Biochemical Pharmacology, Vol. 39, No. 3, pp. 527–535, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
© 1990. Pergamon Press plc

EFFECTS OF HEPATIC PEROXISOME PROLIFERATORS AND 12-*O*-TETRADECANOYL PHORBOL-13-ACETATE ON CATALASE AND OTHER ENZYME ACTIVITIES OF EMBRYONIC CELLS *IN VITRO*

SVEIN-OLE MIKALSEN,* BENTE RUYTER† and TORE SANNER

Laboratory for Environmental and Occupational Cancer, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 Oslo 3 and †Institute for Nutrition Research, University of Oslo, N-0316 Oslo 3, Norway

(*Received* 22 *November* 1988; *accepted* 3 *September* 1989)

Abstract—The effects of the hepatic peroxisome proliferators (HPPs) clofibrate, di-(2-ethylhexyl)-phthalate (DEHP), mono-(2-ethylhexyl)phthalate (MEHP) and 2,4-dichlorophenoxy acetic acid (2,4-D) on the activities of some peroxisome-associated enzymes and marker enzymes for other organelles, have been studied in primary Syrian hamster embryo (SHE) cells and Wistar rat embryo (WRE) cells. The majority of the cells are fibroblast-like. 12-*O*-Tetradecanoyl phorbol-13-acetate (TPA) was included as it has been suggested that it may act as a peroxisome proliferator. The specific activities of catalase, fatty acyl-CoA oxidase (FAO) and peroxisomal β-oxidation were approximately 100-fold lower in the embryonic cells than in rat hepatocytes. Other peroxisome-associated oxidases were not detected. The dihydroxyacetone-phosphate acyltransferase (DHAPAT) activity was comparable to that in rat liver. Marker enzymes for other organelles had specific activities comparable to rat hepatocytes. Catalase was shown by digitonin titration to be contained in a peroxisome-like compartment in both SHE and WRE cells. Clofibrate, DEHP and MEHP increased the catalase activity, which might suggest peroxisome proliferation. However, the findings that FAO and peroxisomal β-oxidation did not increase or only very slightly, argue against peroxisome proliferation. 2,4-D and TPA induced no or only a very slight increase in the catalase activity.

A number of important chemicals have been found to induce hepatic neoplasms in rodents although they appear to be inactive in short-term genotoxic tests. Several of these chemicals induce hepatic peroxisome proliferation [1, 2]. Hepatic peroxisome proliferators (HPPs‡) are a diverse group of chemical compounds consisting of hypolipidemic drugs [3, 4], industrial plasticizers [5], herbicides [6, 7] and several other substances [8, 9]. Concurrently with the induction of hepatic peroxisomes, the HPPs also induce a 10- to 30-fold increase in the peroxisomal β-oxidation of fatty acids and a more modest increase in the catalase activity, 1.5- to 2-fold [10, 11]. The activities of some of the other peroxisome-associated enzymes such as urate oxidase, L-α-hydroxyacid oxidase and D-amino acid oxidase might decrease after HPP-exposure [11, 12]. On the basis of such measurements, it has been proposed that HPP-mediated hepatocarcinogenesis is caused by oxidative stress due to the much greater increase in H_2O_2-generating β-oxidation than in the H_2O_2-degrading catalase [1, 13].

Differences in species sensitivities have been found for some HPPs. The hamster seems less sensitive for di-(2-ethylhexyl)phthalate (DEHP), clofibrate- and bezafibrate-induced peroxisome proliferation than the rat [4, 5, 14] and may also be less sensitive for DEHP-induced hepatocarcinogenesis [15]. It is therefore interesting that DEHP [16–18], clofibrate and tiadenol§ induced morphological transformation (altered growth pattern of cells in colonies) of Syrian hamster embryo (SHE) cells *in vitro*.

In the present work we have studied the effects of some known HPPs and of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) on the activities of a range of peroxisomal and other marker enzymes in SHE cells. For comparison, we also studied embryonic cells from a more HPP-susceptible species, the rat. TPA was included as it has been suggested that TPA might be classified as a peroxisome proliferator [19] since it increases some peroxisome-associated enzymes in C3H10T1/2 cells.

MATERIALS AND METHODS

Chemicals

Clofibrate, 2,4-dichlorophenoxy acetic acid (2,4-D), TPA, PDD and digitonin were obtained from Sigma Chemical Co. (St Louis, MO). DEHP was purchased from Aldrich Chemical Co. (Steinheim, F.R.G.). Mono-(2-ethylhexyl)phthalate (MEHP) was obtained from Tokyo Kasei Kogyo (Tokyo,

* To whom correspondence should be addressed.

‡ Abbreviations: CHO, Chinese hamster ovary; clofibrate, ethyl-2-(*p*-chlorophenoxy) isobutyrate; 2,4-D, 2,4-dichlorophenoxy acetic acid; DEHP, di-(2-ethylhexyl)phthalate; DHAPAT, dihydroxyacetone-phosphate acyltransferase; FAO, fatty acyl-CoA oxidase; HPP, hepatic peroxisome proliferator; MEHP, mono-(2-ethylhexyl)phthalate; Me_2SO, dimethyl sulfoxide; PDD, phorbol-12,13-didecanoate; SHE, Syrian hamster embryo; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate: WRE, Wistar rat embryo.

§ Mikalsen SO, Holen I and Sanner T, submitted.

Japan). The chemicals were dissolved in dimethylsulfoxide (Me_2SO) before addition to the culture medium. The maximum final concentration of Me_2SO was 0.2% (v/v). L-[U-^{14}C]glycerol-3-phosphate (170 mCi/mmol) and [1-^{14}C]palmitoyl-CoA (59.2 mCi/mmol) was purchased from Amersham (Amersham, U.K.).

Rat hepatocytes

Isolated hepatocytes were obtained from Department of Tissue Culture, Institute for Cancer Research. The hepatocytes were prepared from 18 hr starved male Wistar rats, 250–300 g [20]. The hepatocytes were homogenized by sonication and stored at −70°.

Cell cultures

All cells were grown in Dulbecco's modification of Eagle's medium (Flow Laboratories, Irvine, U.K.) supplemented with 10% fetal calf serum (GIBCO, Glasgow, U.K.) at 37° in a 10% CO_2 atmosphere.

