

# Hepatic Effects of Phthalate Esters and Related Compounds—*In Vivo* and *In Vitro* Correlations\*

by Brian G. Lake,<sup>†</sup> Tim J. B. Gray,<sup>†</sup> and Sharat D. Gangolli<sup>†</sup>

The hepatic effects of phthalate esters and related compounds on peroxisomal and microsomal enzyme activities were investigated in the intact animal and in primary hepatocyte cultures. *In vitro* studies with a series of phthalate monoesters demonstrated structure activity differences in the induction of the specific peroxisomal marker KCN-insensitive palmitoyl-CoA oxidation and also of carnitine acetyltransferase. These effects could be reproduced *in vivo*, and potency differences were also observed between di(2-ethylhexyl) phthalate (DEHP) and its straight-chain isomer, di-*n*-octyl phthalate. In *in vivo* studies, DEHP, mono(2-ethylhexyl) phthalate, and clofibrate/clofibric acid produced large increases in liver size and peroxisomal enzyme activities in Sprague-Dawley rats and Chinese hamsters, but had less effect in Syrian hamsters. These effects could be largely reproduced *in vitro* where good responses were obtained with rat and Chinese hamster hepatocytes, but either little or no effect with Syrian hamster and Dunkin-Hartley guinea pig hepatocytes. Both DEHP and clofibrate appeared to induce similar form(s) of microsomal cytochrome P-450 which exhibited a high specificity towards lauric acid hydroxylation. With a range of hypolipidemic agents, including phthalate monoesters, a good correlation was observed between the induction of peroxisomal and microsomal enzyme activities in rat hepatocyte cultures. These results thus demonstrate a good relationship between the *in vivo* and *in vitro* effects of phthalate esters and related compounds and suggest that hepatocyte cultures may be useful for further studies on the hepatic effects of peroxisome proliferators.

## Introduction

A number of compounds are known to produce liver enlargement and hepatic peroxisome proliferation in rodents and certain of these compounds have also been found to increase the incidence of liver tumors in rats and/or mice (1,2). Although the precise relationship between peroxisome proliferation and tumor formation remains to be established, it has been suggested that peroxisome proliferators constitute a novel class of chemical carcinogens (2,3). The phthalate ester plasticizer di(2-ethylhexyl) phthalate (DEHP) has also been shown to produce liver enlargement and hepatic peroxisome proliferation in rats (4,5) and mice (6) and to increase the incidence of liver tumors in these two species (7).

Apart from increasing peroxisomal enzyme activities, and in particular the enzymes of the peroxisomal fatty acid  $\beta$ -oxidation cycle (8), certain peroxisome proliferators are known to enhance the microsomal oxidation of fatty acids (9). Indeed, Gibson and co-workers (10,11)

have isolated a novel form of cytochrome P-450 from the livers of clofibrate [ethyl 2-(*p*-chlorophenoxy) isobutyrate] treated rats which has a high specificity for the  $\omega$ - and ( $\omega$  - 1)-hydroxylation of lauric acid, but correspondingly low specificity towards typical mixed function oxidase substrates. Both DEHP and clofibrate also induce cytosolic epoxide hydratase and glutathione S-transferase activity in rodent liver (12).

We have been interested in elucidating the hepatic effects of phthalate esters both in the intact animal and *in vitro* employing primary hepatocyte cultures. Using these systems we have investigated the structure activity requirements for induction of peroxisomal enzyme activities by phthalate esters. The effects on microsomal enzyme activities have also been determined. Finally, as species differences may be important in assessing the relevance of the rat and mouse carcinogenicity studies (7) to man we have also examined the effect of DEHP and other peroxisome proliferators in the rat, Syrian and Chinese hamsters.

## Materials and Methods

### Materials

Samples of mono-*n*-octyl, mono(1-ethylhexyl), mono-(2-ethylhexyl), mono-*n*-hexyl, mono(1-ethylbutyl),

\*This paper was presented at the Conference on Phthalic Acid Esters, the proceedings of which were published in this journal, volume 65.

<sup>†</sup>The British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, SM5 4DS, England.

mono(2-ethylbutyl) and monoethyl phthalates were synthesized by the method of Albro et al. (13) for phthalate monoesters. Their purity was established by thin layer chromatography, nuclear magnetic resonance spectroscopy and by elemental analysis. DEHP (99.5% pure) was the generous gift of BP Chemicals Ltd., Sully, South Glamorgan, Wales, U.K. All other peroxisome proliferators, enzyme substrates and cofactors and tissue culture materials were obtained from the sources cited previously (14–17).

## Animals and Treatment

Male animals were used for all studies. Sprague-Dawley rats (100–120 g) were purchased from Olac (1976) Ltd., Blackthorn, Bicester, Oxon, U.K., and Dunkin-Hartley guinea pigs (400–450 g) from Porcellus Animal Breeding Ltd., Heathfield, East Sussex, U.K. Syrian hamsters (70–80 g) were obtained from Intersimian Ltd., Milton Trading Estate, Milton, Oxon, U.K., and Chinese hamsters (20–25 g) from Bantin and Kingman Ltd., Grimston, Hull, U.K. The animals were housed in rooms maintained at  $22 \pm 3^\circ\text{C}$  with a relative humidity of 40–70% and allowed free access to appropriate laboratory animal diets and water. After acclimatizing to these conditions for 7 days, the animals were treated with the phthalate esters and related compounds at the dose levels described in the text by daily gastric intubation for either 7 or 14 days. Control animals received corresponding quantities (5 mL/kg) of the corn oil vehicle. Following the last dose, the animals were starved overnight, killed by cervical dislocation and the livers immediately excised for biochemical studies. Whole liver homogenates (0.25 g fresh tissue/mL) were prepared in 0.154 M KCl containing 50 mM Tris-HCl pH 7.4, employing a Potter-type, Teflon-glass, motor-driven homogenizer (15).

