Biochem. J. (1981) **198**, 177–186
Printed in Great Britain

Induction, immunochemical identity and immunofluorescence localization of an 80000-molecular-weight peroxisome-proliferation-associated polypeptide (polypeptide PPA-80) and peroxisomal enoyl-CoA hydratase of mouse liver and renal cortex

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(*Received 22 January 1981/Accepted 6 April 1981*)

The hypolipidaemic drugs methyl clofenapate, BR-931, Wy-14643 and procetofen induced a marked proliferation of peroxisomes in the parenchymal cells of liver and the proximal-convoluted-tubular epithelium of mouse kidney. The proliferation of peroxisomes was associated with 6–12-fold increase in the peroxisomal palmitoyl-CoA oxidizing capacity of the mouse liver. Enhanced activity of the peroxisomal palmitoyl-CoA oxidation system was also found in the renal-cortical homogenates of hypolipidaemic-drug-treated mice. The activity of enoyl-CoA hydratase in the mouse liver increased 30–50-fold and in the kidney cortex 3–5-fold with hypolipidaemic-drug-induced peroxisome proliferation in these tissues, and over 95% of this induced activity was found to be heat-labile peroxisomal enzyme in both organs. Sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analysis of large-particle and microsomal fractions obtained from the liver and kidney cortex of mice treated with hypolipidaemic peroxisome proliferators demonstrated a substantial increase in the quantity of an 80000-mol.wt. peroxisome-proliferation-associated polypeptide (polypeptide PPA-80). The heat-labile peroxisomal enoyl-CoA hydratase was purified from the livers of mice treated with the hypolipidaemic drug methyl clofenapate; the antibodies raised against this electrophoretically homogeneous protein yielded a single immunoprecipitin band with purified mouse liver enoyl-CoA hydratase and with liver and kidney cortical extracts of normal and hypolipidaemic-drug-treated mice. These anti-(mouse liver enoyl-CoA hydratase) antibodies also cross-reacted with purified rat liver enoyl-CoA hydratase and with the polypeptide PPA-80 obtained from rat and mouse liver. Immunofluorescence studies with anti-(polypeptide PPA-80) and anti-(peroxisomal enoyl-CoA hydratase) provided visual evidence for the localization and induction of polypeptide PPA-80 and peroxisomal enoyl-CoA hydratase in the liver and kidney respectively of normal and hypolipidaemic-drug-treated mice. In the kidney, the distribution of these two proteins is identical and limited exclusively to the cytoplasm of proximal-convoluted-tubular epithelium. The immunofluorescence studies clearly complement the biochemical and ultrastructural observations of peroxisome induction in the liver and kidney cortex of mice fed on hypolipidaemic drugs. In addition, preliminary ultrastructural studies with the protein-A–gold-complex technique demonstrate that the heat-labile hepatic enoyl-CoA hydratase is localized in the peroxisome matrix.

In addition to the metabolic role of H_2O_2 detoxification, presumably a general function of peroxisomes in all cell types (Reddy, 1973), the studies of Reddy & Krishnakantha (1975) with

Abbreviations used: SDS, sodium dodecyl sulphate; methyl clofenapate, methyl 2-[4-(*p*-chlorophenyl)-phenoxy]-2-methylpropionate; BR-931, [4-chloro-6-(2,3-xylidino)pyrimidin-2-ylthio]-*N*-(β-hydroxyethyl)-acetamide; Wy-14643, [4-chloro-6-(2,3-xylidino)pyrimidin-2-ylthio]acetic acid; procetofen, isopropyl 3-(*p*-chlorobenzoyl)-2-methyl-2-phenoxypropionate; polypeptide PPA-80, an 80000-mol.wt. peroxisome-proliferation-associated polypeptide; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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hypolipidaemic-drug-induced hepatic-peroxisome proliferation in rodents suggested that peroxisomes are involved in lipid metabolism. Subsequently, Lazarow & de Duve (1976) found that rat liver peroxisomes possess a cyanide-insensitive fatty-acyl-CoA-oxidizing system that is essentially identical with the β-oxidation system identified previously in plant peroxisomes [glyoxysomes of the castor-bean (*Ricinus communis*) endosperm] by Cooper & Beevers (1969). More recently, the presence of a fatty-acyl-CoA-oxidizing system in human liver peroxisomes was reported by Bronfman *et al.* (1979), which suggests that peroxisomal β-oxidation system possibly exists in the livers of all species.

The peroxisomal β-oxidation system in rat liver, which differs from the well-known mitochondrial fatty-acid-oxidation system (Lazarow, 1978), has been shown to be enhanced severalfold by hypolipidaemic drugs (Lazarow, 1977; Inestrosa *et al.*, 1979; Reddy *et al.*, 1981) and the industrial plasticizer di-(2-ethylhexyl) phthalate (Osumi & Hashimoto, 1978), all of which are known hepatic peroxisome proliferators (Hess *et al.*, 1965; Svoboda *et al.*, 1967; Reddy, 1973; Reddy & Krishnakantha, 1975; Reddy *et al.*, 1976). Whether hypolipidaemic compounds induce peroxisomal β-oxidation in the liver of other species is not known. Furthermore, very little information is currently available regarding the existence of fatty-acyl-CoA-oxidizing system in peroxisomes of non-hepatic tissues, with the possible exception of brown adipose tissue, intestine and heart in rats (Kramar *et al.*, 1978; Norseth, 1980; Small *et al.*, 1980). Whether or not the hypolipidaemic drugs that increase peroxisomal β-oxidation in rat liver similarly enhance this enzyme system in other tissues remains to be investigated. The present studies were undertaken to ascertain whether the hypolipidaemic drugs increase the fatty-acid-oxidizing capacity of peroxisomes in the mouse liver and kidney. The results demonstrate that hypolipidaemic drugs methyl clofenapate, BR-931, Wy-14643 and procetofen increase the peroxisomal-palmitoyl-CoA-oxidizing capacity as well as the heat-labile peroxisomal enoyl-CoA hydratase activity in the liver and kidney cortical homogenates of mice. In addition, we show evidence for the distribution and induction of polypeptide PPA-80 and the heat-labile peroxisomal enoyl-CoA hydratase in the mouse liver and kidney by an indirect immunofluorescence technique employing antibodies raised against these two proteins.