Primary cell cultures of Syrian hamster (Wright, Chelmsford, U.K.) embryos at 14 days of gestation were prepared and cryopreserved in liquid nitrogen as described by Pienta *et al.* [21]. Wistar Kyoto rat (Møllegaard, Ejby, Denmark) embryo cells were prepared and handled exactly in the same manner as for the SHE cells, except that preparation was done at day 17 of gestation. For the present experiments it is important to notice that all intestines, including liver, were removed before trypsinization of the carcasses.

Ampoules with cryopreserved cells were used as stock cultures for the enzyme measurements. Mass cultures were grown for 5 days in 100-mm Petri dishes (Costar, Cambridge, MA, U.S.A.) before trypsinization and reseeding at different cell densities in 100-mm dishes. The number of cells were varied in such a way that the dishes were approximately confluent at harvesting of the cells. The cells were exposed to chemicals 24 hr after reseeding, and harvested at 1, 2, 3, 4 or 6 days of exposure. The cells were trypsinized, centrifuged at 200 *g* for 10 min and washed twice in 0.9% NaCl buffered with 20 mM Hepes (pH 7.4), suspended in 0.25 M sucrose at 25×10^6 cells/mL, homogenized and stored at −70°.

For DEHP and clofibrate the concentrations used were limited by the solubility of the chemicals (at higher concentrations they precipitated and produced etchings in the dishes). MEHP was used at equimolar concentration with DEHP. It has been shown that these concentrations of DEHP and clofibrate induced morphological transformation of SHE cells.* This also applies for the concentration of TPA used.

Enzyme assays

All enzyme measurements except digitonin titration (see below) were performed with the cell homogenate, usually after freezing. Freezing and thawing of the cells did not change the activity of any of the measured enzymes. Dihydroxyacetonephosphate acyltransferase (DHAPAT) and cyanide-resistant (peroxisomal) β-oxidation were only measured in cells subjected to freezing and thawing. If not otherwise stated, the enzyme activities (except DHAPAT) were measured in the presence of 0.1–0.3% Triton X-100.

Catalase was measured by the titanoxy sulphate method essentially as described by Baudhuin [22]. *Acid phosphatase* was measured according to Barrett [23]. The *glucose-6-phosphatase* assay was modified from Leighton *et al.* [24] using 80 mM glucose-6-phosphate in 0.1 M acetate buffer pH 6.5 as substrate buffer. *Malate dehydrogenase* was measured as described by Englard and Siegel [25]. *Dihydroxyacetonephosphate acyltransferase* (DHAPAT) was assayed according to published procedures [26, 27]. DHAPAT may have a bi- or trimodal distribution in the cells [28, 29]. It has been proposed that only peroxisomal DHAPAT activity is measured in the presence of 5 mM glycerol-3-phosphate [28]. Extra-peroxisomal DHAPAT has different optimum requirements than peroxisomal DHAPAT and therefore only a very rough estimation can be obtained by this method. In some measurements 5 mM glycerol-3-phosphate was included in the assay for estimation of the peroxisomal part of the total DHAPAT activity. *Peroxisomal β-oxidation* was assayed by the radioactive method of Lazarow [30] with the modification that 1 mM KCN was included in the assay. The activities in SHE and WRE cells were low. Only above 30 μg protein/assay a reproducible activity was found, thereafter the activity was linear to well above 100 μg protein/assay (not shown). In the reported experiments we have used 75–85 μg protein/assay to avoid different levels of protein interaction with the assay. Using this amount of protein, the activity was linear for more than 45 min (not shown). Due to the low activity, there were difficulties in obtaining reproducible results with incubation times shorter than 15 min. For the results reported here an incubation time of 30 min was used. *Peroxisomal* H_2O_2*-producing oxidases* were measured by several methods. D-Amino acid oxidase, L-α-hydroxy acid oxidase, urate oxidase and fatty acyl-CoA oxidase (FAO) were measured by the 2′,7′-dichlorofluorescin method, slightly modified from Small *et al.* [31]. The substrate concentrations were 25 mM D-alanine, 25 mM glycolic acid, 50 μM uric acid and 50 μM palmitoyl-CoA, respectively. Higher concentrations of uric acid was found to give unacceptable high background reaction. D-Amino acid oxidase [32–34] and L-α-hydroxy acid oxidase [32, 35, 36] were also measured by alternative methods as described. *Palmitoyl-CoA hydrolase* was measured by using 5,5′-dithio-bis-(2-nitrobenzoic acid) [37].

Other enzymes. Glutamate dehydrogenase [38] was measured as described. Glutamate dehydrogenase was also measured as described by Wanders *et al.* [32] with the exception that the reaction was followed directly by the oxidation of NADH at 340 nm. Succinate dehydrogenase was measured by the $K_3Fe(CN)_6$-method [39]. Lactate dehydrogenase was assayed using 500 μL 0.2 M Tris–HCl pH 8.0, 200 μL 5 mM pyruvate, 200 μL 1.5 mM NADH and 100 μL sample.

Digitonin titration. The intracellular compartmentations of the marker enzymes were indicated by

* Mikalsen SO, Holen I and Sanner T, submitted.

digitonin titration [32, 40]. Digitonin was dissolved in Me_2SO to 100 mg/mL. When digitonin titration studies of the various enzymes were performed, Triton X-100 was not included in the incubation medium and cells were used freshly from harvesting. The maximum levels of the enzyme activities were determined in the presence of Triton X-100.

Protein. Protein was determined using the Bio-Rad protein assay kit. Standard protein was bovine gamma globulin.

Western blotting. Reduced samples were run on 10% sodium dodecyl sulphate–polyacrylamide gels (0.75 mm thick), and blotted onto nitrocellulose membranes. Rabbit anti-catalase antiserum (courtesy of Drs E. Wiemer and J. M. Tager, Department of Biochemistry, University of Amsterdam) was diluted 1:250. Goat anti-rabbit antiserum conjugated with horseradish peroxidase (Bio-Rad) and the Bio-Rad developer were used to visualize the catalase bands. Parallel gels were silver stained. Crystallized bovine liver catalase (×2) (Sigma) was applied as a standard for catalase. It was diluted 10-fold in water. Residual crystals were spun down before measuring protein in the supernatant.

Calculations and statistics

The relative enzyme activities are always calculated as the ratio of (specific enzyme activity in exposed cells)/(specific enzyme activity in unexposed control cells) in cells seeded and harvested at the same time in the same experiment.

Where $N \geq 4$, the statistical significance was tested by a computerized version (Tadpole; Elsevier Biosoft, Cambridge, U.K.) of Mann-Whitney U test (2-sided) on the relative enzyme activities.

