Biochemical Pharmacology, Vol. 35, No. 17, pp. 2941–2947, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

EFFECTS OF MONO (2-ETHYLHEXYL) PHTHALATE AND ITS STRAIGHT CHAIN ANALOGUES MONO-*n*-HEXYLPHTHALATE AND MONO-*n*-OCTYL PHTHALATE ON LIPID METABOLISM IN ISOLATED HEPATOCYTES

FIONA E. MITCHELL*, JAMES W. BRIDGES and RICHARD H. HINTON†

Robens Institute of Industrial and Environmental Health and Safety, University of Surrey, Guildford, Surrey, GU2 5XH, U.K.

(*Received* 12 *March* 1986; *accepted* 12 *March* 1986)

Abstract—In cultured hepatocytes, as *in vivo*, mono-2-ethylhexyl phthalate (MEHP) and its straight chain analogues mono-*n*-hexyl phthalate (MnHP) and mono-*n*-octyl phthalate (MnOP) each cause accumulation of lipid but only MEHP produces significant induction of peroxisomal fatty acid oxidizing enzymes. To elucidate the mechanisms underlying this lipid accumulation we investigated the effects of these phthalates and the drug clofibric acid on fatty acid metabolism in suspensions of isolated hepatocytes. The effects were found to be markedly dependent on the nutritional state of the animals from which the hepatocytes were isolated. In hepatocytes isolated from animals fasted overnight, or animals fed *ab libitum* but killed at approximately 2.30 p.m., MEHP, MnHP, MnOP and clofibric acid each caused a marked rapid stimulation of fatty acid oxidation and the synthesis of triglycerides in hepatocytes when incubated in Hanks saline. Export of very low density lipoprotein (VLDL) from the cells was either unchanged or somewhat reduced. In contrast, in hepatocytes isolated from rats fed *ad libitum* but killed at approximately 9.30 a.m. MEHP and clofibric acid did not alter fatty acid oxidation or triglyceride synthesis, while MnOP and MnHP increased triglyceride synthesis but decreased fatty acid oxidation. The effects of fasting were largely abolished by incubations of the cells in a complete tissue culture medium (Liebowitz L-15). The results suggest that MEHP and its straight chain analogues can, either as the free acid or the CoA ester, mimic the action of fatty acids in the allosteric regulation of fatty acid metabolism.

A number of structurally unrelated compounds, including the hypolipidaemic drugs exemplified by clofibrate, environmental contaminants, such as the plasticiser di-(2-ethylhexyl) phthalate (DEHP), and industrial chemicals, such as trichloroethylene produce liver tumours in rats and mice [1, 2]. These compounds have been classified as non-genotoxic carcinogens for they give negative results in standard mutagenicity tests and there is no evidence of covalent binding to DNA [2]. All have a similar pharmacological action—lowering serum triglyceride with a limited and more variable action on serum cholesterol [1]. Each provokes a rapid although temporary increase in lipid deposition in the liver [3, 4] and produces a pronounced proliferation of peroxisomes, particularly of the peroxisomal enzymes catalysing β-oxidation of fatty acids in the rat liver [2]. Hence it would seem possible that a change in lipid metabolism may be the first step in a chain of events which eventually results in tumour formation.

Although the mechanism of therapeutic action of clofibrate is not well understood both it and its hydrolysis product clofibric acid appears to produce a direct effect on lipid metabolism in several tissues [5]. Lipid initially accumulates in the liver as small droplets [6], similar to those observed in animals receiving an excess of dietary fat [7]. Treatment of freshly isolated rat hepatocytes with clofibric acid causes a prompt increase in the rate of esterification of radiolabelled palmitate the greatest increase being in the rate of triglyceride formation [5]. It also increases the rate of oxidation of long, medium and short chain fatty acids. Hence, clofibric acid, or a derivative which is rapidly formed can directly and rapidly modulate lipid metabolism. DEHP also has pronounced effects on lipid metabolism *in vivo*. As with clofibrate [8] it causes a pronounced inhibition of cholesterol synthesis (see ref. 9) together with some fall in fatty acid and triglyceride synthesis. Under certain conditions there is a zone-dependent accumulation of fat [3, 4].