Materials and methods

Chemicals

The hypolipidaemic compounds were obtained from the following sources: (i) procetofen from UCB-SMIT S.p.A., Torino, Italy; (ii) Wy-14643 from Wyeth Laboratories, Radnor, PA, U.S.A.; (iii) BR-931 from LPB Istituto Farmaceutico S.p.A., Milan, Italy; and (iv) methyl clofenapate from Neal Handly, California Institute of Technology, Pasadena, CA, U.S.A. Crotonyl-CoA, NAD^+, $NADP^+$, CoA, palmitoyl-CoA and antimycin A were obtained from Sigma, St. Louis, MO, U.S.A. [1-^{14}C]Palmitoyl-CoA (sp. radioactivity 59 mCi/mmol) was obtained from Amersham Corp., Arlington Heights, IL, U.S.A. Purified acrylamide, *NN′*-methylenebisacrylamide and dithiothreitol were purchased from Eastman Kodak Co., Rochester, NY, U.S.A. Molecular-weight standards for SDS/polyacrylamide-gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Animals

Swiss Webster male mice, 5–7 weeks of age, obtained from Charles River, Wilmington, MA, U.S.A., were used in these experiments. To study the time course of induction of peroxisomal palmitoyl-CoA oxidation in the mouse liver and kidney, the hypolipidaemic drug methyl clofenapate was administered in the diet at 0.1% (w/w) and the animals killed at predetermined intervals up to 6 weeks. Additional groups of mice were fed hypolipidaemic compounds in powdered chow for 6 weeks, at the dietary concentrations indicated (w/w): procetofen, 0.2%; Wy-14643, 0.2%; BR-931, 0.2%; and methyl clofenapate, 0.05 or 0.1%. The control mice were fed the chow without the drug. At the end of the treatment period the animals were killed by cervical dislocation; the liver and kidneys were removed, washed free of blood, weighed and processed as described below.

Enzyme assays

The kidneys were freed of capsule and medulla in order to obtain enriched renal cortex for enzyme studies. The kidney cortex thus obtained, as well as the livers from normal and treated mice, were homogenized in ice-cold 0.25 M-sucrose containing 0.1% ethanol by using Potter–Elvehjem homogenizer with a Teflon pestle. These homogenates were used for the measurement of enzyme activities. Palmitoyl-CoA-oxidizing activity in homogenates was measured by the method described by Neat & Osmundsen (1979). The 0.5 ml incubation mixture contained 80 μM-[^{14}C]palmitoyl-CoA, 68 mM-KCl, 5 mM-Hepes, 0.5 mM-EGTA, 10 mM-P_i, 0.4% (w/v) bovine serum albumin, 0.5 mM-$NADP^+$, 0.5 mM-NAD^+, 0.1 mM-CoA and 1 mM-dithiothreitol, pH 7.2, to which 50 or 100 μl of 5% homogenate was added. Antimycin A (10 μg/ml) or KCN (1 mM) was used to block β-oxidation due to mitochondria (Cooper & Beevers, 1969; Lazarow, 1978; Neat & Osmundsen, 1979). The peroxisomal palmitoyl-CoA oxidation was measured in the absence or presence

of 0.4% bovine serum albumin in studies on the time course of induction with methyl clofenapate. After 10min incubation at 37°C, the appearance of $HClO_4$-soluble radioactivity in the supernatant was measured by liquid-scintillation spectrometry in a Beckman LS-9000 liquid-scintillation counter. Enoyl-CoA hydratase (EC 4.2.1.17) activity was assayed as described by Steinman & Hill (1975), with 200μM-crotonyl-CoA as substrate in 0.3M-Tris/HCl, pH 7.4, containing 5mM-EDTA, and 0.05mg of bovine serum albumin/ml in a 1ml mixture. The heat-labile peroxisomal enoyl-CoA hydratase activity was inactivated when the homogenates, diluted with 50mM-potassium phosphate, pH 7.0, were heated at 57°C for 5min (Osumi & Hashimoto, 1979). Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Subcellular fractionation

The homogenates of mouse kidney cortex [10% (w/v) in 0.25M-sucrose] were used for subcellular fractionation by the method outlined by Kurup *et al.* (1970). The subcellular fractionation of mouse livers was performed essentially as previously described (Reddy *et al.*, 1980). The postnuclear large-particle and microsomal fractions thus obtained from kidney cortex and liver were used for the SDS/polyacrylamide-gel electrophoresis as described below.

SDS/polyacrylamide-gel electrophoresis and isoelectric focusing

The electrophoresis was performed on polyacrylamide slab or cylindrical gels by the method of Laemmli (1970). Gels of 7.5% (w/v) polyacrylamide were prepared from a stock of 30% acrylamide and 0.8% *NN'*-methylenebisacrylamide as previously described (Reddy & Kumar, 1977). The stacking gel was 4.5% acrylamide with 0.125M-Tris/HCl, pH 6.8, 0.1% SDS, 0.024% tetramethylethylenediamine and 0.1% ammonium persulphate. The electrode buffer used was 0.025M-Tris/glycine, pH 8.3, which contained 0.1% SDS. The samples of subcellular fractions were solubilized in a solution containing 20% glycerol, 2% SDS, 5% mercaptoethanol and 0.001% Bromophenol Blue in 0.0625M-Tris/HCl, pH 6.8, by heating for 3min in boiling water (Laemmli, 1970). Samples of dissolved proteins (10–150μg) were loaded on to a stacking gel and electrophoresed at 30mA/slab until the marker dye had reached the bottom of the gel. The proteins were then fixed in the gel by immersing the gels in 10% (w/v) trichloroacetic acid and then stained with 0.1% Coomassie Brilliant Blue as described previously (Reddy *et al.*, 1980). The protein bands of the cylindrical gels were scanned at 540nm; quantification of the peaks was made by determining the surface area of each peak. Isoelectric focusing was performed by using slab gels (pH 3.5–10.0 range) or tube gels (pH 9–11 range).

Purification of peroxisomal enoyl-CoA hydratase and production of antibodies in rabbits

The heat-labile peroxisomal enoyl-CoA hydratase was purified from livers of mice treated with 0.1% methyl clofenapate by the method of Osumi & Hashimoto (1979) as previously described (Reddy *et al.*, 1981). Electrophoretically homogeneous enoyl-CoA hydratase was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously in several places on the backs of male New Zealand rabbits. This procedure was repeated four times at weekly intervals. The animals were bled 5 days after the last injection and antisera were prepared and stored at −20°C.

Ouchterlony double-diffusion analysis

Immunodiffusion analyses were performed as described by Ouchterlony & Nilsson (1973). After the immunoprecipitates had developed, the Ouchterlony plates were photographed either before or after staining.