RESULTS

Enzyme activities in unexposed cells

The activities of several peroxisome-associated enzymes were measured. Catalase was readily measurable in SHE and WRE cells, but the specific activities were approximately 100-fold lower than in rat liver (Table 1). The difference in catalase activity is consistent with the differences in the amount of catalase protein as detected by Western blotting (not shown).

A low activity of the total peroxisomal β-oxidation cycle was found in both SHE and WRE cells by the radioactive assay (Table 1). The specific activity is 100-fold lower than in rat liver, consistent with the low catalase activities in the embryo cells. The first enzyme in the peroxisomal β-oxidation, the FAO, showed a specific activity approximately 10-fold higher than the activity of the peroxisomal β-oxidation cycle in both SHE and WRE cells, while in rat hepatocytes there was a good agreement between the two assays (Table 1).

The possibility exists that the observed low peroxisomal β-oxidation is partly caused by interferences by palmitoyl-CoA hydrolase. The palmitoyl-CoA hydrolase activity in WRE cells and rat hepatocytes were comparable (Table 1), while it was 5-fold lower in SHE cells. The activity was disproportionally high in SHE and WRE cells compared to the activity of the β-oxidation cycle. However, when ATP (10 mM) and Mg^{2+} (5 mM) were added to the incubation mixture in the β-oxidation assay, no increase in the activity was found even at a low palmitoyl-CoA concentration (1/10 of normal concentration, not shown). Although the palmitoyl-CoA synthetase activity has not been specifically studied, the results above suggest that the high palmitoyl-CoA hydrolase activity did not affect the observed activity for peroxisomal β-oxidation. The results for the linearities of peroxisomal β-oxidation (see Materials and Methods) also support this conclusion.

The other peroxisomal oxidases tested (D-amino acid oxidase, L-α-hydroxy acid oxidase and urate oxidase) were easily detected in rat hepatocytes by the 2′,7′-dichlorofluorescin method (which was found to be the most sensitive of the tested oxidase assays), while none of these activities were detected in the embryonic cells (Table 1).

DHAPAT showed activities comparable (2- to 3-fold lower) to that of rat liver homogenate (Table 1). A 35–80% inhibition of the DHAPAT activity was found for 5 mM glycerol-3-phosphate in the embryonic cells (not shown).

The activities of the marker enzymes for lysosomes (acid phosphatase) and for endoplasmic reticulum (glucose-6-phosphatase) showed activities in the same order of magnitude in SHE and WRE cells and in rat liver homogenate (Table 1). The mitochondrial marker enzymes succinate dehydrogenase and glutamate dehydrogenase (not shown) showed very low or not detectable levels of activities, while malate dehydrogenase showed activities comparable to rat liver (Table 1). The cytosolic marker enzyme lactate dehydrogenase was easily detectable (Table 1).

In cells cultured for several days, the specific enzyme activities often showed systematic changes. In SHE cells, the catalase activity increased by more than 30% from day 1 to day 6, and acid phosphatase and glucose-6-phosphatase increased by 75%. For the WRE cells, acid phosphatase increased 35%. The other enzymes showed minor or inconsistent changes.

Digitonin titration

An impression of the compartmentation of the different marker enzymes was obtained by digitonin titration of marker enzymes on freshly harvested cells. The results of digitonin titration of SHE cells and WRE cells are shown in Figs 1 and 2, respectively. With the exception for the two phosphatases in WRE cells, the enzyme activities found with no digitonin present (10–20% of the maximum activities) corresponded to the activities in aliquots of the cell free supernatants (not shown), and are therefore probably due to enzymes released from broken cells and organelles. The phosphatase activities in WRE cells behaved differently, but the high activities found with no digitonin present in the assay can be explained by a relatively unspecific trypsin-insensitive cell membrane-associated phosphatase in WRE cells (not shown).

Catalase was less sensitive to digitonin unmasking than the other enzymes in both cell types, while lactate dehydrogenase was more sensitive (Figs 1 and 2). At 10 μg digitonin/mL, 80–90% of the lactate dehydrogenase activity was unmasked, while the

Table 1. A comparison of enzyme activities in SHE cells,* WRE cells* and rat hepatocytes†

Enzyme	Specific enzyme activities		
	SHE cells	WRE cells	Rat liver
Catalase‡	0.24	0.65	90
Peroxisomal β-oxidations§‖	0.025	0.025	3.25
Fatty acyl-CoA oxidase§	0.30	0.35	3.80
Palmitoyl-CoA hydrolase§	1.9	10	10
D-Amino acid oxidase§	ND	ND	2.0
L-α-Hydroxy acid oxidase§	ND	ND	6.0
Urate oxidase§	ND	ND	5.0
DHAPAT§	0.030	0.030	0.085
Acid phosphatase§	10	12	33
Glucose-6-phosphatase§	4.0	17.5	25
Malate dehydrogenase‡	0.20	0.10	0.10
Lactate dehydrogenase§	1.2	0.80	NT

* In cells cultured for several days, the enzyme activities often showed systematic changes (see text). The data represent the activities of unexposed control cells 48 hr after reseeding (corresponding to day 1 of Figs 3 and 4). The specific enzyme activities were found to vary somewhat, depending on both batch of cells used and as well as unknown variables.

† Isolated hepatocytes from three rats were used for all measurements, with the exception of DHAPAT where homogenate from one rat liver was used. The number given is the mean of the measurements. The range for all measurements were within ±15%.

‡ μmol/min/mg protein.

§ nmol/min/mg protein.

‖ Measured by the radioactive method [30].

ND, not detected.

NT, not tested.

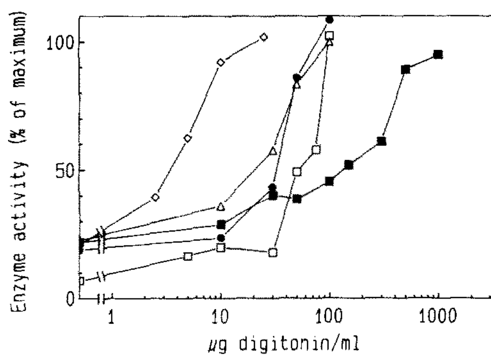

Fig. 1. Digitonin latency of marker enzymes in SHE cells. (■) Catalase, N = 5–8, SD ≤ ±7%. (□) Malate dehydrogenase, N = 3–4, SD = ±20% for 50, 75 and 100 μg digitonin/mL, SD ≤ ±7% for the rest. (●) Acid phosphatase, N = 4–5, SD ≤ ±18%. (△) Glucose-6-phosphatase, N = 3, SD ≤ ±10%. (◇) Lactate dehydrogenase, N = 4, SD = ±14% for 2.5 μg digitonin/mL, SD = ±26% for 5 μg digitonin/mL, SD ≤ ±8% for the rest.