## Hepatocyte Isolation and Culture

Hepatocytes were obtained from rats, hamsters, and guinea pigs by a collagenase perfusion technique described previously (14);  $2.5 \times 10^6$  viable cells were seeded in 60 mm tissue culture plates in 3 mL RPMI 1640 medium containing 5% fetal calf serum, 50  $\mu\text{g}/\text{mL}$  gentamicin, 1  $\mu\text{M}$  insulin, and 0.1 mM hydrocortisone-21-sodium succinate. Cultures were maintained at  $37^\circ\text{C}$  in 5%  $\text{CO}_2/95\%$  air and after 2 hr treatment was commenced by replacing the medium with either medium containing 0.4% (v/v) dimethyl sulfoxide (control cultures) or medium containing the test compounds dissolved in dimethyl sulfoxide (test cultures). Subsequently the medium was changed and the cells redosed every 24 hr. After 70 hr the hepatocytes were harvested in 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4, and whole homogenates prepared by sonication (14).

## Biochemical Determinations

Hepatocyte whole homogenate and/or liver whole homogenate fractions were assayed for activities of KCN-

insensitive palmitoyl-CoA oxidation, carnitine acetyltransferase, total and heat labile enoyl-CoA hydratase, and protein content as described previously (14–16). The activity of lauric acid hydroxylation (measured as the combined 11- and 12-hydroxylation) in hepatocyte whole homogenates was measured by the method of Lake et al. (18). Liver whole homogenate fractions were also fractionated by differential centrifugation (16) to obtain washed microsomal fractions which were assayed for content of cytochrome P-450 and protein and activities of ethylmorphine *N*-demethylase and 7-ethoxycoumarin *O*-deethylase (16). Microsomal lauric acid 11- and 12-hydroxylation was assayed as described previously (16) but employing high performance liquid chromatography to separate the two metabolites (9).

## Results

### Structure–Activity Studies

Rat hepatocytes were cultured for 70 hr with a series of six phthalate monoesters and the activities of KCN-insensitive palmitoyl-CoA oxidation and carnitine acetyltransferase determined. Whereas the former enzyme is a specific peroxisomal marker (8), carnitine acetyltransferase is located in peroxisomal, mitochondrial, and microsomal fractions (19). However, unlike the specific mitochondrial marker carnitine palmitoyltransferase (20), carnitine acetyltransferase activity is markedly induced in rat primary hepatocyte cultures by a number of peroxisome proliferators (14). The addition of mono(2-ethylhexyl) phthalate (MEHP), which is the primary metabolite of DEHP (13), produced dose-related increases in the activities of palmitoyl-CoA oxidation (Fig. 1) and carnitine acetyltransferase (Fig. 2). MEHP was more potent than either its one substituted isomer mono(1-ethylhexyl) phthalate (M1EHP) or its straight-chain isomer mono-*n*-octyl phthalate (MNOP). Similar effects were obtained with the three hexyl isomers examined, where mono(2-ethylbutyl) phthalate (M2EBP) was more potent than either mono(1-ethylbutyl) phthalate (M1EBP) or mono-*n*-hexyl phthalate (MNHP). In general, the octyl isomers were more potent than the hexyl isomers, as they stimulated enzyme activities at lower concentrations in the culture medium. In order to provide an expression of potency the concentrations of the monoesters required to elicit a 3-fold (i.e., 300% of control) increase in palmitoyl-CoA oxidation were calculated. For the straight-chain, 1- and 2-ethyl-substituted octyl isomers the values were 201, 260, and 51  $\mu\text{M}$ ; and for the straight-chain, 1- and 2-ethyl-substituted hexyl isomers the values were 508, 536, and 156  $\mu\text{M}$ . The hepatocyte culture studies would thus suggest that MEHP may be a more potent inducer of peroxisomal enzyme activities *in vivo* than M1EHP. The effect of administration of these two compounds to young male Sprague-Dawley rats for 7 days at oral dose levels of 50–500 mg/kg/day is shown in Figure 3. With both enzyme activities measured, dose-dependent effects were observed, MEHP being the more potent inducer *in vivo*.

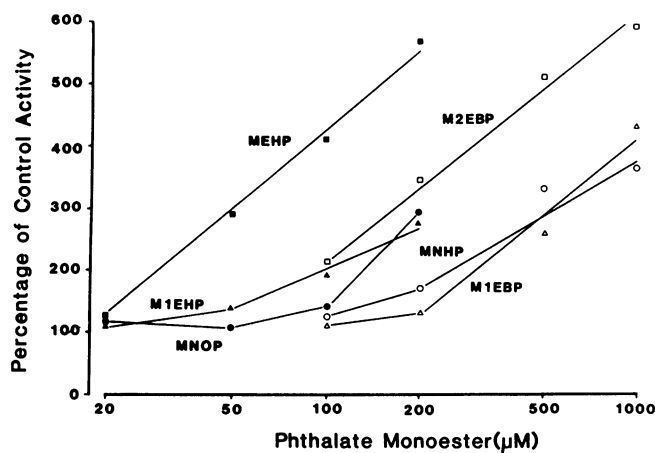


FIGURE 1. Effect of phthalate monoesters on palmitoyl-CoA oxidation in primary rat hepatocytes. Cells were cultured for 70 hr with either 20–200  $\mu\text{M}$  of the octyl isomers (closed symbols), namely mono-*n*-octyl phthalate (MNOP), mono(1-ethylhexyl) phthalate (M1EHP) and mono(2-ethylhexyl) phthalate (MEHP); or 100–1000  $\mu\text{M}$  of the hexyl isomers (open symbols), namely, mono-*n*-hexyl phthalate (MNHP), mono(1-ethylbutyl) phthalate (M1EBP), and mono(2-ethylbutyl) phthalate (M2EBP). All results are expressed as percentage of control activities and each point represents the mean of at least three experiments.