Immunofluorescence procedure

For the immunofluorescence method, small (3mm × 4mm × 3mm) pieces of liver and kidney cortex from normal and methyl clofenapate-treated rats were fixed in ice-cold 96% ethanol for 24h and then in cold xylene for 40h before embedding in paraffin wax (Sainte-Marie, 1962). Paraffin was removed from the sections (approx. 2μm thick), which were hydrated and washed with 0.15M-phosphate-buffered saline containing 2% bovine plasma albumin for 2h at room temperature. The sections were then incubated with rabbit antiserum (1:10 dilution) to polypeptide PPA-80 or to enoyl-CoA hydratase for 1h at room temperature in a moist chamber. After three consecutive 15min washes with phosphate-buffered saline, the sections were covered with goat anti-(rabbit γ-globulin) conjugated with fluorescein isothiocyanate and permitted to react for 30min. The sections were then thoroughly washed with phosphate-buffered saline, mounted in 50% glycerol in the above buffer and examined in a Leitz fluorescence microscope. The specificity of immunofluorescence was ascertained by using: (i) non-immunized rabbit serum; (ii) by omitting the incubation with specific antibody; and (iii) by absorption with specific antibodies.

Electron microscopy and ultrastructural localization of heat-labile enoyl-CoA hydratase in liver by the protein-A–gold-complex technique

The samples of kidney cortex and liver were minced into blocks of 1mm³ in 2.5% glutaraldehyde in 0.1M-sodium cacodylate buffer, pH 7.4, and fixed

for 30 min. They were then washed and postfixed in 1% OsO_4 in 0.1 M-*s*-collidine buffer, pH 7.4, for 1 h at 4°C, dehydrated and embedded in Epon. Thin sections were cut and examined in a Hitachi HU12 electron microscope. For the ultrastructural localization of enoyl-CoA hydratase in aldehyde-fixed and Epon-embedded liver tissue, ultrathin sections were exposed to specific antisera and then observed by using the protein-A–gold-complex method outlined by Roth *et al.* (1978).

Results

Inducibility of kidney and liver peroxisomal β-oxidation in mice

The time course of methyl clofenapate-induced peroxisomal palmitoyl-CoA oxidation in mouse renal-cortical and liver homogenates is shown in Fig. 1. The assay conditions employed in these studies ensure measurement mostly of the peroxisomal β-oxidation system (Lazarow, 1978; Mannaerts *et al.*, 1979; Thomas *et al.*, 1980). The liver and renal-cortical homogenates of methyl clofenapate-treated mice displayed significantly more palmitoyl-CoA-oxidizing activity than the respective control homogenates. It is also evident from Fig. 1 that the palmitoyl-CoA-oxidation rates were maximal when albumin was absent in the assay mixture (Fig. 1*b*). The omission of albumin from the incubation mixture has been reported to impair drastically mitochondrial β-oxidation (Mannaerts *et al.*, 1979).

Effect of other hypolipidaemic drugs on peroxisomal palmitoyl-CoA oxidation and enoyl-CoA hydratase activity in mouse liver and renal cortex

Administration of the hypolipidaemic drugs BR-931, Wy-14643 and procetofen in the diet for 6 weeks resulted in a significant elevation of peroxisomal β-oxidation system in the mouse liver and kidney cortex similar to that observed with methyl clofenapate. A marked induction of the peroxisomal palmitoyl-CoA-oxidizing system in mouse liver similar to that reported in the livers of hypolipidaemic-drug- or plasticizer-treated rats was observed (Lazarow, 1977; Inestrosa *et al.*, 1979; Osumi & Hashimoto, 1978). A 4–7-fold increase in peroxisomal palmitoyl-CoA-oxidizing system in the kidney cortex of hypolipidaemic-drug-treated animals was also observed. All hypolipidaemic drugs also caused a marked increase in the activity of enoyl-CoA hydratase in liver and renal-cortical homogenates, which consisted predominantly of the heat-labile peroxisomal enzyme (Table 1).

Induction of polypeptide PPA-80 in subcellular fractions of liver and kidney cortex

The SDS/polyacrylamide-gel-electrophoretic patterns of large-particle and microsomal pellets obtained from the liver and cortex of normal mice and mice treated with hypolipidaemic drugs for 6 weeks are illustrated in Figs. 2–4. The hypolipidaemic peroxisome proliferators, BR-931, Wy-14643, procetofen and methyl clofenapate caused a substantial

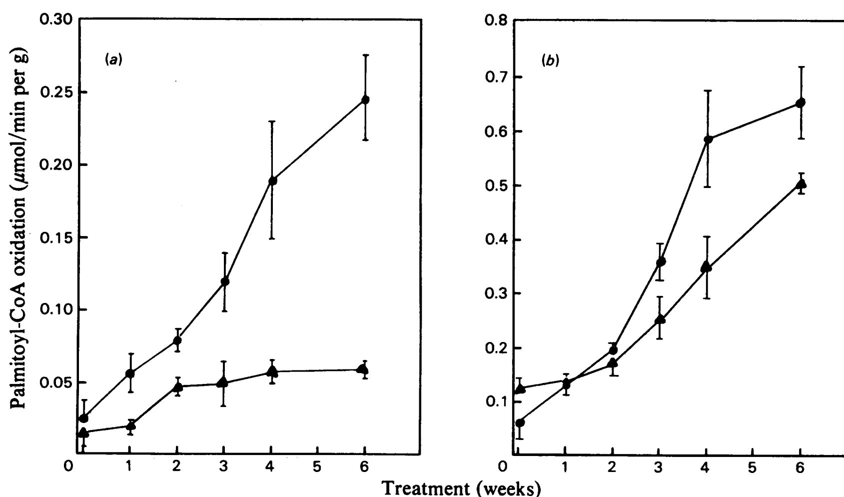


Fig. 1. *Time course of methyl clofenapate-induced peroxisomal β-oxidation system in mouse liver and renal-cortical homogenates*

Methyl clofenapate was administered in the diet *ad libitum* at 0.1% (w/w) and the animals were killed at predetermined intervals. The [1-^{14}C]palmitoyl-CoA oxidation was measured in the presence (*a*) or absence (*b*) of 0.4% bovine serum albumin in the incubation medium. ●, Liver; ▲, kidney cortex.