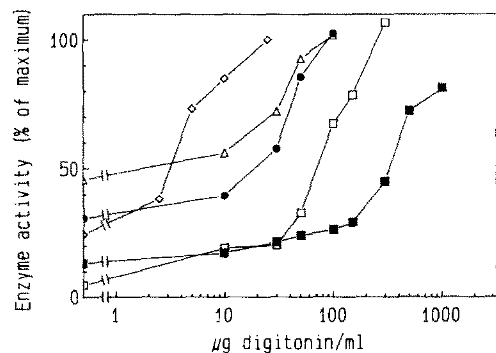

Fig. 2. Digitonin latency of marker enzymes in WRE cells. (■) Catalase, N = 4, SD ≤ ±13%. (□) Malate dehydrogenase, N = 3, SD ≤ ±15%. (●) Acid phosphatase, N = 5, SD = ±23% for 10 μg digitonin/mL, SD ≤ ±15% for the rest. (△) Glucose-6-phosphatase, N = 4–6, SD ≤ ±14%. (◇) Lactate dehydrogenase, N = 3, SD ≤ ±10%.

other enzymes had increased their free activities with maximum 10–15% at this digitonin concentration. Thus, with the exception of lactate dehydrogenase, the enzymes had little, if any, cytosolic location. At 100 μg digitonin the activity of catalase was only slightly elevated, while the activities of the other enzymes were nearly completely unmasked.

Effects of HPPs and TPA

The induction of catalase by HPPs and TPA is shown in Figs 3 and 4. The catalase activity in SHE cells (Fig. 3) increased with time in the presence of DEHP (more than 1.4-fold increase at day 6 of exposure). A single experiment with MEHP indicated that also MEHP increased the catalase activity in SHE cells.

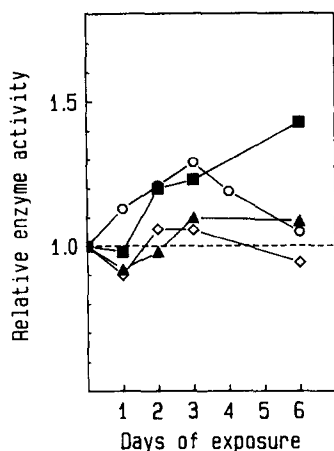

Fig. 3. Catalase activity in SHE cells exposed to (■) 77 μM DEHP (30 μg/mL, N = 5, SD ≤ ±0.20); (○) 206 μM clofibrate (50 μg/mL, N = 4–7, SD ≤ ±0.13); (◇) 135 μM 2,4-D (30 μg/mL, N = 3, SD ≤ ±0.07); and (▲) 16 nM TPA (0.01 μg/mL, N = 4, SD ≤ ±0.10) relative to unexposed cells. P ≤ 0.05: TPA days 3 and 6; clofibrate day 4. P ≤ 0.01: DEHP days 2, 3 and 6. P ≤ 0.001: clofibrate days 2 and 3.

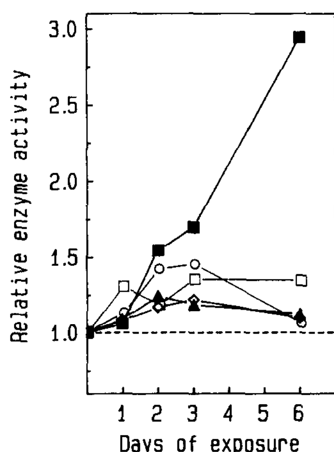

Fig. 4. Catalase activity in WRE cells exposed to (■) 77 μM DEHP (N = 3, SD = 1.0 at day 6, SD ≤ ±0.35 for the rest); (□) 77 μM MEHP (N = 3, SD ≤ ±0.25); (○) 206 μM clofibrate (N = 3, SD ≤ ±0.25); (◇) 135 μM 2,4-D (N = 2, range ≤ ±0.15); and (▲) 16 nM TPA (N = 2, range ≤ ±0.12) relative to unexposed cells.

In the presence of clofibrate the maximum activity occurred at day 3 (1.3-fold), and was back to control level at day 6. Refeeding experiments indicate that this may be due to metabolism of clofibrate to pharmacologically inactive products.* 2,4-D and TPA induced no or only a very small increase in the catalase activity in SHE cells (Fig. 3).

In WRE cells the induction of catalase was much more pronounced by DEHP, more than 2-fold after 6 days of exposure (Fig. 4). MEHP also induced an increase in the catalase activity, but the increase was relatively constant over the time studied and it was lower than for DEHP (Fig. 4). As for SHE cells, clofibrate was found to increase the catalase activity in WRE cells during the first 3 days, whereafter the activity declined to control level at day 6 (Fig. 4). 2,4-D and TPA induced a weak increase in the catalase activity (about 1.2-fold). To see if the low response was caused by rapid metabolizing of TPA, the WRE cells were refed every 24 hr with fresh TPA, as these cells seemed somewhat more sensitive for catalase induction. The relative catalase activity did not, however, increase over 1.2 (not shown). Moreover, for PDD which is less rapidly metabolized than TPA, we also found a relative catalase activity of about 1.2 (not shown).

The location of the catalase activity in DEHP-exposed cells was identical to that of control cells as determined by digitonin titration, even though the catalase activity was 40–60% above unexposed control cells (not shown).

The peroxisomal β-oxidation of palmitoyl-CoA was only slightly affected by clofibrate, DEHP or MEHP (Tables 2 and 3) compared to the 10- to 30-fold increase reported in rat liver. The effect of MEHP was not greater than the effect of DEHP, although MEHP is probably the main active metabolite of DEHP. The relative activities of FAO were very similar to the results of the radioactive method (Tables 2 and 3). For palmitoyl-CoA hydrolase, no consistent increase was found when WRE cells were exposed to HPPs (Table 2), while there seemed to be a slight increase in SHE cells (Table 3). The DHAPAT activity was found to be somewhat increased in clofibrate and DEHP-exposed cells (Tables 2 and 3), but no consistent change in the relative peroxisomal activity of DHAPAT was found in exposed cells as judged by 5 mM glycerol-3-phosphate-insensitive activity (not shown). In contrast to clofibrate and DEHP, TPA seemed to lower the total DHAPAT activity in both WRE (Table 2) and SHE (Table 3) cells.