We have also observed *in vivo* potency differences in the induction of peroxisomal enzyme activities with phthalate diesters. The hypolipidemic drug clofibrate, DEHP, the straight-chain isomer of DEHP di-*n*-octyl

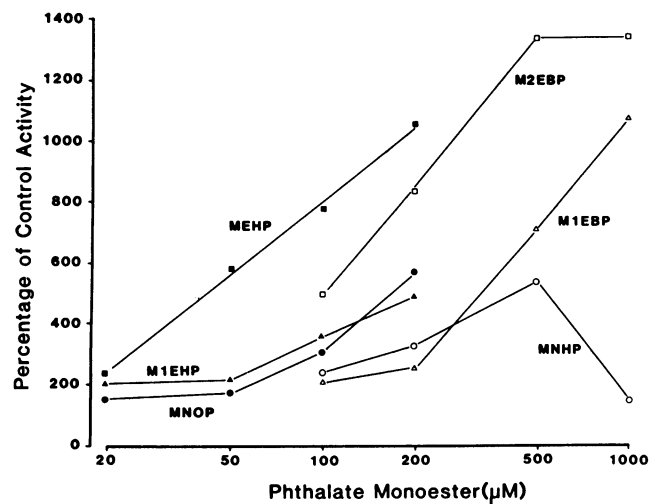


FIGURE 2. Effect of phthalate monoesters on carnitine acetyltransferase in primary rat hepatocytes. Cells were cultured for 70 hr with either 20–200  $\mu\text{M}$  of the octyl isomers (closed symbols), namely, mono-*n*-octyl phthalate (MNOP), mono(1-ethylhexyl) phthalate (M1EHP), and mono(2-ethylhexyl) phthalate (MEHP), or 100–1000  $\mu\text{M}$  of the hexyl isomers (open symbols), namely, mono-*n*-hexyl phthalate (MNHP), mono(1-ethylbutyl) phthalate (M1EBP), and mono(2-ethylbutyl) phthalate (M2EBP). All results are expressed as percentage of control activities and each point represents the mean of at least three experiments.

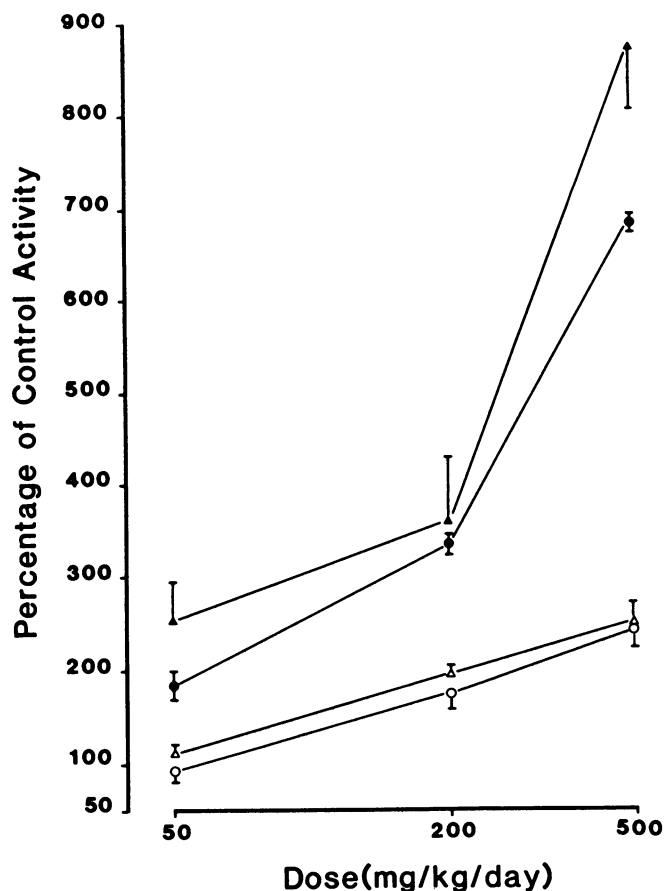


FIGURE 3. Effect of administration of phthalate monoesters on rat hepatic palmitoyl-CoA oxidation ( $\circ$ ,  $\bullet$ ) and carnitine acetyltransferase ( $\Delta$ ,  $\blacktriangle$ ) activities. Rats were treated orally with 50–500 mg/kg/day of either mono(1-ethylhexyl) phthalate (open symbols) or mono(2-ethylhexyl) phthalate (closed symbols), for 7 days. All results are expressed as percentage of control (corn oil-treated) activities and each point represents the mean  $\pm$  SEM of five animals.

phthalate (DNOP), and mono-*n*-octyl phthalate (MNOP) — which is the primary metabolite of DNOP (21) — were administered to young male Sprague-Dawley rats for 14 days. The dose levels of the phthalate esters were equimolar. Although all four compounds increased the relative liver weight (i.e., liver weight/100 g body weight) only DEHP and clofibrate produced large increases in palmitoyl-CoA oxidation (Table 1). Similarly, only DEHP and clofibrate increased total (mitochondrial and peroxisomal) and heat labile (peroxisomal) (22) enoyl-CoA hydratase activity.