Table 1. *Effect of hypolipidaemic peroxisome proliferators on the heat-labile peroxisomal enoyl-CoA hydratase activity in mouse liver and kidney cortex*

Male Swiss Webster mice were fed hypolipidaemic peroxisome proliferators in diet for 6 weeks at the doses indicated below. Homogenates of liver and renal cortex were assayed for heat-labile peroxisomal enoyl-CoA hydratase as described in the Materials and methods section. Heating the homogenates for 5 min at 57°C inactivated the peroxisomal enoyl-CoA hydratase activity. One unit of enoyl-CoA hydratase activity is defined as a decrease of one absorbance unit/min at 280 nm in a volume of 1 ml and corresponds to 0.278 μmol/min. Values are means ± S.D. for four to six animals in each group. All mean values for the hypolipidaemic-drug-treated groups are statistically different from those of the control group ($P < 0.005$, except *$P < 0.05$).

Proliferator	Dietary dose (%, w/w)	Enoyl-CoA hydratase (μmol/min per mg of protein)			
		Liver		Kidney	
		Total	Peroxisomal	Total	Peroxisomal
Control	—	1.0 ± 0.16	0.6 ± 0.26	2.0 ± 0.66	1.9 ± 0.60
BR-931	0.2	49.1 ± 6.32	48.4 ± 6.34	9.1 ± 0.70	8.8 ± 0.77
Wy-14643	0.2	48.0 ± 17.33	47.6 ± 17.20	13.3 ± 4.00	13.2 ± 4.00
Procetofen	0.2	48.7 ± 4.00	48.3 ± 4.31	9.9 ± 0.15	9.8 ± 0.23
Methyl clofenapate	0.05	43.8 ± 8.20	43.1 ± 8.34	6.8 ± 2.90	6.4 ± 3.50*
	0.10	32.9 ± 4.29	32.0 ± 4.50	7.9 ± 4.45	7.2 ± 2.50*

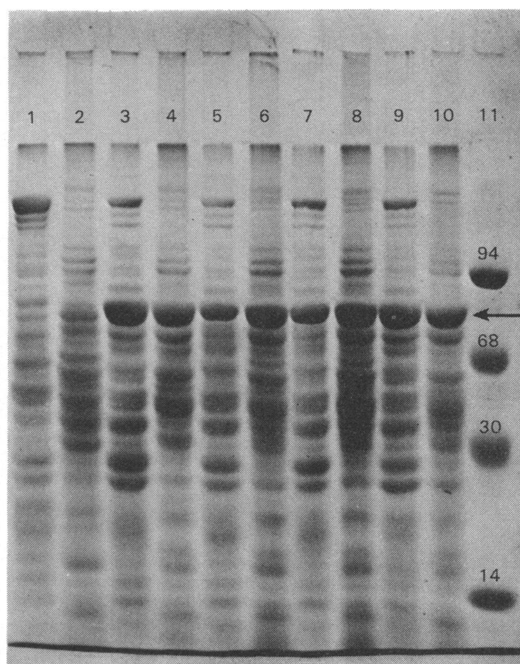


Fig. 2. *SDS/polyacrylamide-slab-gel-electrophoretic profiles of proteins in large-particle fractions (odd numbers) and microsomal fractions (even numbers) obtained from the livers of normal mice and mice treated with hypolipidaemic compounds for 6 weeks*

Subcellular fractions were prepared and electrophoresed as described in the text. Slots 1 and 2, normal liver; 3 and 4, Wy-14643 (0.2% in diet); 5 and 6, BR-931 (0.2% in diet); 7 and 8, procetofen (0.2% in diet); and 9 and 10, methyl clofenapate (0.1% in diet). The arrow indicates the position of polypeptide PPA-80. The protein concentration was 20 μg/slot. Slot 11, standard proteins with molecular masses in kDa.

increase in the intensity of polypeptide PPA-80 in both the large-particle and microsomal fractions of mouse liver (Fig. 2). The treatment of mice with these hypolipidaemic drugs also resulted in a similar increase in an 80000-mol.wt. protein in the large-particle and microsomal fractions obtained from kidney cortex. Figs. 3 and 4 represent respectively the typical densitometric tracings of the polypeptide composition of large-particle and microsomal fractions obtained from the kidney cortex of normal and hypolipidaemic drug-treated mice.

Purity of heat-labile mouse liver enoyl-CoA hydratase

The purity of the final enoyl-CoA hydratase enzyme preparation was assayed by SDS/polyacrylamide-gel electrophoresis (Fig. 5). The purified preparation yielded one distinct band with a minimum approximate mol.wt. of 80000 (Fig. 5, slot 1). The molecular weight of this preparation was identical with that of heat-labile enoyl-CoA hydratase purified from rat liver (Fig. 5, slot 2) and the peroxisome-proliferation-associated 80000-mol.wt. protein induced in the mouse liver (Fig. 5, slot 4) and kidney cortex.

The isoelectric point of purified mouse liver heat-labile enoyl-CoA hydratase was determined to be at pH 9.9. The observed isoelectric point of the mouse liver heat-labile enoyl-CoA hydratase was similar to the isoelectric point of the rat liver enoyl-CoA hydratase and polypeptide PPA-80 (Reddy *et al.*, 1981).

Ouchterlony double-diffusion analysis

The specificity of the anti-(mouse liver peroxisomal enoyl-CoA hydratase) antiserum was evaluated by Ouchterlony double-diffusion analysis.

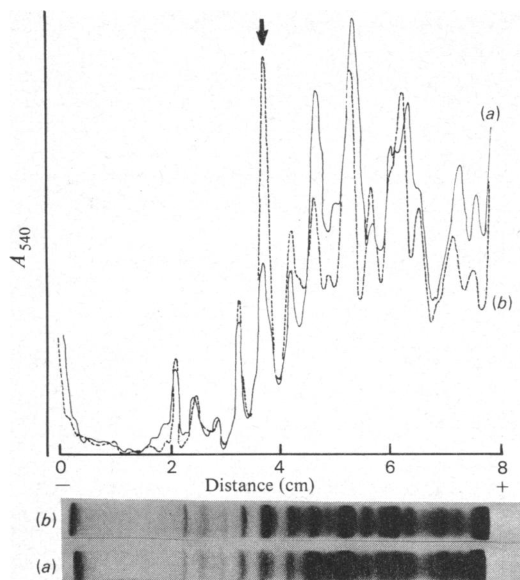


Fig. 3. *Comparison of the polypeptide composition of the large-particle fraction isolated from the renal cortex of normal (gel a, ——) and methyl clofenapate-treated (gel b, ----) mouse*

Methyl clofenapate was fed *ad libitum* for 6 weeks at 0.1% in the diet. Approx. 50μg of protein was applied to 7.5%-polyacrylamide disc gels. The arrow indicates the position of peroxisome-proliferation-associated 80000-mol.wt. polypeptide.