The activities of glucose-6-phosphatase and acid phosphatase did not show any increase for the studied chemicals in neither SHE (Table 4) nor WRE cells (not shown). The activity of malate dehydrogenase in SHE cells exposed to DEHP seemed to increase, but due to the relatively large deviations a statistical significance was not found (Table 4). Although only enzyme activities for day 3 and 6 are shown in Tables 2–4, all activities have also been measured for day 1 and 2. No unexpected or divergent results were found for day 1 and 2.

DISCUSSION

The peroxisome-associated enzyme activities, with the exception of DHAPAT, were much lower in the embryo cells studied than in rat liver. In fetal rat hepatocytes the peroxisomes first appeared at day 13–14 of gestation [41], and the number of peroxisomes were lower than in neonatal hepatocytes [41, 42]. Measurable activities of catalase and peroxisomal β-oxidation in fetal rat liver have first been found at day 15 [43], and D-amino acid oxidase and urate oxidase at day 18–21 of gestation [41, 42]. A low number of peroxisomes in SHE and WRE cells

* Mikalsen SO, Holen I and Sanner T, submitted.

Table 2. Effects of DEHP, MEHP, clofibrate and TPA on the relative activities of lipid metabolizing enzymes in WRE cells after 3 and 6 days of exposure

Chemical	Days	Peroxisomal β-oxidation	FAO	DHAPAT	Palmitoyl-CoA hydrolase
Clofibrate	3	1.44 ± 0.04(3)	1.55 ± 0.74(3)	NT	0.83 ± 0.34(3)
(206 μM)	6	1.17 ± 0.01(3)	1.03 ± 0.54(3)	NT	0.82 ± 0.10(3)
DEHP	3	1.27 ± 0.19(5)*	0.85 ± 0.43(3)	0.93, 1.50	0.99 ± 0.39(3)
(77 μM)	6	1.38 ± 0.26(5)*	1.03 ± 0.54(3)	1.27, 1.45	1.18 ± 0.15(3)
MEHP	3	1.21 ± 0.04(3)	1.43 ± 0.09(3)	NT	1.09 ± 0.17(3)
(77 μM)	6	1.17 ± 0.19(3)	0.77 ± 0.29(3)	NT	1.27 ± 0.18(3)
TPA	3	1.50	NT	0.88	NT
(16 nM)	6	1.20	NT	0.92	NT

Where two experiments have been done, the results for both experiments are shown. Where one number is given, it is the result of a single experiment assayed in 3 to 6 parallels. For the other results, the number of experiments are given in parentheses.
* $P \leq 0.01$.
NT, not tested.

Table 3. Effects of clofibrate, DEHP, MEHP and TPA on relative activities of lipid metabolizing enzymes in SHE cells after 3 and 6 days of exposure

Chemical	Days	Peroxisomal β-oxidation	FAO	DHAPAT	Palmitoyl-CoA hydrolase
Clofibrate	3	1.03 ± 0.23(4)	1.43, 1.57	1.21, 1.45	1.27, 1.00
(206 μM)	6	0.94 ± 0.11(4)	0.88, 1.68	1.44, 1.66	1.35, 0.88
DEHP	3	1.78 ± 0.55(5)*	1.35, 1.26	1.92	1.88, 1.47
(77 μM)	6	1.74 ± 0.38(4)†	1.04, 1.77	1.72	1.30, 1.70
MEHP	3	1.11, 0.71	1.06, 1.79	NT	1.24, 1.26
(77 μM)	6	1.00, 1.90	1.17, 1.32	NT	0.91, 1.08
TPA	3	1.24, 1.92	NT	0.72, 0.92	NT
(16 nM)	6	0.55, 1.73	NT	0.86, 0.94	NT

Where two experiments have been done, the results for both experiments are shown. Where one number is given, it is the result of a single experiment assayed in 3 to 6 parallels. For the other results, the number of experiments are given in parentheses.
* $P \leq 0.01$.
† $P \leq 0.05$.
NT, not tested.

Table 4. Effects of clofibrate, DEHP, 2,4-D and TPA on relative activities of non-peroxisomal enzymes in SHE cells after 3 and 6 days of exposure

Chemical	Days	Acid phosphatase	Glucose-6-phosphatase	Malate dehydrogenase
Clofibrate	3	0.98 ± 0.13(7)	0.94 ± 0.06(5)	1.16 ± 0.33(7)*
(206 μM)	6	0.92 ± 0.09(5)	0.90 ± 0.11(4)	0.97 ± 0.13(5)
DEHP	3	0.80 ± 0.13(4)*	0.82 ± 0.09(5)*	1.68 ± 0.52(5)
(77 μM)	6	0.87 ± 0.09(4)*	0.81 ± 0.06(3)	1.56 ± 0.07(3)
2,4-D	3	1.04 ± 0.07(3)	1.07 ± 0.13(3)	1.09 ± 0.06(3)
(135 μM)	6	1.02 ± 0.04(3)	1.08 ± 0.13(3)	0.99 ± 0.31(3)
TPA	3	0.90 ± 0.07(4)*	0.90 ± 0.10(4)	0.84 ± 0.13(4)*
(16 nM)	6	0.90 ± 0.06(4)*	0.98 ± 0.06(4)	0.95 ± 0.10(4)

The number of experiments are given in parentheses.
* $P \leq 0.05$.

and low peroxisome-associated enzyme activities might therefore be anticipated.

The digitonin titration results for catalase indicate the presence of peroxisomes in SHE and WRE cells. Furthermore, they suggest that less than 10% of the catalase activity is cytosolic and that the majority of the catalase is located in organelles with a peroxisome-like character. This is consistent with the results in normal human fibroblasts [32]. In rat hepatocytes 30% of the catalase may be cytosolic [40], although the compartmentation has been disputed [40].

The presence of DEHP, MEHP or clofibrate in the culture medium increased the catalase activity in both SHE and WRE cells compared with unexposed cells. These increases are comparable to the increase

of catalase activity found in rat liver after administration of HPPs, and might thus suggest peroxisome proliferation in the embryonic cells. It has been reported that in HPP-exposed mouse [44] and rat [12] liver the cytosolic activity of catalase is much more increased than the peroxisomal activity. If this was the case in HPP-exposed SHE and WRE cells, a large increase in catalase unmasking should be detected already at 10 μg digitonin/mL. This was not found. The increase in catalase activity may thus mainly be due to catalase in the peroxisomes.