In view of the fact that DEHP, like clofibrate, both causes accumulation of small droplets of lipid in the periportal zone of the liver lobule and inhibits cholesterol biosynthesis it seemed of interest to determine whether mono 2-ethylhexyl phthalate (MEHP), the form in which DEHP is present in plasma [10], could, like clofibrate, modulate the esterification and the oxidation of long chain fatty acids. We also hoped to explore the relevance of any such modulation of lipid metabolism to peroxisome proliferation by comparing the action of MEHP with its straight chain analogues mono-*n*-hexyl phthalate (MnHP) and mono *n*-octyl phthalate (MnOP), for the straight chain phthalate esters [11], like chlorpromazine [12], cause lipid accumulation in the liver, albeit in the centrilobular zone, with little or no

* Present address: Imperial Cancer Research Fund, London.

† To whom all correspondence should be addressed.

necrosis but do not cause any proliferation of liver peroxisomes.

MATERIALS AND METHODS

Male Wistar albino rats (University of Surrey strain) weighing between 200 and 250 g and fed Spratts Laboratory Rodent pelleted diet (Spratts Barking, Essex, U.K.) were used in these experiments. Unless otherwise specified animals were allowed food and water *ab libitum*. [1-^{14}C]palmitate (58.2 mCi/mmole) was obtained from Amersham International Limited (Amersham, Bucks, U.K.) Fatty acid poor fraction V-Bovine Serum Albumin (BSA) was purchased from Sigma Chemical Company (Poole, Dorset, U.K.) as were all other biochemicals. Stock solutions of [1-^{14}C]palmitate (0.22 mM)–BSA (5% w/v) were prepared as described by Capuzzi *et al.* [5]. Picofluor-36 was purchased from Packard Instrument Co., Inc. (Caversham, Berks, U.K.). Falcon Primaria Culture flasks were purchased from Becton Dickinson and Co. (Oxford, U.K.). Liebowitz-L15 culture medium serum and Kanamycin were obtained from Flow Laboratories Ltd. (Irvine Scotland). Collagenase was purchased from Boehringer Corporation (Lewes, Sussex U.K.). MEHP, MnOP and MnHP were kindly donated by BP Chemicals (Penarth, Wales, U.K.). DEHP, DnOP and DnHP were supplied by BP Chemicals (Hull Works, North Humberside, U.K.). The monoesters of phthalic acid and the sodium salt of clofibrate were used in the majority of experiments as these are the forms in which the compounds are present in plasma [5, 12].

Clofibric acid as its sodium salt was purchased from the Sigma Chemical Co. (Poole, Dorset).

Hepatocyte isolation and culture. Hepatocytes isolated using an adaption of the method of Rao *et al.* [13]. The hepatocytes were either used immediately or cultured as follows: cells 2×10^6 (more than 90% viable by trypan blue exclusion) were seeded in 40 ml tissue culture flasks in 4 ml of Liebowitz L15 medium containing 10% foetal calf serum, 2 mM glutamine, 10% tryptose phosphate broth, 100 μg/ml Kanamycin, 10^{-6} M insulin and 10^{-5} M hydrocortisone. Cultures were maintained at 37°. The medium was changed 2–4 hr after seeding and subsequently every 24 hr. Test compounds freshly dissolved in dimethylformamide (final concentration 0.04% v/v) were added after 24 hr and at subsequent medium changes. The total treatment period was 72 hr.

Biochemical determinations on cultured hepatocytes. The cell monolayer was harvested into 1 ml of 0.25 M sucrose, pH 7.4, containing 5 mM Tris and 5 mM EDTA, and homogenized by two 10 sec bursts in a Dawe sonicator (Dawe Instruments, Shipley, West Yorkshire). Cyanide-insensitive palmitoyl-CoA oxidation was measured by the method of Bronfman *et al.* [14] and α-glycerophosphate dehydrogenase by the method of Lee and Lardy [15]. Lactate dehydrogenase was measured by the method of Bergmeyer [16]. Oil red O staining was carried out after Culling [17].