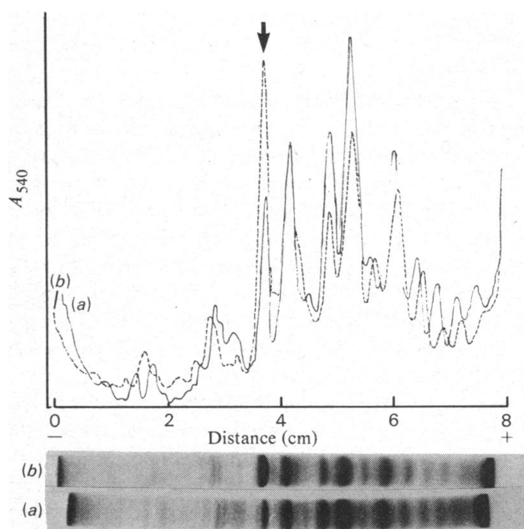


Fig. 4. *Comparison of the polypeptide composition of the microsomal fraction isolated from the renal cortex of normal (gel a, ——) and methyl clofenapate-treated (gel b, ----) mouse*

Methyl clofenapate was administered *ad libitum* for 6 weeks at 0.1% in the diet. Other details are the same as those in Fig. 3.

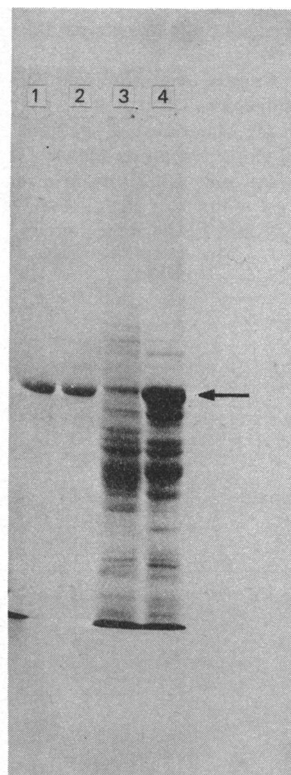


Fig. 5. *SDS/polyacrylamide-slab-gel-electrophoretic pattern of heat-labile peroxisomal enoyl-CoA hydratase purified from mouse and rat liver*

Heat-labile peroxisomal enoyl-CoA hydratase was purified from the livers of mice (slot 1, 5μg of protein) treated with 0.1% methyl clofenapate and rats (slot 2; 5μg of protein) treated with 0.1% Wy-14643 as described by Osumi & Hashimoto (1979). Slots 3 and 4 represent microsomal fractions of normal and 0.1%-methyl clofenapate-treated mouse livers respectively (20μg of protein). The arrow indicates the position of polypeptide PPA-80.

As shown in Fig. 6, the antiserum formed a single precipitin band with purified mouse liver peroxisomal enoyl-CoA hydratase or with liver and kidney cortical extracts of mice treated with the peroxisome proliferators. A trace amount of peroxisomal enoyl-CoA hydratase is apparently present in control mouse kidney cortical and liver extracts. Three- to six-fold greater amounts of control kidney cortical and liver extracts respectively were needed to obtain precipitin bands whose intensity was comparable with those obtained with treated extracts. These results thus indicate a quantitative increase in the amount of peroxisomal enoyl-CoA hydratase in the liver and kidney cortex of treated animals.

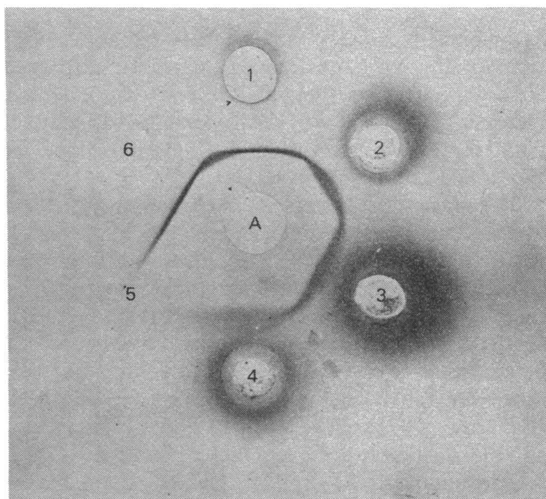


Fig. 6. *Ouchterlony double-diffusion analyses of liver and kidney extracts with rabbit anti-(mouse liver peroxisomal enoyl-CoA hydratase) antiserum*

Mouse liver and kidney cortex were homogenized (10%, w/v) in a buffer containing 10mM-K_3PO_4, 0.1% hexamethyl phosphoric triamide, 2mM-mercaptoethanol and 5mM-EDTA, and centrifuged at 17000*g* for 30min. The supernatants were used for double-diffusion analyses. The centre well (A) contained 10μl of antiserum containing antibody against mouse liver peroxisomal enoyl-CoA hydratase. Wells 1 and 2 contained liver extract (equivalent to 25μg of total protein) and kidney-cortex extract (equivalent to 48μg of total protein) respectively of a mouse treated with 0.1% methyl clofenapate for 6 weeks. Wells 3 and 4 contain liver extract (equivalent to 143μg of protein) and kidney extract (equivalent to 127μg of protein) respectively of a control mouse. Well 5 contains the buffer and well 6 contains approx. 9.6μg of purified mouse liver peroxisomal enoyl-CoA hydratase. The immunoprecipitin bands were stained with Coomassie Brilliant Blue and photographed.

Immunochemical comparison of the mouse and rat liver peroxisomal enoyl-CoA hydratase with polypeptide PPA-80

Ouchterlony double-diffusion analysis revealed a single precipitin line when the anti-(mouse peroxisomal enoyl-CoA hydratase) antibody was allowed to react with enoyl-CoA hydratase purified from mouse liver. The enoyl-CoA hydratase purified from rat liver also gave a single precipitin line, but showed only partial identity. The polypeptide PPA-80 purified from mouse liver appears antigenically identical with the mouse liver peroxisomal enoyl-CoA hydratase (results not shown).

Proliferation of peroxisomes

As expected, the hypolipidaemic drugs BR-931, Wy-14643, procetofen and methyl clofenapate all caused a profound increase in the number of peroxisomes in the mouse liver parenchymal cells. Examination of kidneys of mice treated with these hypolipidaemic drugs for 6 weeks also revealed a marked increase in the number of peroxisomes in all three segments (P_1, P_2 and P_3) of the proximal-convoluted-tabular epithelium. The division of proximal-convoluted-tubular epithelium into these segments was made by using the descriptions of Ericsson (1964).