Although the catalase data suggest HPP-induced peroxisome proliferation, the results for peroxisomal β-oxidation and FAO do not support this notion. Only very modest increases were found. The relative increase is comparable to the increase found for catalase, but is in no way comparable to the increase in peroxisomal β-oxidation found in the livers of HPP-exposed rats and mice. As all the other peroxisomal oxidases had non-detectable activities, this indicates that the observed morphological transformation of SHE cells caused by HPP-exposure is not due to a peroxisome-generated oxidative stress.

Unexpectedly, the first enzyme of the peroxisomal β-oxidation, the H_2O_2-producing FAO, had a more than 10-fold higher specific activity than the total cycle. FAO is generally assumed to be the rate-limiting step of the peroxisomal β-oxidation in rodent liver [45], but the present results for FAO indicate that in SHE and WRE cells the activity of the peroxisomal β-oxidation cycle is limited by the bifunctional enzyme (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase) or the thiolase. Whether this is a general feature of non-hepatic cells, is not known to us. Since FAO is not rate-limiting, measurements of the peroxisomal β-oxidation by the NAD^+-reduction assay [46] might be expected to give an estimate of the activity of the bifunctional enzyme. However, no activity was detected in unexposed SHE cells or cells exposed to clofibrate (not shown). This could be due to the fact that the NAD^+-reduction assay is approximately 50-fold less sensitive than the 2′,7′-dichlorofluorescin assay [31]. The reason for the high FAO activity in embryonic cells is not known.

The palmitoyl-CoA hydrolase activity is also elevated in HPP-treated rat liver [46]. In fact, it has been suggested that the peroxisomal β-oxidation and palmitoyl-CoA hydrolase are regulated by a common mechanism [47, 48], but the relative increase in palmitoyl-CoA hydrolase is usually smaller than the increase in peroxisomal β-oxidation [47, 48]. A more detailed study is needed to see if the small increases in peroxisomal β-oxidation, FAO and palmitoyl-CoA hydrolase in SHE cells are coordinately regulated, especially since there seemed to be no increase in palmitoyl-CoA hydrolase in WRE cells.

Lillehaug and Berge [19] have suggested on the basis of experiments with the mouse cell line C3H10T1/2, that TPA may act as a peroxisome proliferator. TPA had little effect on the catalase activity of SHE and WRE cells, and refeeding of WRE cells with TPA every 24 hr did not increase the catalase activity more than a single exposure did. It is possible that TPA is a peroxisome proliferator in the mouse, and less so in other species. However, if TPA is a peroxisome proliferator, it does not behave like the more classical HPPs in all aspects, as it slightly decreased the DHAPAT activity, while clofibrate and DEHP increased the activity. This is consistent with other reports of the HPP-effects on glycerolipid synthetic enzymes [49, 50] and phospholipid composition in the peroxisomal membrane [51]. Ether lipids have also been shown to inhibit protein kinase C [52, 53]. HPPs may thus influence cell-to-cell communication [54] and signal transduction indirectly, or even directly [55].

Even though peroxisomes have been found in virtually every mammalian cell and tissue that has been studied, peroxisome proliferation *in vivo* is a phenomenon largely restricted to liver and kidney, and to rodents more than to other animals. Peroxisome proliferation has been shown in rat hepatocytes in culture [56, 57]. Lillehaug and coworkers [19, 58] suggested the possibility of peroxisome proliferation also occurs in extrahepatic cells *in vitro*. The low peroxisome-associated H_2O_2-producing oxidase activities and the non-inducibility of FAO and cyanide-resistant β-oxidation disfavour the hypothesis that the reported morphological transformation of SHE cells induced by DEHP [16–18], clofibrate and tiadenol* is due to oxidative stress caused by peroxisome proliferation.

Acknowledgements—The efficient technical assistance of P. Gorton is gratefully acknowledged. The following persons are thanked for various aspects: Dr E Rivedal for valuable discussions; Dr R. K. Berge and N. Aarsæther for their performance of the NAD^+-assay of cyanide-resistant β-oxidation; Dr E. N. Christiansen for his kind cooperation during the measurements of DHAPAT and parts of the cyanide-resistant β-oxidation; Dr M. Santos for leading the attention of S.O.M. towards the 2′,7′-dichlorofluorescin assay, and for advice regarding this assay; Drs E. Wiemer and J. M. Tager for supplying anticatalase antiserum; Drs P. Gordon, A. Kovacs and I. Holen for supplying isolated rat hepatocytes. S.O.M. is a research fellow of The Norwegian Cancer Society.

REFERENCES

1. Reddy JK, Azarnoff DL and Hignite CE, Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. *Nature* **283**: 397–398, 1980.
2. Hess R, Stäubli W and Riess W, Nature of hepatomegalic effect produced by ethyl-chlorophenoxy-isobutyrate in the rat. *Nature* **208**: 856–858, 1965.
3. Kluwe WM, McConnell EE, Huff JE, Haseman JK, Douglas JF and Hartwell WV, Carcinogenicity testing of phthalate esters and related compounds by the National Toxicology Program and the National Cancer Institute. *Environ Health Perspect* **45**: 129–133, 1982.
4. Reddy MK, Lalwani ND, Qureshi SA and Reddy JK, Induction of hamster hepatic peroxisomal β-oxidation and peroxisome proliferation-associated 80000 mol wt. polypeptide by hypolipidemic drugs. *Human Toxicol* **1**: 135–147, 1982.
5. Lake BG, Gray TJB, Foster JR, Stubberfield CR and Gangolli SD, Comparative studies on di(2-ethylhexyl)phthalate induced hepatic peroxisome proliferation in rat and hamster. *Toxicol Appl Pharmacol* **72**: 46–60, 1984.

* Mikalsen SO, Holen I and Sanner T, submitted.