### Species Difference Studies

Previous studies have demonstrated that species differences exist in the response to the hepatic effects of peroxisome proliferators (1). For example, clofibrate has been reported to increase peroxisome numbers in the rat but apparently not in the hamster (23). Accordingly, we compared the effects of DEHP and MEHP in

**Table 1. Effect of oral administration of DEHP, DNOP, MNOP, and clofibrate on liver size and some hepatic parameters in the rat.**

| Parameter                                     | Compound and dose (mg/kg/day) <sup>a,b</sup> |                |                |               |                     |
|---|--|----------------|----------------|---------------|---------------------|
|   | Control<br>(corn oil)                        | DEHP<br>(1000) | DNOP<br>(1000) | MNOP<br>(715) | Clofibrate<br>(500) |
| Relative liver weight, g liver/100 g body wt. | 3.6 ± 0.1                                    | 6.2 ± 0.1*     | 4.2 ± 0.1*     | 4.3 ± 0.1*    | 6.1 ± 0.1*          |
| Palmitoyl-CoA oxidation <sup>c</sup>          | 5.6 ± 0.3                                    | 28.3 ± 1.3*    | 6.9 ± 0.5      | 7.9 ± 0.8     | 33.2 ± 0.8*         |
| Enoyl-CoA hydratase <sup>c</sup>              |  |                |                |               |                     |
| Total activity                                | 8.6 ± 0.4                                    | 29.1 ± 1.0*    | 8.6 ± 0.5      | 9.3 ± 1.1     | 29.4 ± 2.4*         |
| Heat labile activity                          | 1.6 ± 0.1                                    | 22.1 ± 0.9*    | 2.6 ± 0.3      | 3.9 ± 0.6     | 22.9 ± 2.4*         |

<sup>a</sup> Rats were treated by daily gastric intubation for 14 days, the doses of the phthalate esters were equimolar.

<sup>b</sup> All results are expressed as the means ± SEM for groups of six animals.

<sup>c</sup> In units of nmol/min/mg whole homogenate protein.

\*Significantly different (Dunnett's test) from control,  $p < 0.01$ .

Sprague-Dawley rats and Syrian and Chinese hamsters. For purposes of comparison studies were also undertaken with clofibrate and/or clofibric acid which is the primary deethylated metabolite of clofibrate (24).

The administration of DEHP, MEHP, and clofibrate produced marked increases in liver size and activities of palmitoyl-CoA oxidation and carnitine acetyltransferase in the rat (Table 2). In contrast, these three compounds and clofibric acid produced little effect in Syrian hamsters. However, Chinese hamsters were more responsive than Syrian hamsters to the hepatic effects of DEHP, MEHP, and clofibric acid (Table 2).

The species differences in induction of peroxisomal enzyme activities observed *in vivo* could also be largely reproduced in primary hepatocyte cultures. The culture of Sprague-Dawley rat hepatocytes for 70 hr with either 20–200  $\mu$ M MEHP (Fig. 4) or 50–1000  $\mu$ M clofibric acid (Fig. 5) resulted in dose-dependent increases in palmitoyl-CoA oxidation activity. In contrast, little effect was observed in hepatocytes from either Syrian hamsters or Dunkin-Hartley guinea pigs. Previous studies have indicated that the guinea pig may be refractory to peroxisome proliferators as negative effects of both DEHP (25) and clofibrate (23) have been reported in this species.

The species difference observed *in vivo* between Syrian and Chinese hamsters was also exhibited in cell culture. The culture of Chinese hamster hepatocytes for 70 hr with either MEHP, clofibric acid, or 4-chloro-6-(2,3-xylydino)-2-pyrimidinylthio(*N*- $\beta$ -hydroxy-ethyl)-acetamide (BR-931) resulted in significant increases in palmitoyl-CoA oxidation activity (Table 3). BR-931 is known to be a potent inducer of peroxisomal enzyme activities both *in vivo* (2) and *in vitro* (14). In contrast to the Chinese hamster, only clofibric acid produced a significant increase in enzyme activity in hepatocytes from Syrian hamsters.

### Microsomal Effects of Phthalate Esters and Related Compounds

The administration of DEHP and clofibrate, but not DNOP, to male Sprague-Dawley rats resulted in small increases in hepatic microsomal cytochrome P-450 content and activities of ethylmorphine N-demethylase and 7-ethoxycoumarin O-deethylase (Fig. 6). However, both DEHP and clofibrate administration produced a marked induction of lauric acid hydroxylation and in particular of the 12-hydroxylation (i.e.,  $\omega$ -hydroxylation) of this substrate. Increased lauric acid hydroxylation was also

**Table 2. Effect of oral administration of DEHP, MEHP, clofibrate, and clofibric acid on liver size and some hepatic parameters in the rat and two hamster species.**

| Species            | Parameter <sup>c</sup> | Compound and dose (mg/kg/day) <sup>a,b</sup> |               |                     |                         |
|--------------------|------------------------|--|---------------|---------------------|-------------------------|
|                    |                        | DEHP<br>(1000)                               | MEHP<br>(500) | Clofibrate<br>(500) | Clofibric acid<br>(250) |
| Sprague-Dawley rat | RLW                    | 176*   | 165*          | 190*                | n.d. <sup>d</sup>       |
|                    | PCoA                   | 1010*  | 1380*         | 1770*               | n.d.                    |
|                    | CAT                    | 1500*  | 2100*         | 1790*               | n.d.                    |
| Syrian hamster     | RLW                    | 122*   | 110†          | 105                 | 116*                    |
|                    | PCoA                   | 187*   | 171†          | 175*                | 158*                    |
|                    | CAT                    | 197*   | 162*          | 251*                | 183†                    |
| Chinese hamster    | RLW                    | 155*   | 138*          | n.d.                | 131*                    |
|                    | PCoA                   | 559*   | 359*          | n.d.                | 438*                    |
|                    | CAT                    | 265*   | 215*          | n.d.                | 313*                    |

<sup>a</sup> Animals were treated by daily gastric intubation for 14 days.

<sup>b</sup> All results are expressed as percentage of control levels and are mean values for groups of three to six animals.