Immunofluorescence localization of polypeptide PPA-80 and peroxisomal enoyl-CoA hydratase in mouse liver and kidney

The availability of antibodies to mouse liver peroxisomal enoyl-CoA hydratase and to rat liver polypeptide PPA-80 facilitated the immunofluorescent localization of both these proteins in the liver and kidney of normal and hypolipidaemic-drug-treated mice. In the liver of normal mouse, polypeptide PPA-80 and enoyl-CoA hydratase were localized as particulate cytoplasmic material; in hypolipidaemic-drug-treated mice the cytoplasmic immunofluorescent staining of the liver cells was rather intense and diffuse, similar to that observed in rat liver (Reddy *et al.*, 1981).

In the kidney, the localization of both polypeptide PPA-80 and peroxisomal enoyl-CoA hydratase, as ascertained by immunofluorescent staining, was essentially identical. The immunofluorescence staining was observed in the cytoplasm of proximal-convoluted-tubular epithelium. In the normal kidney the fluorescence was less intense in P_1 and P_2 segments when compared with the fluorescence staining in P_3 segment of the proximal convoluted tubule (Plates 1*a* and 1*b*). This distribution of polypeptide PPA-80 and enoyl-CoA hydratase appears similar to the distribution of peroxisomes in the nephron made visible by 3,3′-diaminobenzidine staining of peroxisomal catalase (Beard & Novikoff, 1969). In hypolipidaemic-drug-treated mice, the entire proximal-convoluted-tubular epithelium (P_1, P_2 and P_3 segments) showed intense cytoplasmic fluorescence with both anti-(polypeptide PPA-80) and anti-(enoyl-CoA hydratase) (Plates 1*c* and 1*d*) indicative of peroxisome proliferation. The glomeruli and the distal tubules in the renal medulla showed no fluorescence staining either in the normal or in the hypolipidaemic-drug-treated mice. The absence of immunofluorescence staining of the distal convoluted tubule and of cells in the glomeruli, which are devoid of peroxisomes, provides a strong indication of the specificity of the antibodies used in these studies. The absence of immunofluorescence staining in the distal nephron indicates that polypeptide PPA-80 and heat-labile enoyl-CoA hydratase are not mitochondrial in origin, since mito-

chondria are found in the cells throughout the kidney.

Ultrastructural localization of heat-labile enoyl-CoA hydratase in liver by the protein-A–gold-complex procedure

By using the specific anti-(enoyl-CoA hydratase) antiserum with the recently developed protein-A–gold technique (Roth *et al.*, 1978), we have obtained preliminary visual evidence for the localization of this enzyme in the liver cell. As Plate 2 shows, numerous gold particles are present over peroxisomes, indicating that this enzyme is concentrated in the peroxisome matrix. This technique should serve as a useful tool for the study of intracellular synthesis and distribution of various peroxisomal enzymes.

Discussion

The present results demonstrate that the hypolipidaemic drugs methyl clofenapate, BR-931, Wy-14643 and procetofen are capable of enhancing the activity, in mouse liver and kidney cortex, of the peroxisomal-enzyme system responsible for fatty-acid β-oxidation. The magnitude of the increase of palmitoyl-CoA-oxidizing capacity under the experimental conditions used in the present studies was somewhat higher than that produced by clofibrate (Lazarow and de Duve, 1976) and high-fat diet (Ishii *et al.*, 1980; Neat *et al.*, 1980) in rat liver. Exact quantitative comparisons between our results and those published in the literature are not possible because of the variability in the assay conditions used in different studies. The assay system appears to be particularly sensitive to the substrate/albumin ratio (Mannaerts *et al.*, 1979). In addition, the differences may be attributable to the species variation, and to the fact that the drugs used in these studies are many times more effective than clofibrate in inducing hepatic peroxisome proliferation (Reddy, 1974, 1980; Reddy & Krishnakantha, 1975; Reddy *et al.*, 1978). It is also pertinent to note that these drugs were fed for longer periods (6 weeks) in the diet, whereas clofibrate was given for 1 week (Lazarow & de Duve, 1976). The present results demonstrate, for the first time, that the hypolipidaemic drugs that increase hepatic peroxisomal palmitoyl-CoA-oxidizing capacity, also exert a similar effect on the renal cortex. The extent of increase of palmitoyl-CoA-oxidizing capacity in renal cortex was substantially higher in mice fed Wy-14643 and procetofen.

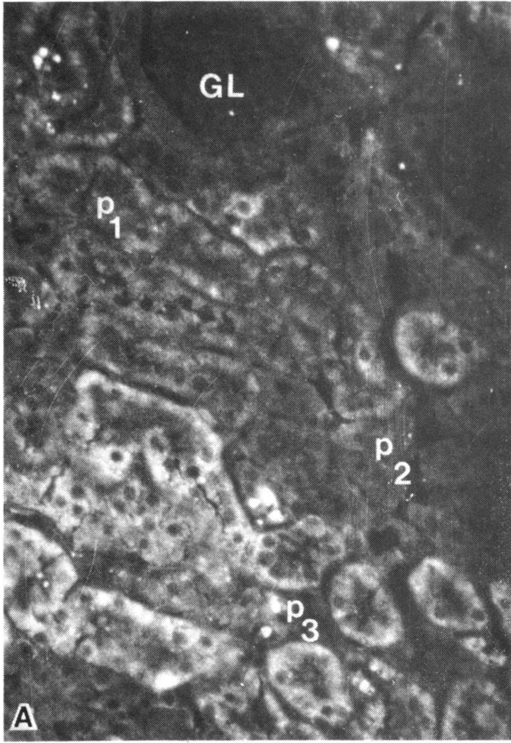
The present studies also demonstrate the existence of heat-labile peroxisomal enoyl-CoA hydratase in the liver and kidney cortex of mice. No significant change in the activity of heat-stable mitochondrial enoyl-CoA hydratase activity was observed either in liver or kidney cortex. A marked increase in peroxisomal enoyl-CoA hydratase activity was noted previously in the livers of rats fed clofibrate (Lazarow, 1978), Wy-14643 (Reddy *et al.*, 1981) and the plasticizer di-(2-ethylhexyl)phthalate (Osumi & Hashimoto, 1979). The present data further extend these observations to the mouse liver and kidney. Since these data provide no indication concerning a possible alteration in the heat-stable mitochondrial enoyl-CoA hydratase in liver and kidney cortex of hypolipidaemic-drug-treated mice, additional studies appear necessary to confirm the present results and examine in detail the comparative induction of peroxisomal and mitochondrial enoyl-CoA hydratase with subcellular-fractionation techniques. It should be pointed out, however, that, in normal mouse liver, the total β-oxidation appears to be distributed equally between the mitochondrial and peroxisomal system (Murphy *et al.*, 1979), in contrast with the rat, where less than 25% of β-oxidation is reported to occur in the peroxisomes (Lazarow, 1978). Since the ratio between peroxisome and mitochondrial number in the normal mouse liver cells is about 1:5 (Reddy, 1974), it would appear that there would be more β-oxidation system per peroxisome compared with a mitochondrion in this species. This relatively high extent of peroxisomal β-oxidation in normal mice, coupled with the fact that hypolipidaemic drugs used in these studies caused a remarkable increase in peroxisome population without appreciably altering the mitochondrial number, both in liver parenchymal cells and proximal-tubular-epithelium of the kidney, may account for the disproportionate induction of heat-labile peroxisomal enoyl-CoA hydratase.