6. Vainio H, Nickels J and Linnainmaa K, Phenoxy acid herbicides cause peroxisome proliferation in Chinese hamsters. *Scand J Work Environ Health* **8**: 70–73, 1982.
7. Vainio H, Linnainmaa K, Kähönen M, Nickels J, Hietanen E, Marniemi J and Peltonen P, Hypolipidemia and peroxisome proliferation induced by phenoxyacetic acid herbicides in rats. *Biochem Pharmacol* **32**: 2775–2779, 1983.
8. Goldsworthy TL and Popp JA, Chlorinated hydrocarbon-induced peroxisomal enzyme activity in relation to species and organ carcinogenicity. *Toxicol Appl Pharmacol* **88**: 225–233, 1987.
9. Hruban Z, Swift H and Slesers A, Ultrastructural alterations of hepatic microbodies. *Lab Invest* **15**: 1884–1901, 1966.
10. Osumi T and Hashimoto T, Enhancement of fatty acyl-CoA oxidizing activity in rat liver peroxisomes by di-(2-ethyl-hexyl)phthalate. *J Biochem* **83**: 1361–1365, 1978.
11. Osumi T and Hashimoto T, The inducible fatty acid oxidation system in mammalian peroxisomes. *Trends Biochem Sci* **9**: 317–319, 1984.
12. Leighton F, Coloma L and Koenig C, Structure, composition, physical properties and turnover of proliferated peroxisomes. A study of the trophic effects of Su-13437 on rat liver. *J Cell Biol* **67**: 281–309, 1975.
13. Reddy JK, Lalwani ND, Reddy MK and Qureshi SA, Excessive accumulation of autofluorescent lipofuscin in the liver during hepatocarcinogenesis by methyl clofenapate and other hypolipidemic peroxisome proliferators. *Cancer Res* **42**: 259–266, 1982.
14. Watanabe T, Horie S, Yamada J, Isaji M, Nishigaki T, Naito J and Suga T, Species differences in the effects of bezafibrate, a hypolipidemic agent, on hepatic peroxisome-associated enzymes. *Biochem Pharmacol* **38**: 367–371, 1989.
15. Schmezer P, Pool BL, Klein RG, Komotowski D and Schmähl D, Various short-term assays and two long-term studies with the plastiziser di(2-ethylhexyl)-phthalate in the Syrian golden hamster. *Carcinogenesis* **9**: 37–43, 1988.
16. Tomita I, Nakamura Y, Aoki N and Inui N, Mutagenic/carcinogenic potential of DEHP and MEHP. *Environ Health Perspect* **45**: 119–125, 1982.
17. Sanner T and Rivedal E, Tests with the Syrian hamster embryo cell transformation assay. In: *Progress in Mutation Research. Vol. 5. Evaluation of Short-term Tests for Carcinogens.* (Eds. Ashby J, deSerres FJ, Draper M, Ishidate M, Margolin BH, Matter BE and Shelby MD), pp. 665–671. Elsevier Science Publishers, Amsterdam, 1985.
18. Barrett JC and Lamb PW, Tests with the Syrian hamster embryo cell transformation assay. In: *Progress in Mutation Research, Vol. 5. Evaluation of Short-term Tests for Carcinogens.* (Eds. Ashby J, deSerres FJ, Draper M, Ishidate M, Margolin BH, Matter BE and Shelby MD), pp. 623–628. Elsevier Science Publishers, Amsterdam, 1985.
19. Lillehaug JR and Berge RK, The tumour promotor 12-*O*-tetradecanoylphorbol 13-acetate increases the activities of some peroxisome associated enzymes in *in vitro* cell culture. *Br J Cancer* **53**: 121–127, 1986.
20. Seglen PO, Preparation of isolated rat liver cells. In: *Methods in Cell Biology* (Ed. Prescott DM), Vol. 13, pp. 29–83. Academic Press, New York, 1976.
21. Pienta RJ, Poiley JA and Lebherz WB, Morphological transformation of early passage golden Syrian hamster embryo cells derived from cryopreserved primary cultures as a reliable *in vitro* bioassay for identifying diverse carcinogens. *Int J Cancer* **19**: 642–655, 1977.
22. Baudhuin P, Isolation of rat liver peroxisomes. In: *Methods in Enzymology* (Eds. Fleischer S and Packer L), Vol. 31, pp. 356–368. Academic Press, New York, 1974.
23. Barrett AJ, Lysosomal enzymes. In: *Lysosomes, a Laboratory Handbook* (Ed. Dingle JT), pp. 46–135. North-Holland Publishing Co., Amsterdam, 1972.
24. Leighton F, Poole B, Beaufay H, Baughuin P, Coffey JW, Fowler S and de Duve C, The large scale separation of peroxisomes, mitochondria and lysosomes from the livers of rats injected with Triton WR-1339. *J Cell Biol* **37**: 482–513, 1968.
25. Englard S and Siegel L, Mitochondrial L-malate dehydrogenase of beef heart. In: *Methods in Enzymology* (Ed. Lowenstein JM), Vol. 13, pp. 99–106. Academic Press, New York, 1969.
26. Schutgens RBH, Romeyn GJ, Ofman R, van den Bosch H, Tager JM and Wanders RJA, Acyl-CoA: dihydroxyacetone phosphate acyl-transferase in human skin fibroblasts: study of its properties using a new assay method. *Biochim Biophys Acta* **879**: 286–291, 1986.
27. Schutgens RBH, Romeyn GJ, Wanders RJA, van den Bosch H, Schrakamp G and Heymans HSA, Deficiency of acyl-CoA: dihydroxyacetone phosphate acyltransferase in patients with Zellweger (cerebro-hepato-renal) syndrome. *Biochem Biophys Res Commun* **120**: 179–184, 1984.
28. Declercq PE, Haagsman HP, Van Veldhoven P, Debeer LJ, Van Golde LMG and Mannaerts GP, Rat liver dihydroxyacetone-phosphate acyltransferases and their contribution to glycerolipid synthesis. *J Biol Chem* **259**: 9064–9075, 1984.
29. Jones GL and Hajra AK, Properties of guinea pig liver peroxisomal dihydroxyacetone phosphate acyltransferase. *J Biol Chem* **255**: 8289–8295, 1980.
30. Lazarow PB, Assay of peroxisomal β-oxidation of fatty acids. In: *Methods in Enzymology* (Ed. Lowenstein JM), Vol. 72, pp. 315–319. Academic Press, New York, 1981.
31. Small GM, Burdett K, and Connock MJ, A sensitive spectrophotometric assay for peroxisomal acyl-CoA oxidase. *Biochem J* **227**: 205–210, 1985.
32. Wanders RJA, Kos M, Roest B, Meijer AJ, Schrakamp G, Heymans HSA, Tegelaers WHH, van den Bosch H, Schutgens RBH and Tager JM, Activity of peroxisomal enzymes and intracellular distribution of catalase in Zellweger syndrome. *Biochem Biophys Res Commun* **123**: 1054–1061, 1984.
33. Holme DA and Goldberg DM, Coupled optical rate determinations of amino acid oxidase activity. *Biochim Biophys Acta* **377**: 61–70, 1975.
34. Wellner D and Lichtenberg LA, Assay of amino acid oxidase. In: *Methods in Enzymology* (Eds. Tabor H and Tabor CW), Vol. 17, pp. 593–597. Academic Press, New York, 1971.
35. Baker AL and Tolbert NE, Glycolate oxidase (ferredoxin-containing form). In: *Methods in Enzymology* (Ed. Wood, WA), Vol. 9, pp. 338–342. Academic Press, New York, 1966.
36. Jorns MS, Glycolic acid oxidase from pig liver. In: *Methods in Enzymology* (Ed. Wood WA), Vol. 41, pp. 337–343. Academic Press, New York, 1975).
37. Berge RK and Døssland B, Differences between microsomal and mitochondrial-matrix palmitoyl-Coenzyme A hydrolase, and palmitoyl-L-carnitine hydrolase from rat liver. *Biochem J* **181**: 119–125, 1979.
38. Strecker H, L-Glutamic dehydrogenase from liver. In: *Methods in Enzymology* (Eds. Colowick SP and Kaplan NO), Vol. 2, pp. 220–225. Academic Press, New York, 1955.
39. Veeger C, DerVartanian DV and Zeylemaker WP, Succinate dehydrogenase. In: *Methods in Enzymology* (Ed. Lowenstein JM), Vol. 13, pp. 81–90. Academic Press, New York, 1969.
40. Fukami MH and Flatmark T, Studies on catalase com-