<sup>c</sup> The parameters measured were relative liver weight (RLW), palmitoyl-CoA oxidation (PCoA) and carnitine acetyltransferase (CAT).

<sup>d</sup> n.d., not determined.

\*Significantly different (Dunnett's test) from control,  $p < 0.01$ .

†Significantly different (Dunnett's test) from control,  $p < 0.05$ .

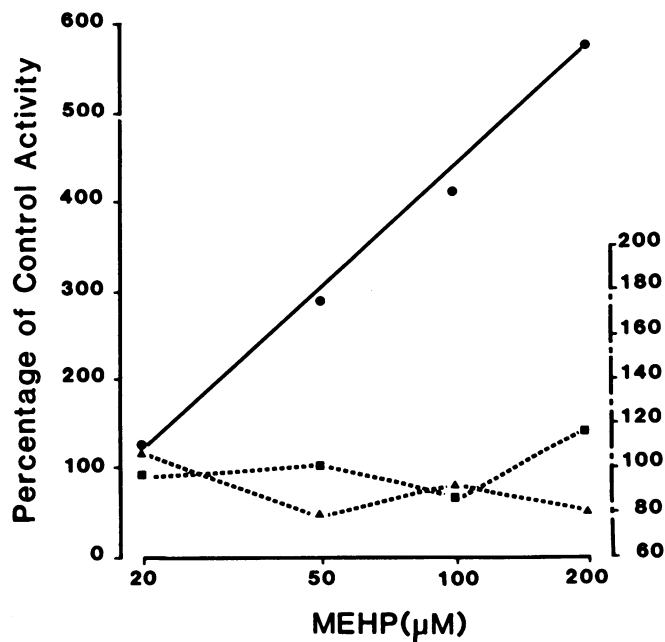


FIGURE 4. Effect of culture with 20–200  $\mu\text{M}$  MEHP for 70 hr on palmitoyl-CoA oxidation in primary hepatocyte cultures. All results are expressed as percentage of control activities for Sprague-Dawley rat (●) hepatocytes (left-hand  $y$  axis) and Syrian hamster (■) and Dunkin-Hartley guinea pig (▲) hepatocytes (right-hand  $y$  axis, i.e., the expanded scale). Each point represents the mean of at least three experiments.

observed after MEHP treatment (data not shown), whereas DNOP and MNOP induced neither peroxisomal (Table 1) nor microsomal (Fig. 6 and data not shown) fatty acid oxidation.

Employing primary hepatocyte cultures we investigated the relationship between the induction of peroxisomal and microsomal enzyme activities by a range of ten hypolipidemic agents including three phthalate monoesters, namely MEHP, MNOP and monoethyl phthalate. Rat hepatocytes were cultured for 70 hr with the compounds and the activities of carnitine acetyltransferase, palmitoyl-CoA oxidation, lauric acid hydroxylation (measured as the combined 11- and 12-hydroxylation) and 7-ethoxycoumarin *O*-deethylase determined. The induction of carnitine acetyltransferase and lauric acid hydroxylation was highly correlated ( $r = 0.980$ ,  $p < 0.001$ ) (Fig. 7), as was the induction of palmitoyl-CoA oxidation and lauric acid hydroxylation ( $r = 0.967$ ,  $p < 0.001$ ) (data not shown). In contrast, the induction of either carnitine acetyltransferase ( $r = -0.253$ ,  $p > 0.05$ ) or palmitoyl-CoA oxidation ( $r = -0.156$ ,  $p > 0.05$ ) did not correlate with a typical mixed function oxidase enzyme activity, namely 7-ethoxycoumarin *O*-deethylase (data not shown).

## Discussion

The structure activity studies indicated potency differences between various phthalate esters. For exam-

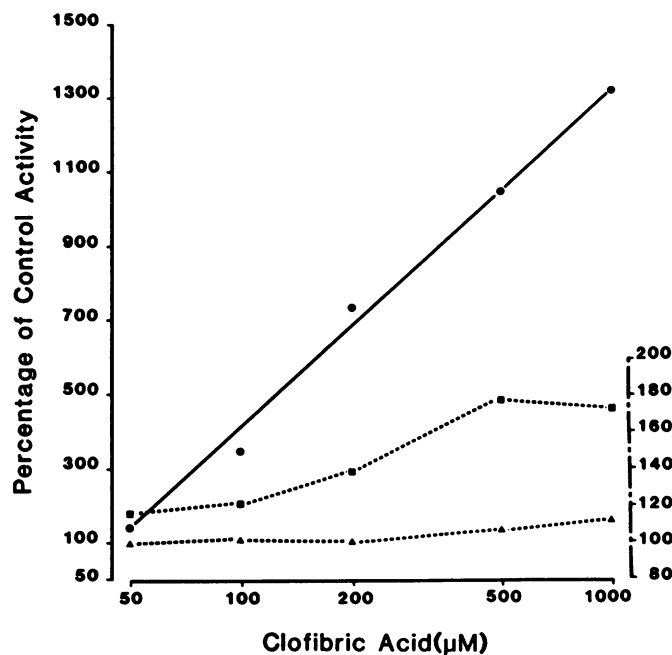


FIGURE 5. Effect of culture with 50–1000  $\mu\text{M}$  clofibric acid for 70 hr on palmitoyl-CoA oxidation in primary hepatocyte cultures. All results are expressed as percentage of control activities for Sprague-Dawley rat (●) hepatocytes (left-hand  $y$  axis) and Syrian hamster (■) and Dunkin-Hartley guinea pig (▲) hepatocytes (right-hand  $y$  axis, i.e., the expanded scale). Each point represents the mean of at least three experiments.