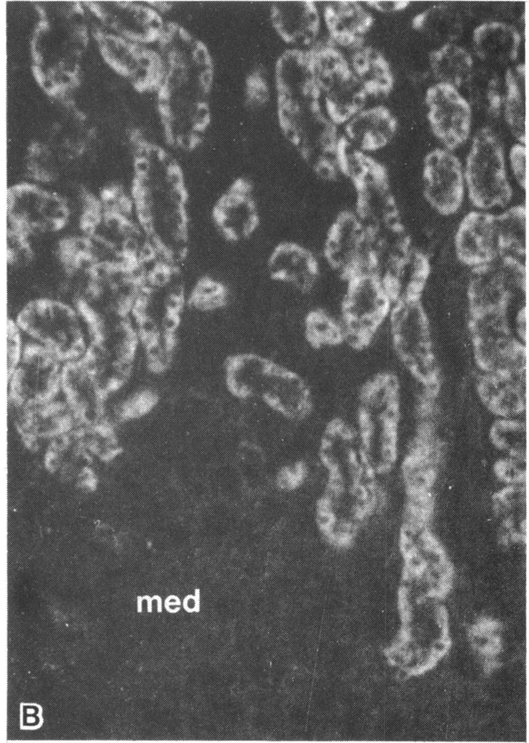
Evidence is also presented to show that treatment

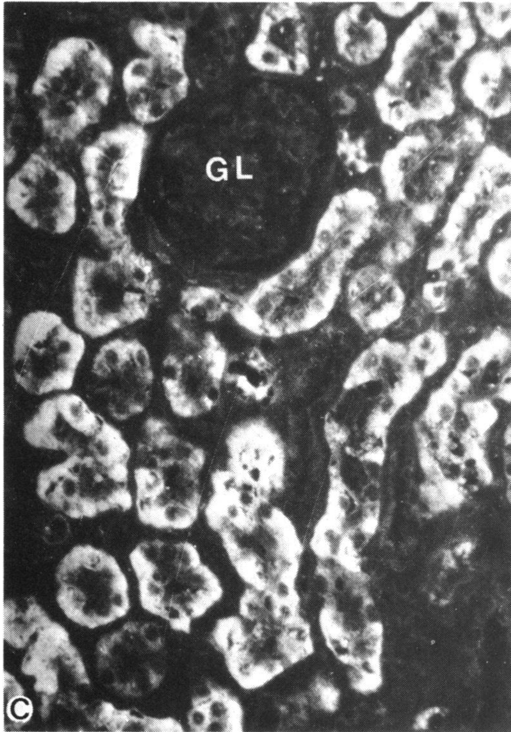
EXPLANATION OF PLATE 1

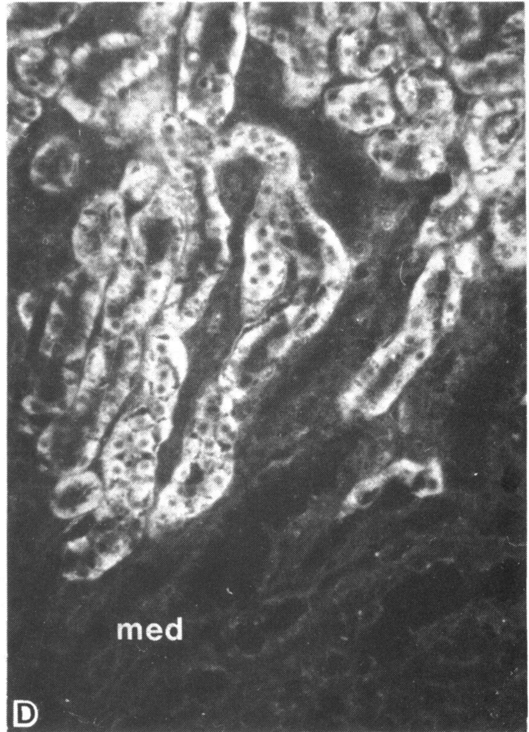
Immunofluorescence localization of heat-labile peroxisomal enoyl-CoA hydratase in the kidney of normal (A and B) and methyl clofenapate-treated (C and D) mouse