Metabolism of palmitic acid by hepatocytes. Hepatocytes were isolated as described earlier, at either 9–10 a.m. or 2–3 p.m. from rats fed *ab libitum* or at 9–10 a.m. from rats starved for 18–24 hr, but provided with water *ad libitum*. Hepatocytes were finally resuspended in bicarbonate free Hanks solution, pH 7.4 containing 1.25 mM $CaCl_2$, at a concentration of 2×10^6 cells per ml medium. To measure incorporation into triglyceride and cholesterol esters 5 ml aliquots were incubated under air at 37° in 50 ml Erlenmeyer flasks in a shaking water bath. The hepatocytes were preincubated for 10 min, then incubated for a further 30 min with various levels of MEHP MnOP MnHP or clofibric acid (sodium salt). [1-^{14}C]palmitate (2.75 μg) and unlabelled palmitate (25.6 μg) were then added in a O 66 mM bovine serum albumin solution and the incubation continued for 30 min. Isolation of labelled lipids was carried out by the method of Capuzzi *et al.* [8] except that samples of the upper phase which contained esterified lipids were dried in glass scintillation vials under nitrogen. Picofluor-36 (4 ml) was added and the radioactivity content determined in a Prias PL counter (Packard Instruments Co., Caversham, Berks, U.K.).

Particles with the density of very low density lipoprotein particles were isolated from the medium as follows: 2 ml of medium (from experiments conducted as described in the previous paragraph) was added to 2 ml of normal human serum (to act as a carrier) mixed and centrifuged at 35,000 rev/min (Prepspin 50, M.S.E., Crawley, U.K.) for at least 15 hr. 1 ml of supernatant (i.e. chylomicrons) was then removed into a scintillation vial containing 4 ml of Picofluor-36 mixed and counted.

The rate of palmitic acid oxidation was measured by assaying the release of $^{14}CO_2$. Aliquots of hepatocyte suspensions (1 ml) were placed in 10 ml Erlenmeyer glass flasks with centre wells containing 100 μl of ethanolamine: ethylene-glycolmonoethylether: toluene (1:8:10). After incubation $^{14}CO_2$ was released from cell suspensions by the addition of 0.25 ml of 12% perchloric acid. The liberated $^{14}CO_2$ was collected during a subsequent 30 min incubation, at 37°. The centre wells were removed placed in scintillation vials containing 4 ml of Picofluor-36 and counted.

RESULTS

Treatment of isolated cultured hepatocytes with MEHP, MnOP or MnHP caused an accumulation of lipid within the cells similar to that observed *in vivo* [4]. Cultured hepatocytes treated with MEHP for 72 hr showed a dose-dependent increase in cyanide-insensitive (peroxisomal) palmitoyl CoA oxidation (Table 1). There was no significant alteration in palmitoyl CoA oxidation in cells treated with MnOP or MnHP. No signs of toxicity were observed in hepatocytes treated with MEHP at concentrations less than 1 mM but hepatocytes treated with MnOP and MnHP showed signs of toxicity such as blebbing, vacuolation and detachment from the culture flasks by 72 hr after commencement of treatment. The phthalic acid diesters were less toxic to cells than the corresponding monoesters. The degree of induction of palmitoyl CoA oxidation by 0.1 mM DEHP was similar to that found with MEHP but the degree of

Table 1. The effect of esters of phthalic acid on cyanide-insensitive palmitoyl CoA oxidation and on α-glycerophosphate dehydrogenase activity in cultured hepatocytes. Hepatocytes were cultured for 3 days in Liebowitz L-15 medium containing 10% foetal calf serum, 10% tryptose-phosphate broth, 100 μg/ml kanamycin. 10^{-6} M insulin and 10^{-5} M hydrocortisone. The media were changed every day. The test compounds were dissolved in dimethylformamide (DMF). An equal volume of DMF was added to control flasks. Results are presented as mean ± SE (number of flasks examined).

