexyl) phthalate (MnHP) were donated by BP chemicals (Sully, Penarth, UK). The hypolipidemic drugs fenofibrate and clofibrate were kindly donated by Laboratories Fournier (Dijon, France) and ICI Pharmaceuticals (Alderley Edge, Cheshire, UK). Clofibric acid, chlorpromazine and fatty acid-poor fraction 5 bovine serum albumin were purchased from the Sigma Chemical Co. Ltd. (Poole Dorset, UK). Liebowitz L15 culture medium, fetal calf serum, and Kanamycin were purchased from Flow Laboratories Ltd. (Irvine, Scotland). Collagenase was purchased from the Boehringer Corporation (Lewes, Sussex, UK) and [1-^{14}C] palmitate (58.3 mCi/mmole) was purchased from Amersham International Ltd. (Amersham, Bucks, UK).

In Vivo Studies

Male and female Wistar albino rats were used, except for a single experiment in which the response of Wistar Albino rats and University of Surrey hooded rats to peroxisome proliferating agents was compared. The rats were obtained either from the University of Surrey Rodent Breeding Unit or from ICI Pharmaceuticals Division. Animals were weighed, inspected, divided at random into control and experimental groups, and transferred from Spratts pelleted diet I to Spratts powdered diet II-expanded (Spratts Ltd., Barking, Essex, UK). After a period of one week, which served to accustom the rats to powdered diets the animals were transferred to experimental diets as follows.

Experiment 1. Male Wistar albino rats of the University of Surrey strain weighing initially between 85 and 115 g were used in this experiment. The rats were divided into four groups which received control diet or diet containing 2% w/w of DEHP, DnHP, or DnOP corresponding approximately to 2000 mg/kg/day. Groups of six control rats and four rats from each experimental group were killed 3, 10, and 21 days after initial offering of the diet.

Experiment 2. Male and female Wistar albino rats of the ICI strain weighing initially about 200 g were used in this experiment. The rats were divided into four groups respectively which received control diet or diet containing 0.05%, 0.2%, or 1.0% w/w of DEHP corresponding approximately to 50, 200, and 1000 mg/kg/day. Groups of six control rats and four rats from each experimental group were killed 3, 7, 14, 28 days and 9 months after initial offering the diet.

Experiment 3. Male Wistar albino rats of the ICI strain aged approximately nine months and weighing 750–800 g were used in this experiment. The rats were divided into four groups which received respectively control diet or diets containing DEHP, clofibrate, or fenofibrate at concentrations designed to produce re-

spectively intakes of 1000, 400, and 200 mg/kg/body weight/day. The rats did not take as readily to the experimental diets as did younger rats. The diet consumption and compound intake for the two periods are presented in the Results section. Groups of four control rats and four rats from each experimental group were killed 3 and 13 days after initial offering of the compounds.

Experiment 4. Male Wistar albino rats of the University of Surrey strain were used in this experiment. The rats were divided into three groups which received respectively control diet or diets containing 0.4% w/w clofibrate or 1% w/w DEHP. Six control rats and six rats from each experimental group were killed 3, 10, and 21 days after initial offering of the diets.

Experiments 5, 6, and 7. University of Surrey Wistar albino rats were used in experiments 5 and 6, which respectively received control diet, diets containing clofibrate to ensure the intake of 400 mg/kg/day or fenofibrate to provide either 13 mg/kg/day, 60 mg/kg/day, or 200 mg/kg/day. Group of six control rats and four rats from each experimental group were killed, in experiment 5 after 24 hr and in experiment 6 after 3, 7, 14, 28 days, 8, 12, and 20 weeks, or 6, 9, 12, and 18 months. In experiment 7 groups of Wistar albino rats and hooded rats were administered diets containing 0.4% clofibrate or 0.2% fenofibrate corresponding to drug intake of 400 or 200 mg/kg/day. Groups of six control rats and four rats from each experimental group were killed 14 days after initial offering of the diet.

In Vitro Studies

Hepatocytes were isolated by an adaption of the method of Rao et al. (*27*). The cells were then either resuspended at a concentration of 2×10^6 cells/mL in bicarbonate-poor Hanks solution, pH 7.4, containing 1.23 mM $CaCl_2$ and used immediately, or, resuspended in Liebowitz L15 medium containing 10% fetal calf serum, 2mM glutamine, 10% tryptose–phosphate broth, 100 g/mL Kanamycin, 10^{-6}M insulin and 10^{-5}M hydrocortisone and seeded (2×10^6 cells in 4 mL medium) in 40 mL Falcon Primaria tissue culture flasks (Becton Dickinson and Co., Oxford). Cultures were incubated at 37°C. The medium was changed 2–4 hr after seeding and subsequently every 24 hr. Test compounds were dissolved in dimethylformamide. The volume added was such that the dimethylformamide concentration was never greater than 0.04% v/v. After incubation the cells were harvested into 1 mL of 0.25 M sucrose containing 5 mM EDTA and 5 mM Tris HCl, pH 7.4, and homogenized by sonication.

Preparation of Tissues; Chemical and Enzyme Determination

Preparation of tissue for light microscopy and details of the preparation of samples for electron microscopy are given elsewhere (*14*). Briefly, samples for light microscopy were fixed in phosphate buffered formalin and samples for electron microscopy were fixed in 4% glutaraldehyde, counterfixed in 2% osmic acid, embedded, cut and counterstained with lead and uranyl acetate as described previously.

Livers were homogenized in 0.25 M sucrose containing 5 mM Tris, pH 7.4, with a Potter-Elvejem homogenizer. The large particulate fraction (150,000*g*-min pellet) and the microsomal fractions were prepared as described by Mann et al. (*14*). Protein and enzyme estimation were carried out as described by Mann et al. (*14*) unless otherwise stated. DNA was estimated by the method of Burton (*28*) and cytochrome P-450 and b_5 by the method of Omura and Sato (*29*). Uricase (*30*), laurate hydroxylase (*31*) and ethoxycoumarin deethylase (*32*) were measured by standard methods. β-D-Galactosidase was measured as described by Hinton and Norris (*33*) and lactate dehydrogenase according to Bergmeyer (*34*).

Results

This paper summarizes a program of work carried out in our laboratories over the last four years, designed to elucidate the hepatic changes which lead to the induction of tumors in rats treated with DEHP and with other peroxisome-proliferating agents. Clearly, it is impossible to present all our results here in detail. We have, accordingly, presented only in summary the results of experiments where detailed descriptions will be available in papers either in the press or which have already been accepted for publication.

Experiment 1 (Comparison of Effects of Diets Containing DEHP and of the Equivalent Straight-Chain Esters of Phthalic Acid)

The aim of this experiment, described in detail elsewhere (*14*), was to determine whether the straight-chain phthalate esters DnOP and DnHP produced in rats hepatic changes similar to those caused by DEHP. The results, summarized in Table 1, show clearly that this is not the case. Treatment with DEHP caused a marked proliferation of peroxisomes, induction of ω-oxidation of fatty acids and a rapid accumulation of fat in small droplets apparently concentrated in the periportal zone. DnOP and DnHP, by contrast, did not induce peroxisome proliferation or ω-oxidation of fatty acids but did cause a slowly developing but very marked accumulation of fat in the centrilobular zone of the liver with evidence of fatty necrosis 21 days after commencement of treatment. In a separate experiment we found that the changes in rat liver caused by administration of diets containing 2% DnOP and DnHP are qualitatively similar to, although somewhat more severe than, those found in rats treated with diets containing 0.025% w/w chlorpromazine (*35,36*).

Table 1. Summary of morphological changes in the livers of rats administered diets containing 2% w/w DEHP, DnOP, or DnHP.

Effect	Treatment		
	DEHP	DnHP	DnOP
Hepatomegaly	+++[a]	+[b]	+[b]
General appearance of liver	Dark	Light, greasy	Light, greasy
Centrilobular loss of glycogen	+	+	++
Total glycogen loss after 21 days treatment	+	+[c]	−
Centrilobular necrosis	−	++	++
Periportal fat accumulation	+	−	−
Centrilobular fat accumulation	−	+	+
Peroxisome proliferation	+++	[b]+[b]	+[b]
Smooth endoplasmic proliferation	++	+	+
Loss of rough endoplasmic reticulum	+	+	+
Increased density of inner mitochondrial matrix	++	−	−
Initial burst of mitosis	++	−	−

[a] Degree of change seen when compared to age-matched controls.
[b] Visible after 21 days but not at earlier times.
[c] Lesion absent.