ple, with a range of monoesters, octyl isomers were generally more potent than hexyl isomers. Branched-chain esters, depending on the position of the side chain, may be more potent than straight-chain esters. However, the observed differences in induction of peroxisomal enzymes by phthalate esters appear, in general, to be dose-related, and thus the observed differences may be purely quantitative rather than qualitative. Certainly, in the cell culture studies no compound was truly negative, in that they all produced some stimulation of enzyme activities. We have previously demonstrated increases in peroxisome numbers in rat hepatocyte cultures after treatment with MEHP and other peroxisome proliferators (14, 26, 27). Although hepatocyte cultures cannot compensate for differences in compound phar-

Table 3. Effect of some peroxisome proliferators on palmitoyl-CoA oxidation in Chinese and Syrian hamster hepatocyte cultures.

|         | Treatment (70 hr culture) <sup>a</sup> |                        |                            |
|---------|--|------------------------|----------------------------|
|         | MEHP (0.2mM)                           | Clofibric acid (0.5mM) | BR-931 (50 $\mu\text{M}$ ) |
| Hamster |  |                        |                            |
| Chinese | 361*                                   | 508*                   | 502*                       |
| Syrian  | 119                                    | 117*                   | 132                        |

<sup>a</sup> All results are expressed as percentage of control levels and are mean values for either three or four experiments.

\* Significantly different (Dunnett's test) from control,  $p < 0.01$ .

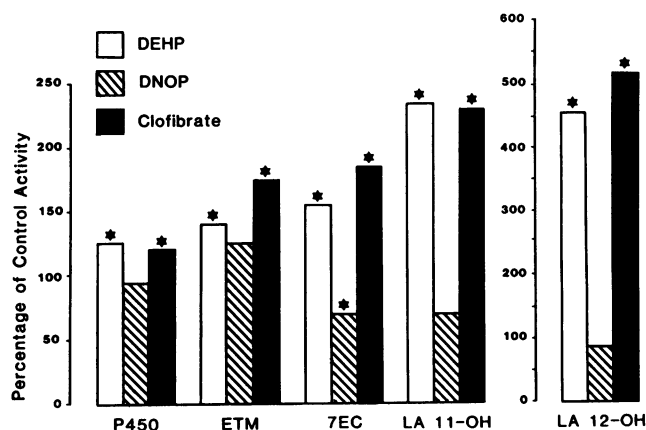


FIGURE 6. Effect of oral administration of DEHP (1000 mg/kg/day), DNOP (1000 mg/kg/day) and clofibrate (500 mg/kg/day) for 14 days on some rat hepatic microsomal parameters. The activities measured were cytochrome P-450 content (P450), ethylmorphine *N*-demethylase (ETM), 7-ethoxycoumarin *O*-deethylase (7EC), lauric acid 11-hydroxylation (LA 11-OH), and lauric acid 12-hydroxylation (LA 12-OH). All results are expressed as percentage of control (corn oil treated) activities and each point represents the mean of at least four animals. Results significantly different (Dunnett's test) from control are, (\*)  $p < 0.05$ .

macokinetics as well as possible differences between *in vivo* and *in vitro* metabolism; the present studies indicate the potential usefulness of cell cultures as a rapid and economical screen for the induction of peroxisomal enzyme activities. Primary hepatocyte cultures also reduce the requirement for experimental animals. In this study, MEHP was found to be more potent than M1EHP in inducing peroxisomal enzymes either *in vivo* or *in vitro*.

Species differences in the magnitude of induction of peroxisomal enzyme activities were observed between the rat and Syrian and Chinese hamsters. In the case of DEHP the observed difference between the Sprague-Dawley rat and Syrian hamster may be partially attributed to differences in rates of intestinal hydrolysis to MEHP and subsequent absorption and excretion (15) as well as to species differences in the subsequent metabolism of MEHP (28). However, that the *in vivo* differences between the rat and Chinese and Syrian hamsters could be largely reproduced *in vitro*, suggests that intrahepatic factors are of major importance in determining species differences in response. As would be expected from the *in vivo* studies, the induction of palmitoyl-CoA oxidation was observed in rat and Chinese hamster hepatocytes after treatment with either MEHP or clofibric acid. In contrast, Syrian hamster hepatocytes responded only weakly to clofibric acid and no significant effect was observed with either MEHP or BR-931. These observations do not correlate well with *in vivo* findings as Syrian hamsters do respond weakly to MEHP (Table 2), and the potent peroxisome proliferator BR-931 has been shown to produce a marked effect in this species after dietary administra-