	Palmitoyl CoA oxidation†						α-Glycerophosphase dehydrogenase‡		
Concentration	Compound: MEHP	MnOP	MnHP	DEHP	DnOP	DnHP	DEHP	DnOP	DnHP
0.01 mM	0.71 ± 0.12 (3)	1.35 ± 0.17 (4)	0.44 ± 0.13 (3)	na	na	na	na	na	na
0.025 mM	1.65 ± 0.31 (4)	0.72 ± 0.34 (3)	0.54 (2)	na	na	na	na	na	na
0.05 mM	2.90 ± 0.57 (4)*	0.35 ± 0.19 (4)	0.80 ± 0.03 (4)*	na	na	na	na	na	na
0.1 mM	5.85 ± 0.27 (4)*	0.37 ± 0.13 (3)*	0.57 ± 0.25 (3)	5.41 ± 0.61 (6)*	2.0 ± 0.20 (6)*	1.69 ± 0.06 (6)*	69 ± 3 (6)*	49 ± 4 (6)*	55 ± 7 (6)*
0.25 mM	10.27 ± 0.30 (4)*	0.38 ± 0.17 (4)	0.46 ± 0.23 (3)	na	na	na	na	na	na
0.5 mM	5.61 ± 0.97 (3)*	na	0.34 ± 0.10 (4)	1.87 ± 0.17 (6)	1.00 ± 0.09 (5)	1.36 ± 0.20 (6)	41 ± 4 (6)*	40 ± 4 (6)*	57 ± 8 (6)*
1 mM	na	na	na	2.85 ± 0.49 (6)*	1.38 ± 0.21 (5)	2.42 ± 0.28 (6)*	56 ± 4 (6)*	45 ± 3 (6)*	48 ± 6 (6)*
1.5 mM	na	na	na	1.36 ± 0.13 (6)	1.61 ± 0.11 (6)	1.29 ± 0.25 (6)*	37 ± 2 (6)*	41 ± 3 (6)*	39 ± 7 (6)
2 mM	na	na	na	1.54 ± 0.16 (6)	1.10 ± 0.12 (5)	1.62 ± 0.16 (6)*	43 ± 4 (6)*	35 ± 3 (6)	39 ± 6 (6)
5 mM	na	na	na	1.13 ± 0.09	na	1.32 ± 0.19 (6)*	41 ± 4 (6)*	28 ± 4 (6)	24 ± 6 (6)
control	1.03 ± 0.02 (3)	0.85 ± 0.01 (3)	0.37 ± 0.11 (4)	1.29 ± 0.21 (5)	§	0.56 ± 0.04 (6)*	28 ± 2 (6)*	§	25 ± 4 (6)

na, not assayed.
* Significantly different from control ($P < 0.05$).
† nmoles NAD^+/min/mg protein.
‡ Change in A_{500}/min/g protein.
§ Assayed in same batch as DEHP.

Table 2. The effect of MEHP, MnOP, MnHP or clofibric acid on [1-^{14}C]palmitate incorporation into triglyceride and cholesterol esters in hepatocytes. (Results are expressed as % of the incorporation observed in control flasks)

Addition to total medium	% Change in [1-^{14}C]palmitic acid incorporation[a]			
Test compound: Concentration (mM)	MEHP	MnOP	MnHP	Clofibric acid
	% Control			
Results from fed rats killed between 9 a.m. and 10 a.m.[b]				
0.05	91 ± 4 (6)	183 ± 14 (3)*	161 ± 5 (3)	nm
0.1	90 ± 4 (6)	212 ± 3 (3)*	147 ± 32 (3)	nm
0.25	99 ± 5 (6)	241 ± 9 (6)*	245 ± 14 (3)*	nm
1	nm	nm	nm	102 ± 11 (6)
Results from fed rats killed between 2 p.m. and 3 p.m.[b]				
0.05	106 ± 6 (6)	nm	nm	nm
0.1	121 ± 6 (6)*	nm	nm	nm
0.25	131 ± 8 (8)*	nm	nm	nm
1	nm	nm	nm	187 ± 20 (60)*
Results from fasted rats[b]				
0.05	137 ± 1 (3)*	144 ± (2)	278 ± 20 (3)*	nm
0.1	153 ± 11 (3)*	327 ± 29 (3)*	458 ± 54 (3)*	nm
0.25	192 ± 7 (6)*	608 ± 25 (3)*	409 ± 28 (3)*	nm
1	nm	nm	nm	349 ± 25 (6)*