Experiments 2, 5, 6, and 7 (Dose- and Time-Response Studies on Young Rats Administered DEHP or Hypolipidemic Drugs)

The aim of these experiments was firstly to determine the dose dependence and the time course of the changes observed in the livers of rats treated with DEHP or with the hypolipidemic drugs clofibrate and fenofibrate, and secondly to determine whether there were significant differences between the responses of male and female rats. Detailed accounts of experiments 2 and 5 have been published elsewhere (*37,38*), and the results are summarized in Table 2. Study of the time course of the alterations showed that the changes could be divided into four groups: changes which occurred immediately after commencement of treatment but which then reversed to the control situation (stage 1), changes which were fully developed by 3 days and which were maintained through the whole period of diet administration (stage 2), changes which developed more slowly but which were fully established by 28 days after commencement of treatment (stage 3), and very slowly developing changes (stage 4). The parameters which fall into these groups are as follows.

Stage 1. Initially there was a very marked increase in the rate of incorporation of ^{3}H thymidine into DNA (Fig. 1A). In rats treated with 200 or 1000 mg/kg/day of DEHP the increase was most marked at 3 days, while in rats treated with 50 mg/kg/day the increase was most pronounced at 7 days. In each case, by 28 days the rate of ^{3}H thymidine incorporation had fallen to below control levels. The increase in incorporate of ^{3}H thymidine into DNA was due to synthesis preceding cell divisions rather than repair as the number of mitotic figures visible by light microscopy increased in parallel (Fig. 1A). There was also an increased incidence of myelin figures in bile canaliculi at 3 days but not at subsequent times, and this linked to a transient increase in bile flow.

Stage 2. The increase in the peroxisomal enzymes responsible for cyanide-insensitive palmitoyl CoA oxidation, particulate α-glycerophosphate dehydrogenase activity, and the P-450 isoenzyme catalyzing laurate ω-hydroxylation were all markedly induced after 3 days of treatment and showed little or no further change at subsequent times. Catalase showed a similar pattern as did the number of peroxisomes seen in male rats. In female rats, on the other hand, the number of peroxisomes did not appear to change greatly in spite of a very marked induction of palmitoyl CoA oxidation. This suggests that much of the increase in activity may have been due to microperoxisomes which are indistinguishable morphologically from endoplasmic reticulum elements unless stained for peroxidase activity.

Stage 3. The endoplasmic reticulum enzyme glucose-6-phosphatase showed a time- and dose-dependent fall in activity, minimum activities being reached at 14 days (Fig. 1C). A similar fall was found in rats treated with hypolipidemic drugs; in the latter experiments histochemical staining for glucose-6-phosphatase activity was performed. This showed that the loss was specifically from cells in the centrilobular zone. As is frequently the case centrilobular loss of glycogen paralleled the fall in glucose-6-phosphatase activity. In addition the P-450 isoenzymes responsible for ethoxycoumarin deethylase activity were not altered at 3, 7, and 14 days in male rats but were somewhat increased after 21 days. In female rats the increase was greater and was observed at both 14 and 28 days. Finally, in male rats a marked hypertrophy arose as assessed by counting nuclear and measuring the DNA content/gram tissue. This developed over the first 14 days. The effect was much less marked in female rats.

Stage 4. The lysosomal enzyme β-D-galactosidase showed no change in activity between 3 and 28 days of treatment but was markedly induced at 9 months (Fig. 1D). The increase in this enzyme activity was paralleled by an increase in the number and size of lysosomes and the formation of lipofuscin deposits. Experiments using hypolipidemic drugs show that the enlarged lysosomes, which have the same staining properties as the lipofuscin deposits, appear after about 8 weeks of treatment but that the lipofuscin deposits did not form until the animals have been receiving these compounds for 6 months.

Three further observations do not fit neatly into the pattern listed above. First, the percentage of catalase activity recovered in the large particulate fraction fell in rats treated with 1000 mg/kg/day of DEHP but rose in rats treated with 50 mg/kg/day. As discussed elsewhere (*38*), we believe this to be due to damage to the membranes of the peroxisomes. Second, the overall levels of cytochrome P-450 rose above control levels at 3

Table 2. Changes in liver enzymes and cellular constituents in male and female Wistar albino rats (ICI strain) treated with varying doses of di(2-ethylhexyl) phthalate or clofibrate.[a]

	DEHP						
	Male			Female			Clofibrate, 400 mg/kg/day
	50 mg/kg/day	200 mg/kg/day	1000 mg/kg/day	50 mg/kg/day	200 mg/kg/day	1000 mg/kg/day	
Body weight, g	102	98	93	101	92	91	101
Liver weight, g	113*	129*	155*	111	106	127*	151
Palmitoyl CoA oxidation	162*	257*	568*	137	205*	550*	> 300*[b]
α-Glycerophosphate dehydrogenase	215*	299*	336*	156	205*	310*	—
Catalase	100	109	122*	100	109	137*	162*
Uricase	94*	78*	89*	100	100	106	—
% Catalase sedimentable							
Glucose-6-phosphatase	91	77*	50*	80*	77*	60*	51*
Cytochrome P-450	110	120	135	111	102	117*	176*
Cytochrome b_5	130	115*	117	104	115	116	99
Laurate hydroxylase	220*	362*	479*	164*	193*	311*	795*
Ethoxycoumarin deethylase	102	114*	110*	148*	151*	193*	—
β-D-galactosidase	119	115	189*	126	133*	187*	216*
Nonprotein SH	125	87*	68*	68*	81*	88	66*

[a] For comparative purposes changes in these parameters in Wistar albino rats of the University of Surrey strain are shown. The results are presented as percentage of control values and are the mean of values obtained after treatment for 14 and 28 days with the exception of the value for β-D-galactosidase which is the change after treatment for 9 months. The results have been abstracted from our published work (*37,38*).

[b] Only determined after 24 hours and 18 months treatment.

*Significantly different from control ($p < 0.05$).

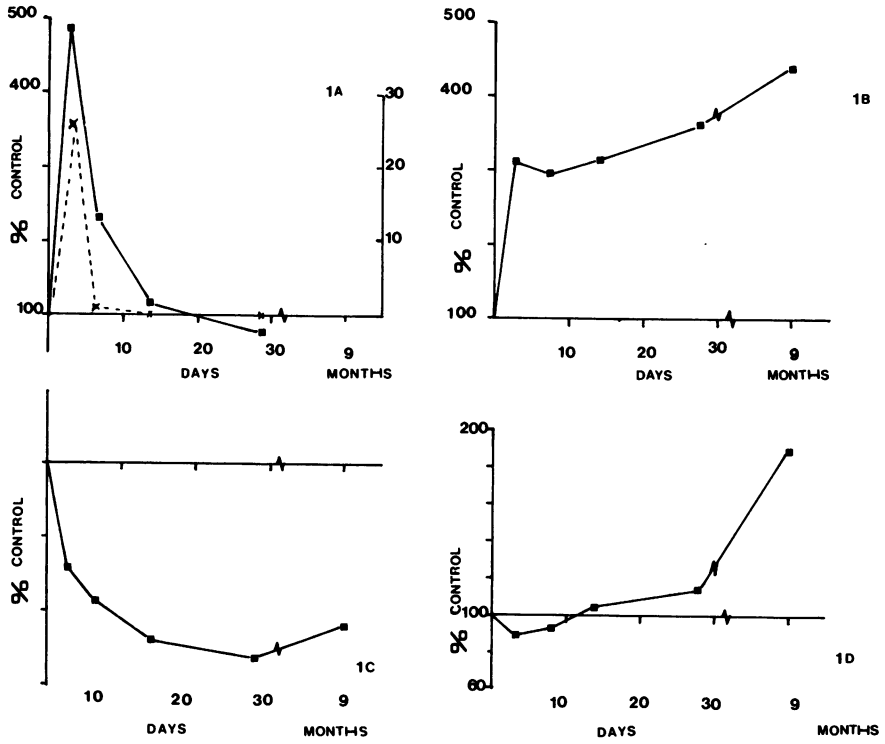


FIGURE 1. Time course of changes in rat liver induced by feeding diets adjusted to ensure an intake of 1000 mg/kg/day of DEHP: (*A*) Changes in (%—) incorporation of ^{3}H-thymidine into DNA and (—) the number of mitotic figures in the liver; (*B*) in CN^--insensitive (peroxisomal) β-oxidation in the liver; (*C*) in glucose-6-phosphatase activity in the liver; (*D*) in acid β-galactosidase activity in the liver.

and 7 days but fell back to below control levels at later times. We consider that the initial increase in total P-450 is due to the induction of the specific P-450 isoenzyme responsible for laurate hydroxylation and that the subsequent loss in activity is due to a loss in the P-450 isoenzymes arising from subtle damage to centrilobular hepatocytes. This is probably the cause of the loss of glucose-6-phosphatase activity and of glycogen. Third, there are marked changes in lipid distribution. In animals treated for three days with 50 mg/kg/day of DEHP there was an overall increase in fat. With increasing time or dose the fat clears from the vicinity of the central vein resulting in an apparent periportal accumulation. In experiments with hypolipidemic drugs a similar biphasic change was apparent and, in this case, was so great that at certain times and doses all the fat disappeared from the liver. We interpret these data as showing that DEHP and hypolipidemic agents initially induce the accumulation of lipid within hepatocytes, and it is the subsequent induction of peroxisomes, known to be concentrated in the centrilobular area (*39*), that is responsible for the clearance of the fat.