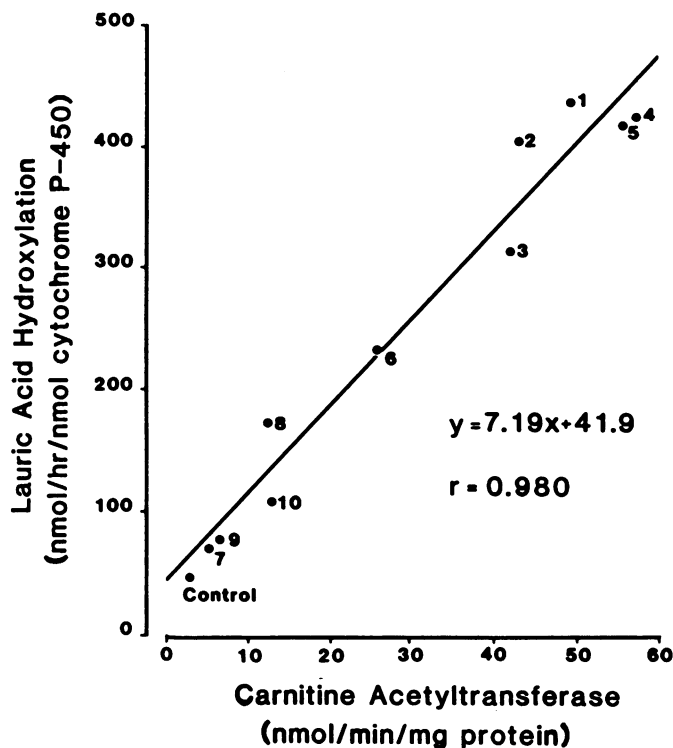


FIGURE 7. Relationship between the induction of carnitine acetyltransferase and lauric acid hydroxylation by hypolipidemic agents in primary rat hepatocytes. Cells were cultured for 70 hr with (1) Wy-14,643 (50  $\mu$ M), (2) tiadenol (50  $\mu$ M), (3) nafenopin (50  $\mu$ M), (4) BR-931 (50  $\mu$ M), (5) clofibrate (0.5 mM), (6) MEHP (0.2 mM), (7) monoethyl phthalate (0.2 mM), (8) mono-*n*-octyl phthalate (0.2 mM), (9) MDL 14,514 (50  $\mu$ M), and (10) acetylsalicylic acid (2.5 mM) or with no treatment (control). Chemical names of the hypolipidemic agents are given elsewhere (17). Each point represents the mean of either six control or four treated dishes.

tion for a period of 6 weeks (29).

Although hepatic peroxisome proliferation has been reported to occur in primate species as well as in rodents (30), controversy exists as to whether this effect occurs in man (1,2). Should further species difference studies confirm a good correlation between the *in vivo* and *in vitro* effects of peroxisome proliferators, then appropriate studies with human hepatocyte cultures may be of great value in predicting the likely human response to these compounds. Species differences could also be employed to probe the proposed relationship between peroxisome proliferation and hepatocarcinogenicity (2,3), by performing long-term studies in species such as the Syrian hamster which is less responsive than the rat to compounds such as DEHP and clofibrate.

Both clofibrate and DEHP appear to induce similar form(s) of rat hepatic microsomal cytochrome P-450 which have a high specificity for the hydroxylation of lauric acid. This was indicated by the present studies comparing effects on a number of mixed function oxidase

activities and by other studies where spectrophotometric and electrophoretic criteria were also employed (16). These effects can also be reproduced in cell culture as both clofibrate and MEHP have been shown to maintain hepatocyte cytochrome P-450 levels and to induce lauric acid hydroxylation (17,18). The cell culture studies also indicated that with a range of hypolipidemic agents, including phthalate monoesters, a close correlation exists between the induction of peroxisomal and microsomal enzyme activities. Whether the induction of novel form(s) of cytochrome P-450 by peroxisome proliferators is involved in the hepatotoxicity of these compounds remains to be elucidated.

In conclusion, the results described in this paper generally demonstrate a good correlation between the *in vivo* and *in vitro* hepatic effects of phthalate esters and related compounds. Thus the factor(s) responsible for the initiation of peroxisome proliferation and induction of peroxisomal and microsomal enzyme activities appear to be intrahepatic in nature and are retained in primary hepatocyte cultures. Hepatocyte cultures may thus be useful for further studies on the relevance of peroxisome proliferation to man (e.g., for structure-activity and species difference studies) and also for investigations into the mechanism of hepatotoxicity of peroxisome proliferators.

We thank Dr R. Purchase for the synthesis of the phthalate monoesters. This work forms part of a research project sponsored by the U.K. Ministry of Agriculture, Fisheries and Food to whom our thanks are due. The results of the research are the property of the Ministry of Agriculture, Fisheries and Food and are Crown Copyright.