[a] Results are presented as mean ± SE (number of observation).
[b] Overall levels of incorporation expressed as cpm/flask employing the same batch of palmitic acid were: fed rats killed between 9 a.m. and 10 a.m. 149634 ± 2855 (3); fed rats killed between 2 and 3 p.m., 69860 ± 3877 (6); fasted rats, 10147 ± 1177 (3).
* Significantly different from control at the 5% level.

induction fell at higher concentrations. Changes in α-glycerophosphate dehydrogenase followed a similar pattern. The straight chain phthalic acid diesters DnOP and DnHP caused a very slight increase in palmitoyl CoA oxidation at a dose of 1 mM but a much more marked increase in α-glycerophosphate dehydrogenase activity.

Neutral lipid synthesis

The results presented in Table 2 show that incubation with MEHP or clofibrate did not significantly alter the incorporation of (1-^{14}C)palmitate into neutral lipid (triglycerides and cholesterol esters) by rats fed *ab libitum* providing that the animals were killed early in the morning (9–10 a.m.). If, however, the rats were killed in the early afternoon the rate of incorporation by control cells fell by about 50% but, in this case, both MEHP and clofibrate caused significant increases in the synthesis of neutral lipids. Both the fall in incorporation in control cells and the stimulation by added clofibrate were accentuated if the animals were fasted overnight prior to isolation of the hepatocytes. An equally marked change in response depending on the nutritional state of the rats from which the hepatocytes were isolated was observed with the straight chain phthalate esters MnHP and MnOP. Unlike clofibrate and MEHP, these did stimulate incorporation in hepatocytes iso-

Table 3. The effect of MEHP or clofibric acid on [1-^{14}C]palmitate incorporation into triglyceride and cholesterol esters in hepatocytes isolated from a rat fed *ad libitum*, at midday and incubated in either bicarbonate-free Hanks solution pH 7.4 or in L-15 medium containing 2 mM glutamine. (Results are presented as mean ± SE for triplicate samples)

Addition to medium	[1-^{14}C]palmitate incorporation (cpm × 10^{-3}/10^7 cells)			
	HCO_3 free Hanks buffer pH 7.4 + 1.25 mM $CaCl_2$	% Control	L-15 medium containing glutamine	% Control
Control	110 ± 4	—	107 ± 4	—
0.25 mM MEHP	177 ± 3*	161	118 ± 15	110
1.0 mM CPIB	173 ± 8*	157	138 ± 4	129

[a] Results are mean ±SEM for triplicate samples.
* Significantly different from control at 5% level.

Table 4. The relation between incubation time and effect of 0.25 mM MEHP on [1-^{14}C]palmitate incorporation into trigylceride and cholesterol esters in hepatocytes isolated from a fasted rat (results are presented as mean ±SE for triplicate samples except where indicated in parentheses. The results for two replicate experiments are presented separately)

Time of preincubation with 0.25 mM MEHP. or with DMF (controls) (min)	[1-^{14}C]palmitate incorporation cpm × 10^{-3}/10^7 cells		
	Control	0.25 mM MEHP	Control
Expt. 1			
0	12.0 ± 1.0	20.5 ± 2.2*	171
7.5	13.9 ± 0.3	21.7 ± 2.3*	156
15	16.3 ± 0.4	29.4 ± 2.8*	180
22.5	20.3 (2)	32.5 ± 3.3*	160
Expt. 2			
0	26.0 ± 1.8	34.0 ± 4.3	131
7.15	33.7 ± 2.3	38.4 ± 0.9	114
15	32.0 (2)	38.7 ± 2.9	121
22.5	35.5 ± 0.7	37.3 ± 2.4	105

[a] Results are mean ±SEM for triplicate samples except where indicated in parentheses.