The conclusion from experiment 2 was that the changes in the livers of rats treated with DEHP do show a distinct time sequence. The findings also demonstrate that the changes which occur in rats treated with DEHP are identical to the changes which occur in the livers of rats treated with the hypolipidemic drugs clofibrate and fenofibrate (Table 2). Thus 1000 mg/kg/day of DEHP produced qualitative and quantitative changes similar to those found in rats treated with 400 mg/kg/day of clofibrate or 60 mg/kg/day of fenofibrate. Finally, the response of female rats to DEHP was for most parameters qualitatively similar to that of male rats but quantitatively slightly less. The exceptions are that although the female rats showed less hepatocellular hypertrophy and , accordingly, less hepatomegaly, there was greater induction of ethoxycoumarin deethylase activity in female rats than in males and that initially in female rats the induction of peroxisomal enzymes was not matched by an increase in the number of peroxisomes recognizable by electron microscopy without special staining.

The results of experiment 7, designed to examine possible differences in response between the Wistar albino and hooded rats, were assessed solely by examination of the gross pathology and by light and electron microscopy. No significant difference between the two strains was detected.

Experiment 3 (Examination of the Effects of DEHP or Hypolipidemic Drugs on Mature Rats)

This experiment was carried out to determine whether the response of fully mature male rats to treatments with DEHP or with hypolipidaemic drugs was the same as that of the adolescent animals normally employed in toxicological studies. The susceptibility of both rats (*40*) and humans (*41*) to hepatotoxins often varies with age and, indeed, age changes (and lowers) the ability of the rat liver to hydrolyze DEHP to MEHP (*42*), although the relevance of the last observation to rats administered DEHP in the diet must be open to doubt as in this case most hydrolysis occurs in the gut (*43,44*).

Mature animals, unlike the adolescent animals used in previous studies, did not take readily to the experimental diet. Food consumption was less than that of control rats resulting in a slight loss of body weight (Table 3). However, after 3 days there was a significant increase in relative liver weight in all groups of experimental rats (Table 3). Examination of sections of liver stained with hemotoxylin and eosin showed no significant difference between experimental and control rats after 3 days of treatment. However, after treatment for 13 days there was slightly increased centrilobular eosinophilia in all groups of rats as compared to the control groups. There was no increase in the number of mitotic figures in any treatment group. In all treated groups there was a marked glycogen loss principally from the centrilobular areas which was most notable after 13 days of treatment. The distribution of neutral fats also showed a clear dose response. After 3 days of treatment the animals showed a periportal accumulation of fat which became more marked after 13 days. The animals treated with fenofibrate showed an almost total loss of lipid from the liver lobule after treatment for 13 days.

Electron microscopic examination (Fig. 2) of the livers of rats treated with fenofibrate, clofibrate, and DEHP revealed changes after only 3 days of treatment. The degree of change was compound-related; fenofi-

Table 3. Effect of the administration of diets containing fenofibrate, clofibrate, or DEHP on food consumption, body weight, and liver weight of mature (9 month old) rats.

	Control	Fenofibrate	Clofibrate	DEHP
Food consumption, g consumed/rat/day				
0–3 days	21.46	20.56	10.00	15.40
3–13 days	24.61	16.80	11.46	16.50
Additive consumed, mg/kg/day[a]				
0–3 days	—	191	101	446
3–13 days	—	140	183	781
Body weight[b]				
3 days	771 ± 19	93 (3)	85 (3)*	97
13 days	775 ± 31	87	73	88
Absolute liver weight[c]				
3 days	20.06 ± 1.96	127	73	110
13 days	20.72 ± 1.22	106	74	111
Relative liver weight[d]				
3 days	0.026 ± 0.003	136*	85	114
13 days	0.029 ± 0.001	121*	101	126*

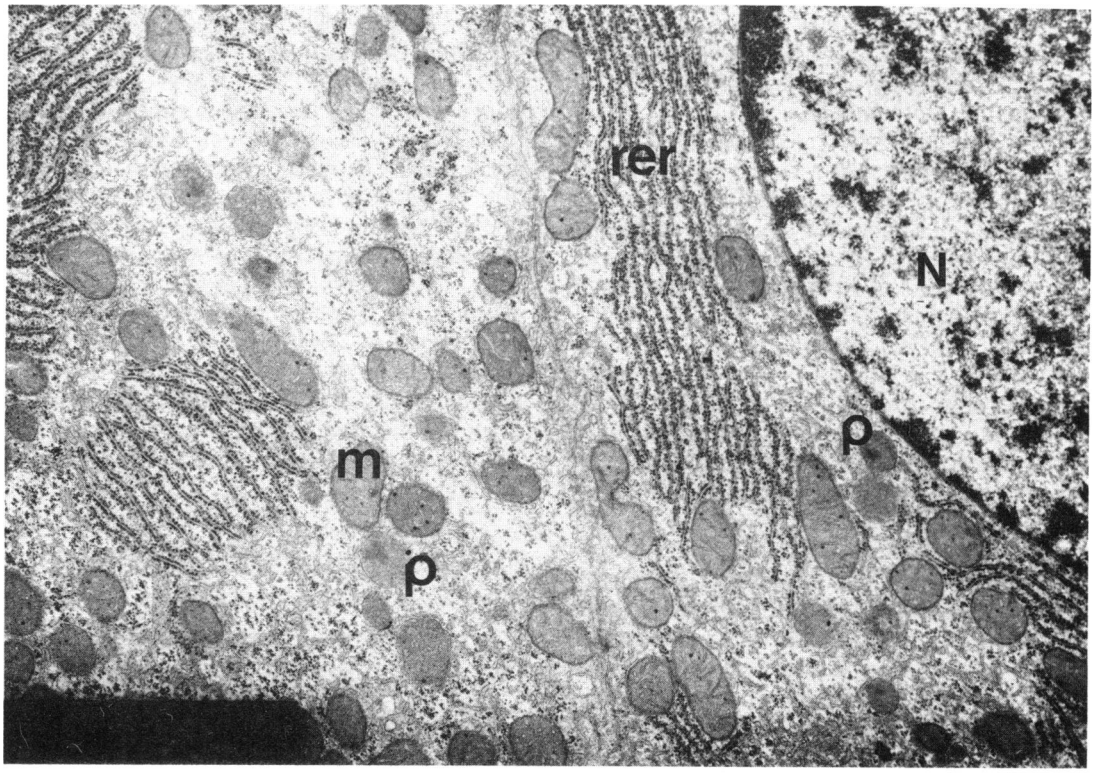
[a] Additive consumed is presented as mg additive consumed/kg body weight/day.

[b] Body weight is presented as weight(g) ± standard error for control group, the results from treated groups are presented as % control.