partmentation in digitonin-treated rat hepatocytes. *Biochim Biophys Acta* **889**: 91–94, 1986.
41. Stefanini S, Farrace MG and Ceru Argento MP, Differentiation of liver peroxisomes in the foetal and newborn rat. Cytochemistry of catalase and D-amino acid oxidase. *J Embryol Exp Morphol* **88**: 151–163, 1985.
42. Tsukuda H, Mochizuki Y and Konishi T, Morphogenesis and development of microbodies of hepatocytes of rats during pre- and postnatal growth. *J Cell Biol* **37**: 231–243, 1968.
43. Cibelli A, Stefanini S and Ceru MP, Peroxisomal β-oxidation and catalase activities in fetal rat liver: effect of maternal treatment with clofibrate. *Cell Mol Biol* **34**: 191–205, 1988.
44. Jones GL and Masters CJ, On the synthesis and degradation of multiple forms of catalase in mouse liver. Effects of aminotriazole and *p*-chlorophenoxyisobutyric acid ethyl ester. *Arch Biochem Biophys* **187**: 431–440, 1978.
45. Inestrosa NC, Bronfman M and Leighton F, Detection of peroxisomal fatty acyl-Coenzyme A oxidase activity. *Biochem J* **182**: 779–788, 1979.
46. Berge RK and Bakke OM, Changes in lipid metabolizing enzymes of hepatic subcellular fractions from rats treated with tiadenol and clofibrate. *Biochem Pharmacol* **30**: 2251–2256, 1981.
47. Berge RK and Aarsland A, Correlation between the cellular level of long-chain acyl-CoA, peroxisomal β-oxidation, and palmitoyl-CoA hydrolase activity in rat liver. Are the two enzyme systems regulated by a substrate-induced mechanism? *Biochim Biophys Acta* **837**: 141–151, 1985.
48. Berge RK, Nilsson A and Husøy AM, Rapid stimulation of liver palmitoyl-CoA synthetase, carnitine palmitoyl-transferase and glycerophosphate acyltransferase compared to peroxisomal β-oxidation and palmitoyl-CoA hydrolase in rats fed high-fat diets. *Biochim Biophys Acta* **960**: 417–426, 1988.
49. Yanagita T, Satoh M, Enomoto N and Sugano M, Di(2-ethyl-hexyl)phthalate enhances hepatic phospholipid synthesis in rats. *Biochim Biophys Acta* **919**: 64–70, 1987.
50. Burke CL and Hajra AK, Clofibrate feeding increases the activities of rat liver dihydroxyacetone phosphate acyltransferase and glycerol-3-phosphate acyltransferase. *Biochem Int* **1**: 312–318, 1980.
51. Crane DI and Masters CJ, The effect on the phospholipid composition of the peroxisomal membranes in mouse liver. *Biochim Biophys Acta* **876**: 256–263, 1986.
52. Daniel LW, Small GW and Schmitt JD, Alkyl-linked diglycerides inhibit protein kinase C activation by diacylglycerols. *Biochem Biophys Res Commun* **151**: 291–297, 1988.
53. Daniel LW, Etkin LA, Morrison BT, Parker J, Morris-Natschke S, Surles JR and Piantadosi C, Ether lipids inhibit the effects of phorbol diester tumor promotors. *Lipids* **22**: 851–855, 1987.
54. Malcolm AR, Mills LJ and McKenna EJ, Inhibition of metabolic cooperation between Chinese hamster V79 cells by tumor promotors and other chemicals. *Ann NY Acad Sci* **407**: 448–450, 1983.
55. Sager G and Little C, The effect of the plasticizers TBEP (tris-(2-butoxyethyl)-phosphate) and DEHP (di-(2-ethylhexyl)-phthalate on β-adrenergic binding to α_1-acid glycoprotein and mononuclear leukocytes. *Biochem Pharmacol* **38**: 2551–2557, 1989.
56. Bieri F, Bentley P, Waechter F and Stäubli W, Use of primary cultures of adult hepatocytes to investigate mechanisms of action of nafenopin, a hepatocarcinogenic peroxisome proliferator. *Carcinogenesis* **5**: 1033–1039, 1984.
57. Gray TJB, Lake BG, Beamand JA, Foster JR and Gangolli SD, Peroxisome proliferation in primary cultures of rat hepatocytes. *Toxicol Appl Pharmacol* **67**: 15–25, 1983.
58. Berge RK and Lillehaug JR, Tiadenol-mediated induction of peroxisomal enzymes in cultured C3H/10T1/2 CL8 cells and in chemically transformed C3H/10T1/2 MCA16 cells. *Int J Cancer* **36**: 489–494, 1985.