* Significantly different from control, at the 5% level.

lated from fed rats but whereas the stimulation with fed rats was around 240%, in the case of fasted rats maximal stimulation of 400%–600% were observed.

In view of the difference in response between hepatocytes isolated from rats in different nutritious states we compared the responses of hepatocytes incubated in simple saline solution (HCO_3-free Hanks buffer) and in a complete tissue culture medium (Liebowitz L-15 containing glutamine). The results showed that incubation in a complete tissue culture medium did not alter the rate of incorporation in control hepatocytes, but did inhibit the stimulation by clofibrate of fatty acid incorporation into neutral lipids (Table 3). Furthermore in view of suggestions [18] that a metabolite of MEHP rather than the parent compound may be the toxicologically active agent, we examined the effect of reducing or omitting preincubation of hepatocytes with the test compound as used in the standard protocol of Cappuzzi *et al.* [8]. The results (Table 4) showed that equally pronounced stimulation occurs whether or not hepatocytes are preincubated with the test compounds. The results of two separate experiments are presented separately in Table 4 to illustrate the marked differences in the degree of stimulation which occurs between experiments. Such differences were also observedly Cappuzzi *et al.* [5] and attributed to differences in protease contamination between collagenase preparation. We observed no correlation with collagenase batches but later experiments with other hypolipidaemic agents [19] have indicated that a low degree of stimulation is associated with a fall in viability in the treated cells. In view of this and the evidence that the nutritional state of the rat influences the properties of the cells, we consider that the experimental variability is due to variation in the energy reserve to the isolated cells.

Fatty acid oxidation

Incubation with MEHP and clofibrate resulted in a dose dependent stimulation in the release of $^{14}CO_2$ from [1-^{14}C]palmitate (Table 5) in hepatocytes from both fed and fasted rats. In view of the close contact between single hepatocytes and the surrounding medium it would appear likely that the amount of $^{14}CO_2$ released into the medium reflects the underlying rate of fatty acid oxidation within the cells. Cells treated with 1 mM clofibrate showed greater changes than cells treated with 0.25 mM MEHP. Addition of MnHP or MnOP caused some stimulation of carbon dioxide release from [1-^{14}C] palmitate in hepatocytes from fasted rats but marked inhibition in hepatocytes isolated from fed rats (Table 5).

Table 5. The effect of MEHP, MnOP, MnHP or clofibrate on the release of $^{14}CO_2$ from 1-^{14}C-palmitic acid in hepatocytes (results are presented as mean ±SE (number of experiments))

Concentration (mM)	% of Control Flasks[a]			
	Addition to medium: MEHP	MnOP	MnHP	Clofibrate
Results from fed rats				
0.05	116 ± 4 (3)*	110 ± 12 (9)	77 ± 15 (9)	nm
0.1	137 ± 4 (6)*	74 ± 10 (7)*	45 ± 6 (9)*	nm
0.25	144 ± 10 (9)*	54 ± 5 (9)*	39 ± 5 (9)*	nm
1	nm	nm	nm	202 ± 33 (6)*
Results from fasted rats[a]				
0.05	116 ± 18 (3)	117 ± 8 (6)	128 ± 9 (5)*	nm
0.1	143 ± 11 (3)*	137 ± 9 (6)*	122 ± 8 (6)*	nm
0.25	147 (2)	162 ± 20 (5)*	118 ± 10 (5)	nm
1	nm	nm	nm	235 ± 17 (6)*

* Significantly different from control at the 5% level.

[a] The results are based on four sets of experiments with fed rats and two sets of experiments with fasted rats. Absolute levels of incorporation in control flasks (counts/min/10^7 cells) were as follows: fed rats—5856 ± 412 (3); 5728 ± 487 (3); 7629 ± 1046 (4); 6866 ± 215 (3); fasted rats—14558 ± 515 (3); 15774 ± 1316 (3).