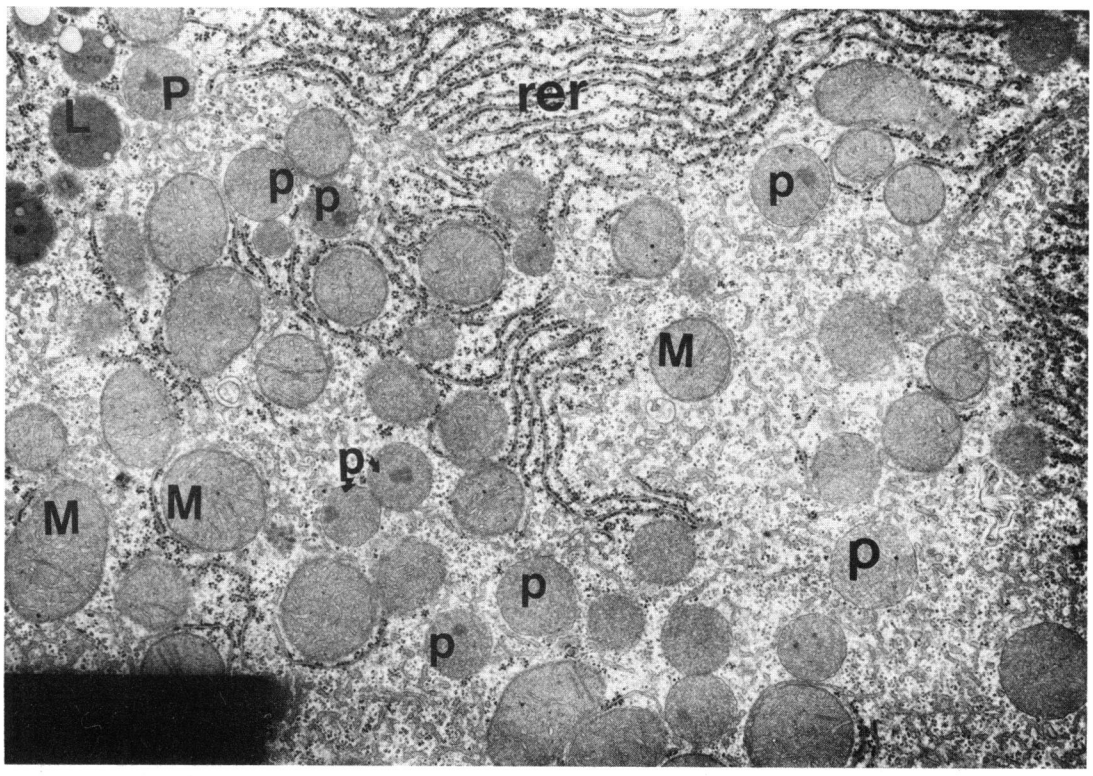
[c] Absolute liver weight is presented as weight (g) ± standard error, for control groups and as % control for treated groups.

[d] Relative liver weight is presented as the liver weight as a % of body weight ± standard error for control groups, and as % of control for treated groups.

* Significantly different from control.



a



b

FIGURE 2. Sections from livers of (*a*) 9-month-old control rat and (*b*) the liver of a 9-month-old control rat fed a diet containing sufficient DEHP to ensure an intake of approximately 600 mg/kg/day for 21 days. (N) nucleus; (M) mitochondria, (P) peroxisome, (L) lysosome and (rer) rough endoplasmic reticulum.

brate showed the greatest degree of alteration followed by clofibrate and DEHP. There was a marked increase of peroxisomes, and many of these peroxisomes lacked the "core" seen in peroxisomes of control animals. There were also changes in mitochondria consisting of increased density of the inner mitochondrial matrix and swelling with loss of internal structure. The treated animals showed proliferation of the smooth endoplasmic reticulum and disorganisation, dilation and degranulation of the rough endoplasmic reticulum. There was also an increase in the number of lipid droplets compared with controls with all agents. This was most marked in animals treated with DEHP. Examination of the livers of animals treated for 13 days with these hypolipidemic compounds showed that the effect found at 3 days all persisted with the exception that there were fewer lipid droplets in the treated animals than at the earlier time although there was still an increase as compared to control animals.

The peroxisome-associated enzyme, cyanide-insensitive palmitoyl CoA oxidase, was significantly induced after only 3 days of treatment with fenofibrate (Table 4). By 13 days all treatment groups showed a significant induction of this enzyme. Total catalase was significantly induced in animals treated with DEHP and fenofibrate, whereas with clofibrate the apparent increase in activity was not statistically significant. Changes in the distribution of catalase activity were less clear cut. The specific activity of catalase in the particulate fractions was decreased after 13 days of treatment in both groups treated with fenofibrate and clofibrate, although the decrease was only significant in rats treated with fenofibrate; rats treated with DEHP showed no apparent change.

Table 4. Effect of the administration of diets containing fenofibrate, clofibrate, or DEHP on liver enzymes of mature rats.[a]

Time	Control	Fenofibrate	Clofibrate	DEHP
Total catalase activity, units/mg homogenate/min				
3 days	1.53 ± 0.12	113	89	100
13 days	1.71 ± 0.10	128*	112	121*
KCN-insensitive PCoA oxidase activity, nmole NAD + reduced/min/kg protein				
3 days	0.60 ± 0.03	282	305*	121
13 days	0.54 ± 0.06	712*	256*	424*
Glucose-6-phosphatase activity, μmole/min/mg homogenate protein				
3 days	27.4 ± 2.4	77	102	83
13 days	31.4 ± 0.7	62*	92	67
Cytochrome P-450 activity, nmole/mg microsomal protein				
3 days	1.10 ± 0.03	118	85	118
13 days	0.70 ± 0.01	150	130	145
Laurate hydroxylase activity, units/mg microsomal protein				
3 days	2.79 ± 0.26	267*	132*	216*
13 days	4.70 ± 0.43	312*	292*	266*
β-D-Galactosidase activity, μmole/min/mg homogenate protein				
3 days	3.31 ± 0.49	87	97	87
13 days	3.78 ± 0.25	81	79	98

[a] The intake of the compounds is as shown in Table 3. Results from treated rats are presented as a percentage of control values. Control results are presented as mean ± standard error.

* Significantly different from control ($p < 0.05$).

Three endoplasmic reticulum-associated enzymes were measured. These were glucose-6-phosphatase, cytochrome P-450, and the specialized P-450 enzyme laurate hydroxylase. Glucose-6-phosphatase activity was depressed in all treatment groups after 13 days although the effect in animals given clofibrate was very small (Table 4). A biphasic change was observed with cytochrome P-450 in animals treated with fenofibrate or with DEHP, i.e., an initial increase after 3 days of treatment was followed by return to near control values after 13 days, although in animals treated with DEHP there appeared to be a slight fall in enzyme activity to below control value. The specific P-450 isoenzyme laurate hydroxylase was, however, induced in all treatment groups (Table 4). The activity of the lysosomal enzyme, β-D-galactosidase remained unaltered by treatment. This result was consistent with the electron microscopic results (Table 4).

Experiment 4 (Effects of DEHP and of Analogous Straight-Chain Esters of Phthalic Acid on the Thyroid)

In experiments with the hypolipidemic drugs fenofibrate and clofibrate we had previously observed a fall in serum thyroxine (T_4), although serum triiodothyronine (T_3) remained unaltered or even slightly increased and the histological alterations in the thyroids were indicative of hyperactivity (*45*). Examination of the thyroids of rats treated with DEHP for nine months showed similar changes. Accordingly serum samples collected in experiments 1 and 2 were reassayed, and an additional experiment performed to examine alterations in the thyroid after short periods of treatment and to compare the response to DEHP and to the hypolipidaemic drug clofibrate showed a fall in plasma T_4 after treatment with DEHP or its straight-chain analogs di(*n*-hexyl) phthalate and di(*n*-octyl) phthalate (DnOP) (Table 5) or with clofibrate (Table 6). The levels of T_3 in the plasma were, however, essentially unaffected by treatment with any of these phthalate esters.

Table 5. Effect of administration of diets containing DEHP (20,000 ppm), DnHP (20,000 ppm), or DnOP (20,000 ppm) on triiodothyronine and thyroxine levels in rat serum.

Time of treatment	Parameter	Control[a]	Experimental[b] DEHP	DnHP	DnOP
3 days	T_3	0.37 ± 0.07 (6)	89	62	73
	T_4	30 ± 6 (6)	66	53	47
10 days	T_3	0.33 ± 0.08 (6)	79	109	91
	T_4	27 ± 3 (6)	85	56	59
21 days	T_3	0.3 ± 0.05 (6)	140	183	133
	T_4	33 ± 5 (6)	64	58	76

[a] Control results are presented as mean (μg/L) ± standard error. Value in parentheses is number of animals examined.