## REFERENCES

- Cohen, A. J., and Grasso, P. Review of the hepatic response to hypolipidaemic drugs in rodents and assessment of its toxicological significance to man. *Food Cosmet. Toxicol.* 19: 585-605 (1981).
- Reddy, J. K., and Lalwani, N.D. Carcinogenesis by hepatic peroxisome proliferators: evaluation of the risk of hypolipidemic drugs and industrial plasticizers to humans. *Crit. Rev. Toxicol.* 12: 1-58 (1983).
- Reddy, J. K., Azarnoff, D. L., and Hignite, C. E. Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. *Nature* 283: 397-398 (1980).
- Lake, B. G., Gangolli, S. D., Grasso, P., and Lloyd, A. G. Studies on the hepatic effects of orally administered di-(2-ethylhexyl)phthalate in the rat. *Toxicol. Appl. Pharmacol.* 32: 355-367 (1975).
- Moody, D. E., and Reddy, J. K. Hepatic peroxisome (microbody) proliferation in rats fed plasticizers and related compounds. *Toxicol. Appl. Pharmacol.* 45: 497-504 (1978).
- Reddy, J. K., Moody, D. E., Azarnoff, D. L., and Rao, M. S. Di-(2-ethylhexyl)phthalate: an industrial plasticizer induces hypolipidemia and enhances hepatic catalase and carnitine acetyltransferase activities in rats and mice. *Life Sci.* 18: 941-945 (1976).
- Kluwe, W. M., Haseman, J. K., Douglas, J. F., and Huff, J. E. The carcinogenicity of dietary di(2-ethylhexyl)phthalate (DEHP) in Fischer 344 rats and B6C3F<sub>1</sub> mice. *J. Toxicol. Environ. Health* 10: 797-815 (1982).
- Lazarow, P. B., and DeDuve, C. A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by clofibrate, a hypolipidemic drug. *Proc. Natl. Acad. Sci. (U.S.)* 73: 2043-2046 (1976).
- Orton, T. C., and Parker, G. L. The effect of hypolipidemic agents on the hepatic microsomal drug-metabolizing enzyme system of the rat. Induction of cytochrome(s) P-450 with specificity toward terminal hydroxylation of lauric acid. *Drug Metab. Dispos.* 10: 110-115 (1982).
- Gibson, G. G., Orton, T. C., and Tamburini, P. P. Cytochrome P-450 induction by clofibrate. Purification and properties of a hepatic cytochrome P-450 relatively specific for the 12- and 11-hydroxylation of dodecanoic acid (lauric acid). *Biochem. J.* 203: 161-168 (1982).
- Tamburini, P. P., Masson, H. A., Bains, S. K., Makowski, R. J., Morris, B., and Gibson, G. G. Multiple forms of cytochrome P-450. Purification, characterization and comparison of a novel clofibrate-induced isozyme with other major forms of cytochrome P-450. *Eur. J. Biochem.* 139: 235-246 (1984).
- Hammock, B. D., and Ota, K. Differential induction of cytosolic epoxide hydrolase, microsomal epoxide hydrolase, and glutathione S-transferase activities. *Toxicol. Appl. Pharmacol.* 71: 254-265 (1983).
- Albro, P. W., Thomas, R., and Fishbein, L. Metabolism of diethylhexyl phthalate by rats. Isolation and characterization of the urinary metabolites. *J. Chromatogr.* 76: 321-330 (1973).
- Gray, T. J. B., Lake, B. G., Beaman, J. A., Foster, J. R., and Gangolli, S. D. Peroxisome proliferation in primary cultures of rat hepatocytes. *Toxicol. Appl. Pharmacol.* 67: 15-25 (1983).
- Lake, B. G., Gray, T. J. B., Foster, J. R., Stubberfield, C. R., and Gangolli, S. D. Comparative studies on di-(2-ethylhexyl)phthalate-induced hepatic peroxisome proliferation in the rat and hamster. *Toxicol. Appl. Pharmacol.* 72: 46-60 (1984).
- Lake, B. G., Pels Rijcken, W. R., Gray, T. J. B., Foster, J. R., and Gangolli, S. D. Comparative studies of the hepatic effects of di- and mono-*n*-octyl phthalates, di-(2-ethylhexyl) phthalate and clofibrate in the rat. *Acta Pharmacol. Toxicol.* 54: 167-176 (1984).
- Lake, B. G., Gray, T. J. B., Pels Rijcken, W. R., Beaman, J. A., and Gangolli, S. D. The effect of hypolipidaemic agents on peroxisomal  $\beta$ -oxidation and mixed-function oxidase activities in primary cultures of rat hepatocytes. Relationship between induction of palmitoyl-CoA oxidation and lauric acid hydroxylation. *Xenobiotica* 14: 269-276 (1984).
- Lake, B. G., Gray, T. J. B., Stubberfield, C. R., Beaman, J. A., and Gangolli, S. D. Induction of lauric acid hydroxylation and maintenance of cytochrome P-450 content by clofibrate in primary cultures of rat hepatocytes. *Life Sci.* 33: 249-254 (1983).
- Bieber, L. L., Krahl, J. B., Clarke, P. R. H., Valkner, K. J., and Tolbert, N. E. Carnitine acetyltransferases in rat liver peroxisomes. *Arch. Biochem. Biophys.* 211: 599-604 (1981).
- Ishii, H., Fukumori, N., Horie, S., and Suga, T. Effects of fat content in the diet on hepatic peroxisomes of the rat. *Biochim. Biophys. Acta* 617: 1-11 (1980).
- Albro, P. W., and Moore, B. Identification of the metabolites of simple phthalate diesters in rat urine. *J. Chromatogr.* 94: 209-218 (1974).
- Osumi, T., and Hashimoto, T. Peroxisomal  $\beta$ -oxidation system of rat liver. Copurification of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase. *Biochem. Biophys. Res. Commun.* 89: 580-584 (1979).
- Svoboda, D., Grady, H., and Azarnoff, D. Microbodies in experimentally altered cells. *J. Cell Biol.* 35: 127-152 (1967).
- Emudianughe, T. S., Caldwell, J., Sinclair, K. A., and Smith, R. L. Species differences in the metabolic conjugation of clofibrac acid and clofibrate in laboratory animals and man. *Drug Metab. Dispos.* 11: 97-102 (1983).
- Osumi, T., and Hashimoto, T. Enhancement of fatty acyl-CoA oxidizing activity in rat liver peroxisomes by di-(2-ethylhexyl)phthalate. *J. Biochem.* 83: 1361-1365 (1978).
- Gray, T. J. B., Beaman, J. A., Lake, B. G., Foster, J. R., and Gangolli, S. D. Peroxisome proliferation in cultured rat hepatocytes produced by clofibrate and phthalate ester metabolites. *Toxicol. Lett.* 10: 273-279 (1982).
- Gray, T. J. B., Lake, B. G., Beaman, J. A., Foster, J. R., and Gangolli, S. D. Peroxisomal effects of phthalate esters in primary cultures of rat hepatocytes. *Toxicology* 28: 167-179 (1983).

28. Albro, P. W., Corbett, J. T., Schroeder, J. L., Jordan, S., and Matthews, H. B. Pharmacokinetics, interactions with macromolecules and species differences in metabolism of DEHP. *Environ. Health Perspect.* 45: 19-25 (1982).
29. Reddy, M. K., Lalwani, N. D., Qureshi, S. A., and Reddy, J. K. Induction of hamster hepatic peroxisomal  $\beta$ -oxidation and peroxisome proliferation-associated 80000 mol. wt. polypeptide by hypolipidemic drugs. *Human Toxicol.* 1: 135-147 (1982).
30. Reddy, J. K., Lalwani, N. D., Qureshi, S. A., Reddy, M. K., and Moehle, C. M. Induction of hepatic peroxisome proliferation in nonrodent species, including primates. *Am. J. Pathol.* 114: 171-183 (1984).