Lipoprotein formation

In addition to measuring triglyceride accumulation in hepatocytes, we examined whether treatment with 1 mM clofibrate or with any of the phthalate esters affected incorporation of [1-^{14}C]palmitate into VLDL particles or the release of such particles from hepatocytes. Duplicate experiments showed no alterations in hepatocytes fed or fasted rats treated with MEHP or clofibrate. Export was somewhat increased in hepatocytes from fed rats treated with 0.25 mM MnOP but no change was observed with MnHP. Export from hepatocytes separated from fasted rats and treated with MnOP or MnHP was reduced to 50% of control values respectively although there was a marked increase (6-fold and 4-fold respectively) in incorporation of [1-^{14}C]palmitate into triglyceride. However, these results cannot be taken as proof that increased triglyceride synthesis is not accompanied *in vivo* by increased export as there may well be a significant lag phase due to the time taken to assemble the lipoproteins particles and, possibly, to a need to induce synthesis of the relevant apoproteins.

DISCUSSION

The results presented in this paper show that MEHP perturbs lipid metabolism in isolated hepatocytes in the same way as clofibrate, increasing the release of $^{14}CO_2$ from [1-^{14}C]palmitic acid in hepatocytes isolated from both fed and fasted rats and increasing the rate of incorporation of palmitic acid into neutral lipid in hepatocytes isolated from fasted rats but not in hepatocytes isolated from fed rats. Comparison with published data [20] indicates that the increased incorporation into neutral lipid is due to an increased rate of triglyceride synthesis, for DEHP depresses acyl CoA: cholesterol acyl transferase activity in the liver. The absence of any need for preincubation indicates that the changes which we observe are due to the direct action of the parent compounds rather than their metabolites and a likely explanation is an allosteric effect on one or more of the regulatory enzymes although, especially in the case of triglyceride synthesis regulation even by endogenous cell constituents is poorly understood. The effects which we observe are, however, consistent with MEHP and clofibric acid acting like fatty acids on the regulatory pathways so that triglyceride assembly continues even in hepatocytes isolated from fasted animals and such an hypothesis would certainly be consistent with the triglyceride accumulation seen both in intact liver [4] and in isolated hepatocytes.

Two important questions remain. First, is there a causal link between these initial effects of the compounds on lipid metabolizing enzymes and the subsequent peroxisome induction and secondly, why are the effects of DEHP *in vivo* so different from the effects of its straight chain analogues DnHP and DnOP.

It is possible that the changes in fatty acid metabolism described above are directly responsible for the peroxisome induction. In support, high fat diets are known to cause both an accumulation of lipid in small droplets [7] and proliferation of peroxisomes [22, 23]. However, the extent of induction of peroxisomal enzymes in rats fed high fat diets is much less than in rats treated with clofibrate or DEHP and it would therefore seem more likely that the link between alteration in lipid metabolism and induction of peroxisomal enzymes is less direct. Induction of the peroxisomal fatty acid oxidizing enzymes and of the P_{450} isoenzyme catalysing ω oxidation of fatty acids is tightly co-ordinated [24]. As these enzymes are all concerned with fatty acid metabolism it would seem likely that fatty acids are the natural inducing agent clofibric acid and MEHP are potent mimics of the action of fatty acids on the regulator protein for the expression of the genes for these enzymes.

There remains the question of why the straight chain esters of phthalate acid do not cause peroxisome proliferation. Our results show that the initial effects of MnHP and MnOP on triglyceride synthesis are generally similar to, but more pronounced than the effects of MEHP. The effects on fatty acid oxidation, however, differ. Straight chain phthalate esters inhibit CO_2 production from 1-^{14}C-palmitate in hepatocytes separated from fed rats. Since at very low concentration MnHP and DnHP and DnOP (which will be hydrolysed within the cells to MnHP and MnOP) cause a slight proliferation of peroxisomes, it would appear likely that MnHP and MnOP are capable of interacting with the regulatory protein for the peroxisomal and microsomal fatty acid oxidizing enzymes but that a quite separate event interferes with expression of the genes. The nature of this event requires further investigation but it is worth noting that (a) MnOP and MnHP, unlike MEHP, are extensively metabolised by β-oxidation [25] releasing acetyl CoA subunits which will, in turn, have a profound effect on fatty acid and lipid metabolism, and (b) both compounds are toxic to hepatocytes at lower doses than MEHP. This toxicity may interfere and restrict the ability of the cell to respond to the straight chain phthalate esters by proliferation of peroxisomes.