[b] Experimental results are presented as a percentage of control values. Each experimental group consisted of four animals. Analysis of variance showed a significant, treatment related decrease in T_4 with all three phthalate esters but no significant effect on T_3.

Table 6. Effect of administration of diets containing DEHP (10,000 ppm) or clofibrate (4000 ppm) in triiodothyronine and thyroxine levels in rat serum.

Time of treatment	Parameter	Control[a]	Experimental DEHP[b]	Experimental Clofibrate[b]
7 days	T_3	0.37 ± 0.05 (6)	54	41*
	T_4	33.3 ± 0.5 (6)	58	36*
21 days	T_3	0.32 ± 0.03 (6)	119	125
	T_4	31 ± 2 (6)	55*	65*

[a] Control results are presented as mean (μg/L) ± standard error. Values in parentheses are number of animals examined.
[b] Experimental results are presented as a percentage of control values. Each experimental group consisted of six animals.
* Significantly different from control ($p < 0.05$).

Electron microscopic examination of the thyroids of rats treated with DEHP or DnOP showed marked ultrastructural changes (Fig. 3); similar changes were found in rats treated with DnHP. There was no significant difference between the effects of the different compounds. In all cases there was a considerable increase in the number and size of lysosomes. The Golgi apparatus was also enlarged, and the mitochondria appeared damaged. These alterations are very similar to those seen in rats after administration of TSH (*46*), polychlorinated biphenyls (*47,48*), or photomirex (*49*) and are indicative of hyperactivity of the thyroid.

Experiments on Isolated Hepatocytes

In agreement with the work of other groups (*50*), we found that treatment of cultured hepatocytes with MEHP resulted in induction of cyanide-insensitive palmitoyl CoA oxidation. Induction was clearly dose-dependent. Maximal induction was observed at a dose of 0.25 mM. In contrast, no systematic change in cyanide-insensitive palmitoyl CoA oxidation was seen in hepatocytes treated with MnHP or, in agreement with Gray et al. (*21*), in hepatocytes treated with MnOP. Hepatocytes treated with 0.25 mM MnHP or MnOP, unlike hepatocytes treated with 0.25 mM MEHP, showed signs of systematic toxicity such as blebbing and vacuolation although there was no increase in cell death as assessed by leakage of lactate dehydrogenase into the medium. Hepatocytes treated with the three phthalate esters showed increased amounts of lipid accumulation, this

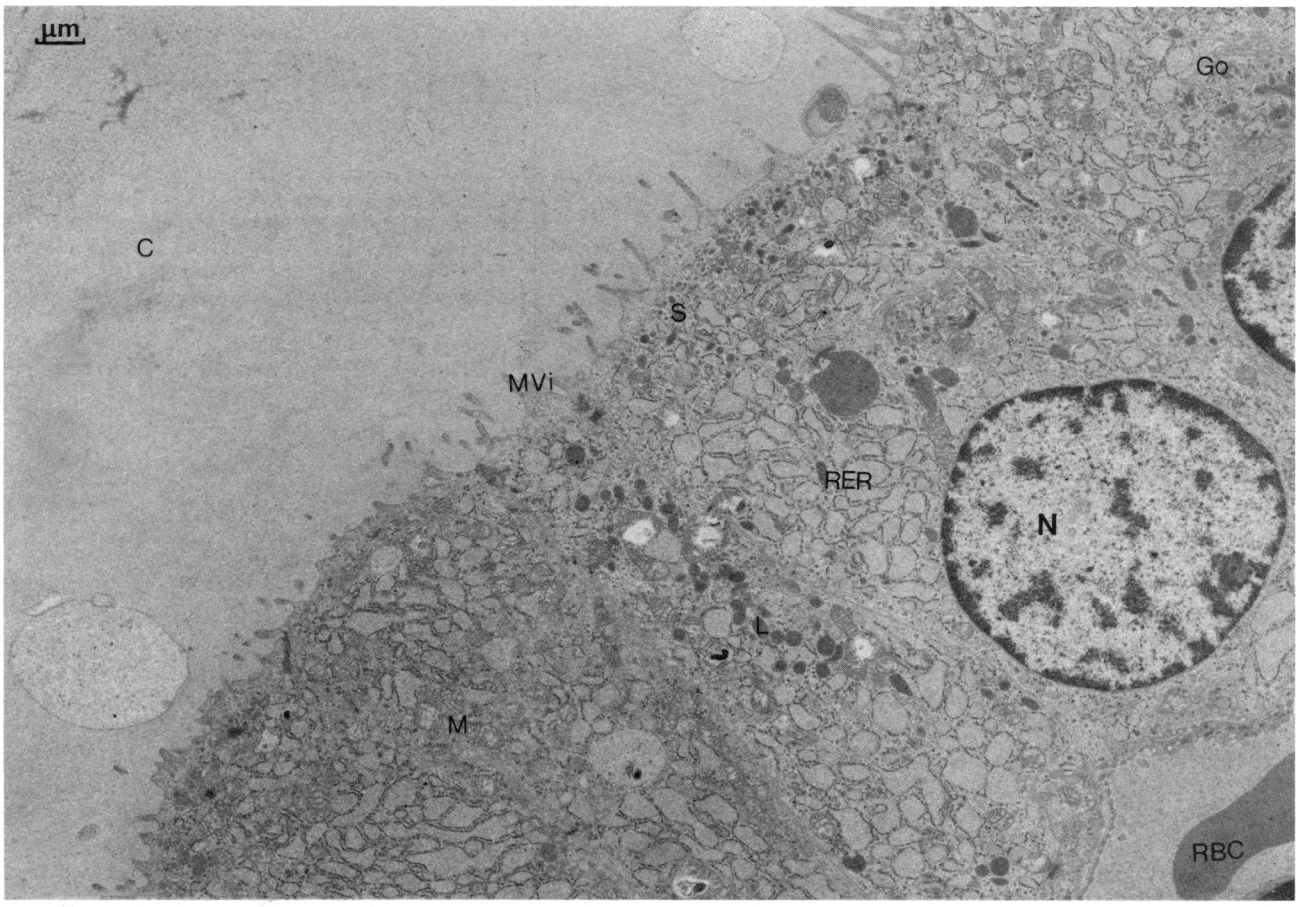


FIGURE 3*a*. Sections of the thyroids of a young (8-week-old) control rat. (N) nucleus; (Go) Golgi apparatus; (L) lysosome; (M) mitochondria; (V) villi; (RER) rough endoplasmic reticulum; (RBC) red blood cell.

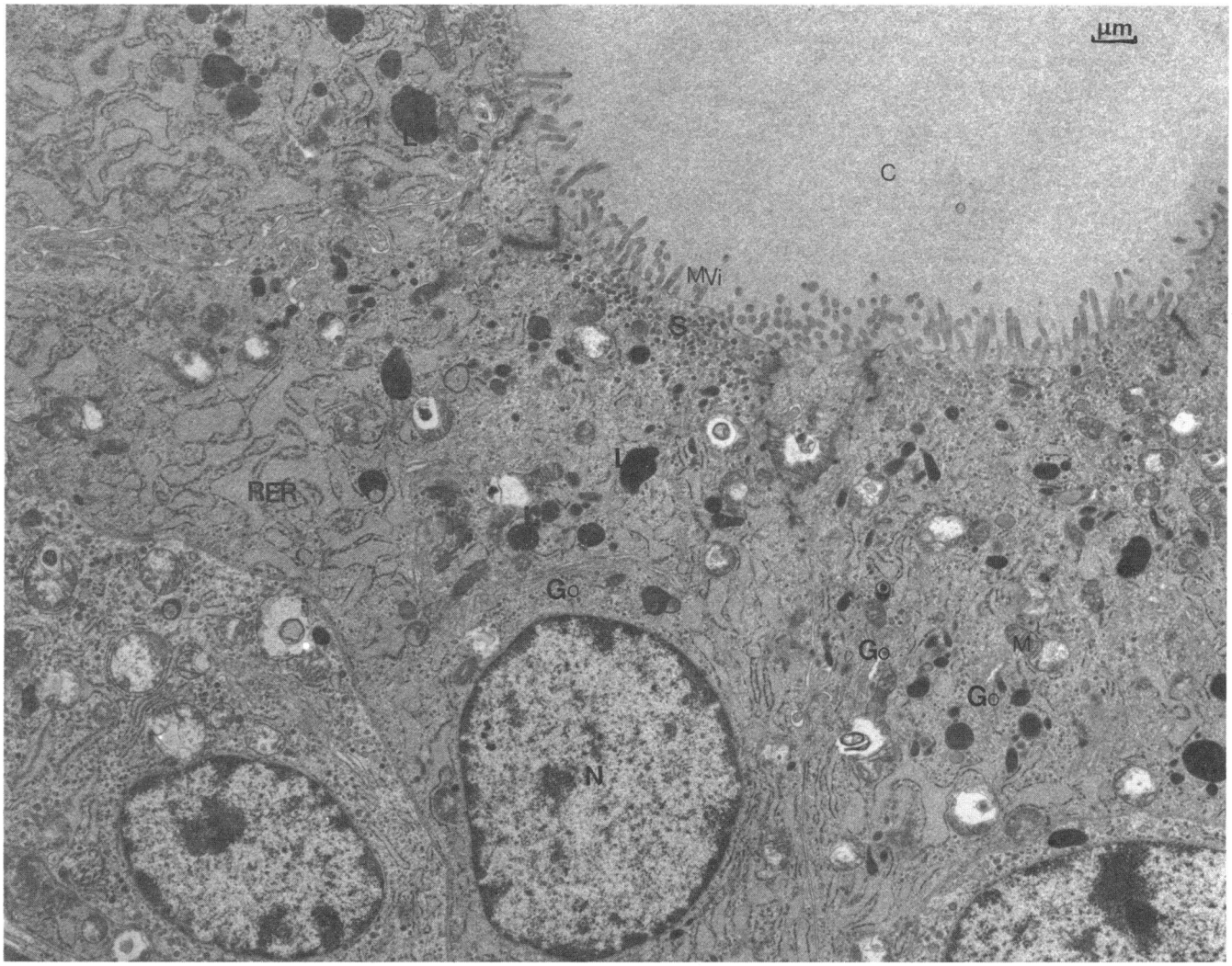


FIGURE 3b. Sections of the thyroids of a young rat administered a diet containing 20,000 ppm of DEHP for 21 days. (N) nucleus; (Go) Golgi apparatus; (L) lysosome; (M) mitochondria; (V) villi; (RER) rough endoplasmic reticulum; (RBC) red blood cell.

was seen as small droplets around the margins of the cell after treatment with MEHP, while cells treated with MnHP or MnOP showed, in addition, some larger some central droplets (*51*). No other signs of toxicity such as a decrease in glucose-6-phosphatase activity or glutathione nor any increase in H_2O_2 production were observed in hepatocytes treated with MEHP (Table 7).

In view of the rapid increase in the amount of lipid in hepatocytes after treatment *in vivo* or MEHP *in vitro*, we decided to investigate whether MEHP and its straight-chain analogs MnHP and MnOP interfered in fat metabolism in freshly isolated hepatocytes. The results of these experiments have already been published (*51,52*). In brief, we found that when hepatocytes were isolated from fasted rats or in the early afternoon from rats fed *ad libitum*, all three phthalic esters caused a rapid and marked increase both in the incorporation of 1-^{14}C-palmitate into triglyceride and cholesterol esters and an increase in fatty acid oxidation similar to that already reported in rats treated with clofibrate (*57*), whereas hepatocytes isolated from fed rats in the morning showed much smaller changes. In no case was there any increase in the rate of export of lipoproteins.

The results obtained in these experiments were compared with the results obtained in parallel experiments with the parent acids (clofibric acid and fenofibric acid) of the hypolipidemic drugs clofibrate and fenofibrate, with ciprofibric acid or with trichloracetic acid, the peroxisome-proliferating (*58*) metabolite of trichlorethylene. The results obtained (*59*) showed that fenofibric acid and ciprofibric acid interfered in lipid metabolism in the same way as did clofibrate and MEHP. However, although both fenofibrate acid and ciprofibrate acid are more potent peroxisome proliferators than clofibric acid or MEHP, the early effects on lipid metabolism were not proportionally greater. Trichloracetic acid had no significant effect, as might be expected from its structure.

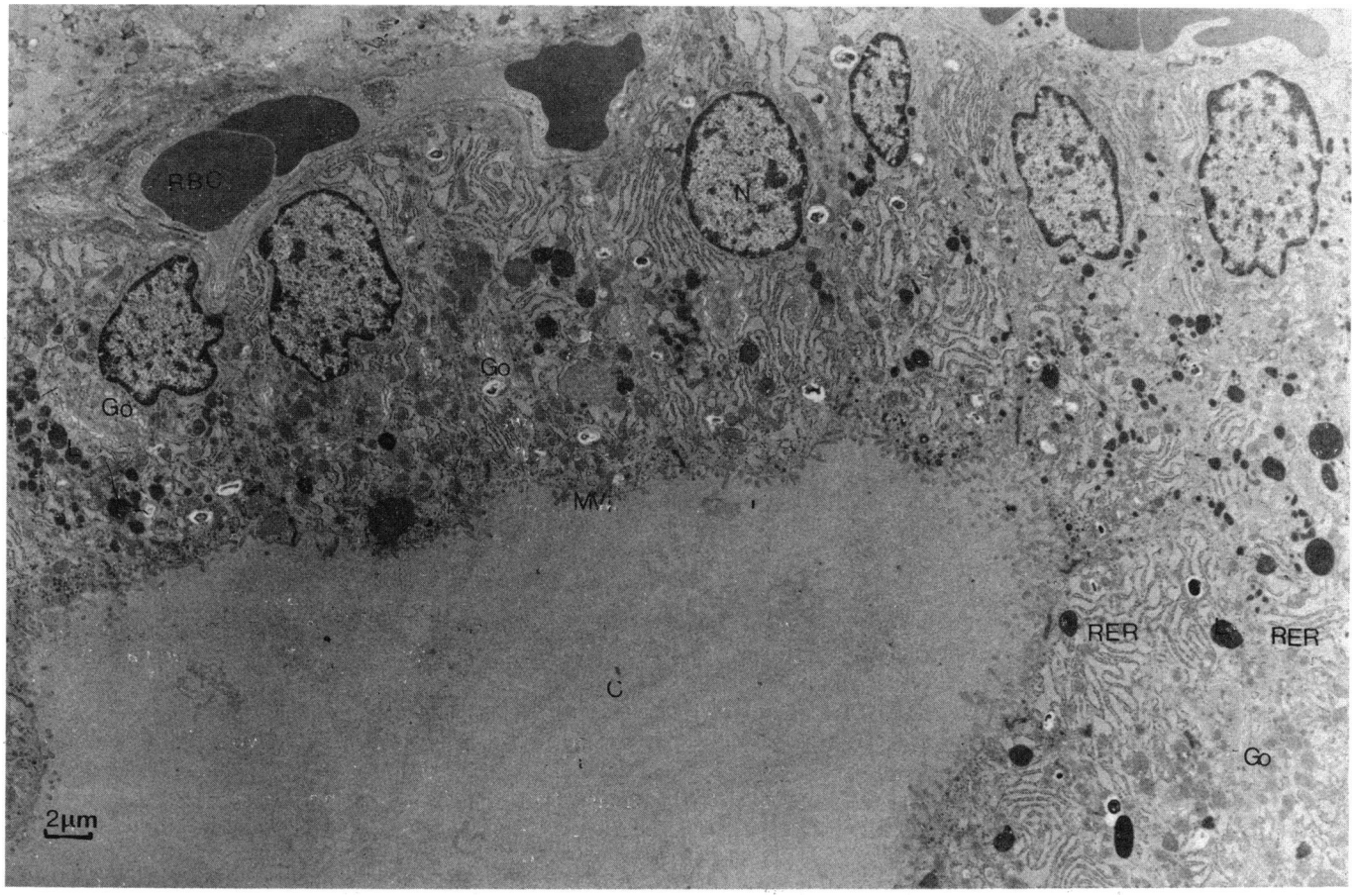


FIGURE 3c. Section of the thyroids of a young rat administered a diet containing 20,000 ppm of DnOP for 21 days. (N) nucleus; (Go) Golgi apparatus; (L) lysosome; (M) mitochondria; (V) villi; (RER) rough endoplasmic reticulum; (RBC) red blood cell.