Acknowledgements—We thank Dr S. C. Price for many helpful discussions and Dr D. Benford and Mrs J. Ng for much help in setting up and carrying out methods for hepatocyte isolation and culture. Financial assistance was provided by the Cancer Research Campaign.

REFERENCES

1. A. J. Cohen and P. Grasso *Food Cosmet. Toxic.* **19**, 585 (1981).
2. J. K. Reddy and N. D. Lalwani, *CRC Crit. Rev. Toxic.* **12**, 1 (1984).
3. M. S. Stein, P. J. Caasi and P. P. Nair, *J. Nutr* **104**, 187 (1974).
4. F. E. Mitchell, S. C. Price, R. H. Hinton, P. Grasso and J. W. Bridges, *Toxic. appl. Pharmac.* **81**, 371 (1985).
5. D. M. Cappuzzi, C. M. Intenzo, R. D. Lackman, A. F. Whereat and D. M. Scott, *Biochem. Pharmac.* **32**, 2195 (1983).
6. S. C. Price, PhD Thesis, University of Surrey (1985).
7. C. E. Neat, M. S. Thomassen and H. Osmundsen, *Biochem J.* **196**, 149 (1981).
8. R. G. Gould, E. H. Swyryd, D. Avoy and B. Coan, *Progr. Biochem. Pharmac.* **2**, 345 (1967).
9. F. P. Bell, *Environ. Health Perspect.* **45**, 41 (1982).

10. I. R. Rowland, R. C. Cottrell and J. C. Phillips, *Food Cosmet. Toxic.* **15**, 17 (1977).
11. A. H. Mann, S. C. Price, F. E. Mitchell, P. Grasso, R. H. Hinton and J. W. Bridges, *Toxic. appl. Pharmac.* **77**, 116 (1985).
12. S. C. Price, D. E. Hall and R. H. Hinton, *Toxic. Lett.* **25**, 11 (1985).
13. M. L. Rao, G. S. Rao, M. Holler, M. Breuer, P. J. Schattenberg and W. D. Stein, *Hoppe Seyler's Z. Physiol. Chem.* **357**, 573 (1976).
14. M. Bronfman, N. C. Inestrosa and F. Leighton, *Biochem. biophys. Res. Commun.* **88**, 1030 (1979).
15. Y. P. Lee and H. A. Lardy, *J. biol. Chem.* **240**, 1427 (1965).
16. M. U. Bergmeyer, *Methods in Enzymatic Analysis*, 2nd edn., pp. 574–579 (1974).
17. C. F. A. Culling, *Handbook of Histopathological and Histochemical Techniques*, 3rd. edn. Butterworths, London (1974).
18. A. M. Mitchell, J-C. Lheuguenot, J. W. Bridges and C. R. Elcombe, *Toxic. appl. Pharmac.* **80**, 23 (1985).
19. S. C. Price, F. E. Mitchell and R. H. Hinton, *Biochem. Soc. Trans.* in press.
20. F. P. Bell and D. A. Buthala, *Bull. Environ. Contam. Toxic.* **31**, 177 (1983).
21. R. M. Bell and R. A. Coleman, *Ann. Rev. Biochem.* **49**, 459 (1980).
22. H. Ishii, N. Fukumori, S. Haru and T. Suga, *Biochim biophys. Acta* **617**, 1 (1980).
23. C. E. Neat, M. S. Thomassen and H. Osmundsen, *Biochem. J.* **186**, 369 (1980).
24. B. G. Lake, T. J. B. Grey, W. F. Pels-Rijcken, J. A. Beamand and S. D. Gangolli *Xenobiotica* **14**, 269 (1984).
25. P. W. Albro and B. Moore, *J. Chromatog.* **94**, 209 (1974).