Table 7. Effect of MEHP on palmitoyl CoA oxidation, H_2O_2 release, glutathione, glutathione peroxidase, glucose-6-phosphatase, and incorporation of ^{3}H-thymidine into DNA in cultured rat hepatocytes.[a]

Addition to medium	CN^--Independent palmitoyl CoA oxidation	H_2O_2, nmole/mg[b]	Glutathione, nmole/mg protein[d]	Glutathione peroxidase, units/mg[c]	Glucose-6-phosphatase, nmole/mg/min[e]	^{3}H-Thymidine incorporated, counts/min/mg protein[f]
Control	1.03 ± 0.03 (3)	14.8 ± 5.2 (4)	79.9 ± 9.6 (8)	0.30 ± 0.07 (4)	133 ± 9 (7)	60 ± 10 (4)
0.05 mM MEHP	2.90 ± 0.57 (4)*	15.0 ± 5.4 (7)	107.6 ± 18.9 (8)	0.27 ± 0.05 (4)	136 ± 24 (4)	68 ± 9 (3)
0.1 mM MEHP	5.85 ± 0.27 (4)*	16.2 ± 3.1 (8)	84.2 ± 11.6 (8)	0.31 ± 0.02 (4)	138 ± 6 (5)	51 ± 6 (4)
0.25 mM MEHP	10.27 ± 0.30 (4)*	13.3 ± 2.1 (8)	114.6 ± 16.4 (8)	0.32 ± 0.04 (4)	144 ± 6 (4)	51 ± 4 (4)

[a] MEHP was dissolved in dimethylformamide (DMF) and added to the hepatocytes to give a final concentration of 0.04% v/v. An equivalent volume of DMF was added to the control cultures. Details of the cell culture methods are as given elsewhere (*52*). Results are presented as mean ± standard error (number of flasks).

[b] Assayed according to Hildebrand et al. (*53*).

[c] Assayed according to Saville (*54*).

[d] Assayed according to Thompson et al. (*55*).

[e] Assayed according to Neville and Arion (*56*).

[f] Hepatocytes were incubated for 48 hr prior to addition of the compounds. After exposure for 24 hr to the compound ^{3}H-thymidine was added and incubated for Lh with the cells. Acid-insoluble radioactivity was used as a measure of DNA incorporation. In other experiments in which the compounds were added 24 hr after plating the cells and then incubated for either 24 or 48 hr, no signifiant change in incorporation was observed.

* Significantly different from control ($p < 0.05$).

Discussion

Development of Hepatic Changes

As a result of these studies we would propose the following mechanism for the development of liver damage in rats treated with DEHP.

Following the hydrolysis of DEHP to MEHP in the gut, the absorbed MEHP causes an inappropriate synthesis of triglyceride in the liver. The most likely explanation for this, given that these effects are seen in hepatocytes immediately after addition of MEHP, is that MEHP, or its CoA ester, is sufficiently similar to a natural fatty acid to alter triglyceride synthesis by allosteric effects on one or more of the enzymes involved. It is worth noting that long-chain fatty acids are known to regulate lipid synthesis and degradation (*60*) and MEHP resembles a C_{13} fatty acid.

Either MEHP or a metabolite or, less probably, the accumulated lipid induces the synthesis of both peroxisomal and microsomal fatty acid oxidases. This again would appear to be due to MEHP (or a metabolite) mimicking a natural fatty acid, in this case acting as inducer rather than as allosteric regulator. Interestingly, a similar, though much smaller, induction of peroxisomal enzymes is found in rats fed a high fat diet (*61,62*) and there is also marked induction in rats treated with CPUC (ethyl-2[5(4-chloro-phenyl)pentyl]oxiran-2-carboxylate) (*63*), valproic acid (*64*), or with citral (*65*) compounds which resemble a fatty acid but with a blocking group which will prevent β-oxidation. This is consistent with the proposal by Masters and Crane (*66*) that medium-length fatty acids ($< C_{14}$) play a principal role in regulating peroxisomal lipid metabolism.

The increased amounts of peroxisomal fatty acid-oxidizing enzymes and of the microsomal P-450 isoenzyme-catalyzing β-oxidation of fatty acids coupled with the increased availability of both "artificial" substrate (about 50% of ingested DEHP is excreted as a metabolite of MEHP produced by ω-oxidation coupled with a single round of β-oxidation) (*67*) and, possibly, of "natural" fatty acyl CoA results in greatly increased release of active oxygen species. This may be due to either inability of peroxisomal catalase to cope with the amount of hydrogen peroxide being generated by β-oxidation of fatty acids and/or release of superoxides due to uncoupling (*68*) of the microsomal P-450 isoenzymes responsible for ω or ω-1 oxidation of fatty acids. Removal of the hydrogen peroxide (either formed in peroxisomes or by the action of superoxide dismutase) may be responsible for the reduction in intracellular SH groups observed in animals treated *in vivo* (Table 1).

The active oxygen species damage membranes by addition across double bonds in unsaturated lipids and oxidation of SH groups, for example. In the case of the peroxisomal membrane this results in the membrane becoming weakened and hence there is increased fragility of peroxisomes during homogenization. Similarly, damage to endoplasmic reticulum membranes may explain the loss of glucose-6-phosphatase activity.

The damaged membranes are removed by autophagy resulting in the accumulation of enlarged, lipid filled, lysosomes. The majority of these, like other lysosomes (*69*), discharge into the bile canaliculi, but some remain—for reasons not yet clear—forming lipofuscin deposits.

There is as yet insufficient data to propose with confidence any single hypothesis to link lipofuscin accumulation and the hyperplastic lesions which are the first signs of developing tumors (*70,71*).

Three tentative hypotheses are as follows: (a) The generation of active oxygen species itself results in DNA damage. This could suggest that the peroxisome-proliferating agents do cause mutations, albeit indirectly. However, the behavior of the hyperplastic nodules, both in their lack of certain enzymes, especially γ-glutamyl transpeptidase (*72*) and, more importantly, their disappearance on cessation of long-term treatment, suggests a difference between these compounds and those which exert their carcinogenic effects directly on the DNA. The liver tumors formed following treatment of rats with DEHP and related compounds are more similar to the benign adenomas which are induced in humans by contraceptive steroids and which may also, in a very few cases; progress to malignant tumors (*73*) than to tumors formed by typical mutagenic carcinogens such as dimethyl nitrosamine. Therefore, although the depletion of nonprotein SH groups and the appearance of lipofuscin suggests that there is release of active oxygen, either from peroxisomes or via uncoupling at the endoplasmic reticulum P-450 isoenzymes, there is no convincing evidence that the tumors form as a consequence of damage to DNA.

(b) A second possible hypothesis is that the tumors form because of DNA damage, e.g., chromosomal breaks, which arise as a result either of the burst of mitosis which occurs immediately after the start of dosing or late in life of the animal. We have observed (*38*) that in rats treated with hypolipidemic drugs for 18 months there are more mitotic figures in test than in control animals, indicating an increased rate of cell death and replacement. The nuclei of a very high proportion of the hepatocytes of aged rats and mice are polyploid (*74,75*); polyploid nuclei divide with about the same frequency as diploid (*76*), and it is believed that an increase in ploidy will increase the risk of chromosome damage during mitosis. Furthermore, this theory is similar to one proposed to explain the carcinogenicity of plastic film implants (*77*). In this case the chromosomal damage appears to occur long before the appearance of an identifiable tumor. Again, however, the reversal of the majority of the preneoplastic foci on cessation of treatment would seem to weaken support for this mechanism.

(c) A third hypothesis would connect the carcinogenesis by DEHP not only with that caused by other peroxisome-proliferating agents but also with nonmutagenic carcinogens such as Safrole, Ponceau MX (*78*), and chlorinated hydrocarbons (*79*), whose principal

short-term effects are hepatomegaly and alteration in the endoplasmic reticulum.

It would seem likely that the burst of cell division seen in young rats represents a natural response to the inducing agent—effectively increasing the capacity of the liver to detoxify the material administered. This capacity is lost in older rats. However, in the case of continual stimulation from chemicals such as MEHP, there is low grade cell damage leading to an increased rate of cell death and hepatocytes division therefore occurs to replace those dying cells. Cells particularly sensitive, because of random somatic mutations, to the inducing agent or particularly insensitive to the natural regulators of liver size will divide preferentially. This will result in clones of cells whose size will, however, be limited by the normal control mechanisms which govern liver growth. Subsequently further spontaneous mutations could then reduce the sensitivity of the cells to natural regulators resulting in a hyperplastic nodule which does, however, like hormone-dependent tumors, remain dependent on the presence of the inducer. Finally, and very infrequently, a further mutation results in dependence from cell growth regulatory factors and the formation of a frank tumor. This scheme is, of course, analogous to the normal model for multistage carcinogenesis. The difference is that there is no increase in chemically induced damage to the DNA, so that DEHP is not acting as a classical initiating agent nor is DEHP acting—like a classical promoting agent—to "fix" damage in the genome. Instead, the continued low grade damage selects clones of cells, particularly sensitive to the inducing agent. It is a prerequisite of this hypothesis that the damage is mild. Hepatocyte division will be regulated by the balance between the cytotoxic effects of the inducer and the natural regulators whose concentration varies inversely with liver size. A large change—caused, for example, by partial hepatectomy—will result in "indiscriminate" cell division. Selection will only occur if the damage is prolonged. This theory, therefore, suggests that formation of the hyperplastic nodules requires firstly the continued stimulation by the inducer and secondly sufficient damage to cells to maintain an elevated rate of cell divisions.

Clearly, there are a number of questions regarding the mechanisms proposed above. Firstly, why do straight-chain phthalate esters which perturb lipid metabolism in a similar way and to a similar extent to MEHP, not cause peroxisome proliferation? There are differences in the metabolism of MEHP and MnOP which might explain the difference in hepatotoxicity between the straight chain phthalates and DEHP. The side chain of each ester is excreted as the product of ω-1 and ω-oxidation. In the case of MnOP, 21% is excreted as the product of ω-1 oxidation and 62% as the product of ω-oxidation followed by two rounds of β-oxidation (*67*). In the case of MEHP, 35% is excreted as the product of ω-1 oxidation and 30% as the product of ω-oxidation, only 5% of which undergoes β-oxidation (*68*). It appears therefore that the presence of the ethyl group on C_2 both blocks β-oxidation and renders MEHP a less suitable substrate for ω-oxidation and subsequent extensive β-oxidation than MnOP. This would affect the intracellular concentrations of the different esters as the concentration of the ester in the hepatocyte will depend on the balance between its rate of entry and its rate of metabolism. Hence, if MnOP is more rapidly metabolized, then it is possible that the intracellular concentration of MEHP is markedly higher than that of MnOP. A further difference is that β-oxidation of the ω-oxidation product of MnOP will release acetyl CoA. Given the difference in structure between a normal fatty acid and the dicarboxylic acid deriving from ω-oxidation of MnOP, it is possible that the latter will evade the normal control which govern oxidation of fatty acids and sugars so that excess acetyl CoA will be formed. A similar modification in metabolism is thought to occur after ingestion of ethanol, and it is, perhaps, not surprising therefore that the lesion produced by DnOP in rats resembles that produced in humans by ethanol.

A second major objection to the proposed mechanism of action is that while the mechanism given above suggests that greatly increased amounts of hydrogen peroxide are evolved following treatment of rats with DEHP, direct experiments with hepatocytes gave no evidence of any such change. It should be noted, however, that cultured hepatocytes showed neither the fall in glucose-6-phosphatase activity found *in vivo* nor do they show any reduction in glutathione levels, whereas *in vivo* nonprotein SH groups are significantly reduced. A possible cause for the discrepancy is that the culture medium provides a predominantly reducing environment (due to the presence of cysteine) as opposed to the essentially oxidizing nature of blood. However, this remains to be substantiated. It does, however, seem clear that hepatocytes *in vitro* do not at the present time serve as an adequate model for the third stage in the development of the lesion.

We would, therefore, conclude that while the pattern of development of liver damage in rats treated with DEHP is consistent with the mechanism discussed by Reddy and Lalwani (*2*), namely, that increased production of hydrogen peroxide arising from increased turnover of the fatty acid-oxidizing system of peroxisomes is an essential part of the process of liver damage. However, there is as yet no direct evidence that peroxisome proliferation or active oxygen generation, in themselves, are linked with the ultimate development of hepatocellular adenomas and carcinomas. We propose that both peroxisome proliferators such as DEHP and agents such as chlorinated hydrocarbons which cause both induction of endoplasmic reticulum oxidases and liver damage be grouped together and that the tumors which they cause, like those caused by certain hormones (*80*), be considered to arise by an epigenetic mechanism involving breakdown of the normal homeostatic mechanism—in the case of DEHP, the balance between hepatocellular proliferation in response to persistent

xenobiotic exposure and the normal controls governing liver size.

Effects of Phthalic Acid Esters on the Thyroid

Our results show that agents which cause peroxisome proliferation in the liver join an already extensive list of chemicals which alter the metabolism of both the liver and of the thyroid gland, other examples being chlorinated hydrocarbons such as the polychlorinated biphenyls (*47*) and barbiturates (*81*). The results of treatment are, in all cases, associated with an increased activity in the thyroid gland, accompanied by a lowering of plasma T_4 but with plasma T_3 remaining close to control values. Our results, which show that short-term thyroid changes are produced both by the peroxisome-proliferating ester di-2(ethylhexyl) phthalate and by its straight-chain analogs di(*n*-octyl) phthalate and di (*n*-hexyl) phthalate which do not cause proliferation of hepatic peroxisomes, emphasize that the risk of thyroidal changes is not restricted to compounds or to species which show proliferation of peroxisomes.

In spite of many studies there is no agreed mechanism for the linkage between hepatic and thyroidal effects of inducers of microsomal oxidases such as polychlorinated biphenyls or barbiturates. There are several indications that the linkage may be associated with the increased rate of excretion of the hormone which has been observed both with groups of compounds (*81–83*); but whereas with polychlorinated biphenyls (*82*) and polycyclic hydrocarbons (*83*) the increase would appear due to increased glucuronyl transferase activity, this is not the case in rats treated with phenobarbital. Another possible correlate is displacement of thyroxine from binding proteins. The association between low plasma T_4 and a clinically euthyroid state is reminiscent of human patients treated with diphenylhydantoin (*84*), and indeed polychlorinated biphenyls appear to displace thyroxine from binding proteins (*82*) as does clofibrate (*85*). There is considerable evidence that whereas for most purposes T_4 is essentially a prohormone, the regulation of TSH synthesis reflects the concentration of T_4 in plasma not of T_3 (*86*). Hence, it is possible that this combination of a clinically euthyroid state with low plasma T_4 and hyperactivity of the thyroid gland is due to the combination of a high proportion of binding sites for thyroxine by the toxin, resulting in a reduced total plasma T_4. This, in turn, results in stimulation of TSH production and, hence, of thyroxine synthesis, but this is counterbalanced by peripheral conversion to T_3 (*87*) and by an increase in excretion through the liver. There are, however, many other mechanisms that may connect alterations in the liver and thyroid and considerably more experimental data is required before more than outline hypothesis can be formulated. It should, however, be noted that, with polychlorinated biphenyls, it is known that thyroidal changes are induced in man (*88*). Our results show that the thyroidal changes induced by phthalate esters is induced by agents which do not induce peroxisome proliferation. Hence, it is not currently possible to use the extensive epidemiological data gathered to hypolipidemic compounds (*89*) to assess whether there is any risk to man.

Conclusion

Our experiments suggest a connection between the early hepatic changes observed in rats (liver enlargement and proliferation of hepatic peroxisomes) and the subsequent development of liver tumors. The changes which occur in the livers are consistent with an epigenetic mechanism of carcinogenesis. This is supported by the appearance of the tumors (well differentiated, with no or few metastases) which resemble that of the benign adenomas which occur occasionally in women taking contraceptive steroids. In view of the apparent connection in rats between the short-term effects in liver and subsequent tumorigenesis, the identification that the no-effect level for pathological changes in rat liver occurs at relatively high doses and the evidence that humans treated with hypolipidemic drugs (*90*) or marmosets treated with DEHP (*91*) do not show the typical short-term liver changes produced by these compounds in rats, it would seem unlikely that the low environmental levels of DEHP to which man is exposed pose a significant risk of liver cancer in humans. For humans exposed over short periods to rather higher levels of DEHP (e.g., by blood transfusions) the situation is less clear-cut, and effects on liver and thyroid function could arise. It would seem unwise to attempt substitution of DEHP by straight-chain analogs without carefully monitoring, for it is clear that although the hepatic effects are different from those of DEHP they may give nonetheless grounds for concern.

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