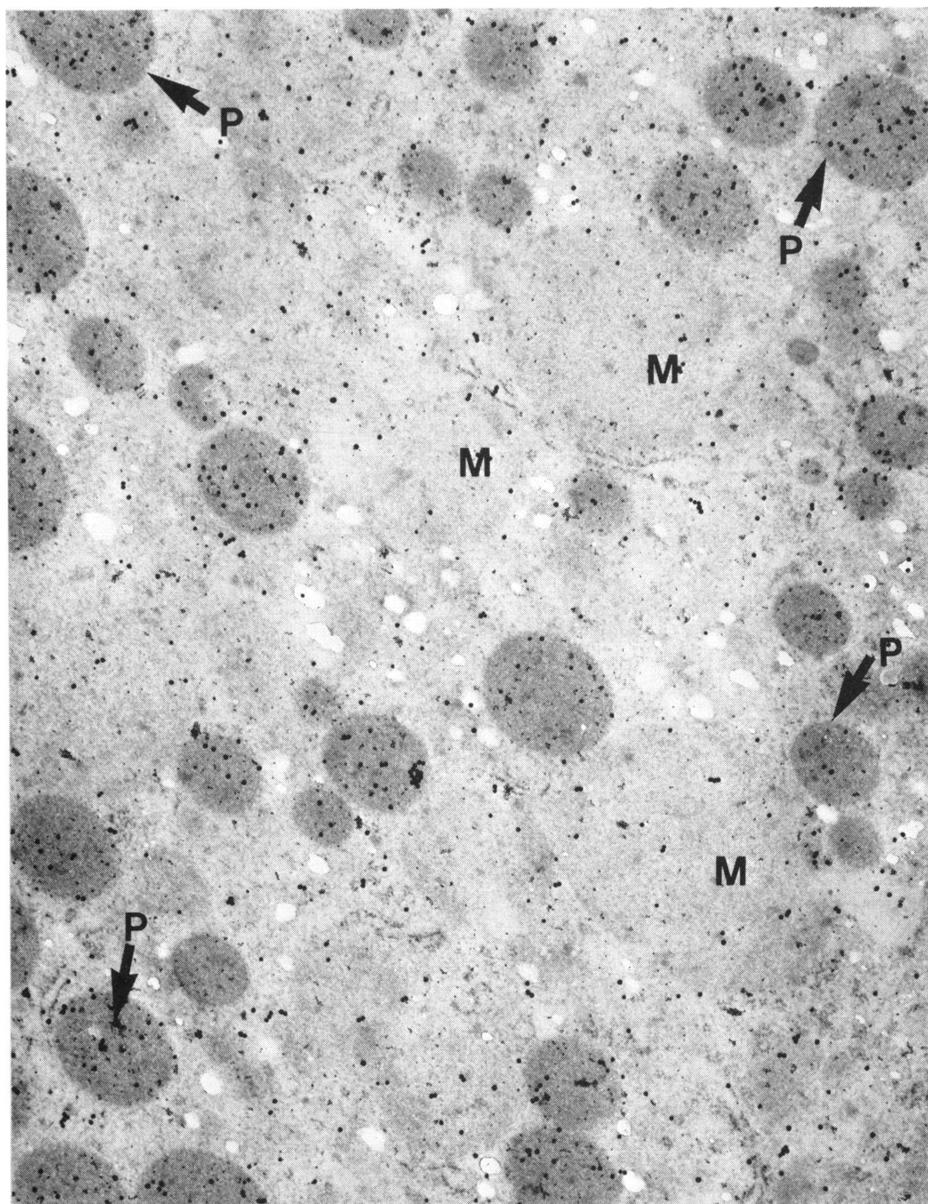
Note the absence of immunofluorescence in the renal medulla (med) and glomerulus (GL). In the normal kidney, the immunofluorescence is somewhat brighter in the peroxisome-rich p_3 segment of the proximal nephron and less intense in p_1 and p_2 segments. In the methyl clofenapate-treated mouse, the cytoplasmic immunofluorescence staining is intense throughout the proximal-convoluted-tubular epithelium. A similar immunofluorescence pattern was obtained with anti-(polypeptide PPA-80). Magnification $\times$ 150.


GL
p1
p2
p3
A


med
B


GL
C


med
D



EXPLANATION OF PLATE 2

Immunocytochemical localization of heat-labile peroxisomal enoyl-CoA hydratase in liver cell by the protein-A–gold technique

Ultrathin sections of aldehyde-fixed Epon-embedded liver tissue from hypolipidaemic-drug-treated animal were exposed to the anti-(enoyl-CoA hydratase) antiserum for 2 h at room temperature, washed and then incubated with the protein-A–gold complex for 1 h. The sections were washed, counterstained with uranyl acetate and examined in an electron microscope. Note the presence of numerous gold particles over peroxisomes (P) and very few over mitochondria (M). Magnification ×34 000.

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of mice with hypolipidaemic drugs results in the induction of peroxisome-proliferation-associated 80000-mol.wt. polypeptide (polypeptide PPA-80) in liver and kidney. Polypeptide PPA-80 accounts for a large increase in peroxisomal protein in the livers of rats (Reddy *et al.*, 1980) and (now) mice treated with peroxisome proliferators and in animals fed a high-fat diet (Ishii *et al.*, 1980). The studies of Ishii *et al.* (1980) show that the induction of polypeptide PPA-80 in livers of rats fed a high-fat diet is also associated with peroxisome proliferation in liver cells. Recent immunocytochemical studies from our laboratory have localized this polypeptide in the peroxisome matrix in rat liver (Reddy *et al.*, 1981) and not in the peroxisome membrane as reported previously by Hüttinger *et al.* (1979). The detection of this protein in SDS/polyacrylamide gel electrophoretograms of peroxisomal membrane fraction may be due to incomplete extraction of luminal-matrix protein of peroxisomes during fractionation. The induction of polypeptide PPA-80 in the renal cortex appears to parallel the increase in peroxisome number in the proximal-convoluted-tubular epithelium of kidney of hypolipidaemic-drug-treated mice. In a previous study we have reported the induction of peroxisomes in the kidney of methyl clofenapate-treated mice (Reddy *et al.*, 1975); the present study clearly shows that three other hypolipidaemic drugs also induce peroxisome proliferation in mouse renal cortex.

The nature of the peroxisome-proliferation-associated polypeptide is not clearly understood. This polypeptide has been shown recently by Inestrosa *et al.* (1980) to co-purify with fatty acyl-CoA oxidase. Furthermore, the immunochemical results presented here indicate that heat-labile peroxisomal enoyl-CoA hydratase is immunologically identical with the peroxisome-proliferation-associated 80000-mol.wt. protein (Reddy *et al.*, 1980). Because of the abundance of this protein in the livers of hypolipidaemic-drug-treated rats (Reddy *et al.*, 1980), hamsters (J. K. Reddy, unpublished work) and mice (the present study), it would appear that this protein, together with other components of the peroxisomal fatty-acid-oxidation system, including fatty-acid-binding protein (Appelkvist & Dallner, 1980), may constitute a multienzyme complex. It would be necessary to compare the immunochemical properties of other enzymes of peroxisomal fatty-acid-β-oxidation system with polypeptide PPA-80. Studies of the synthesis of polypeptide PPA-80 in cell-free translational systems *in vitro* may provide additional insight into the nature of this multienzyme complex.

The immunofluorescence studies of localization of polypeptide PPA-80 and heat-labile enoyl-CoA hydratase clearly complement the biochemical and ultrastructural observations of peroxisome induction in the liver and kidney cortex of mice fed on hypolipidaemic drugs. In the kidney, the distribution of these two proteins is identical and limited exclusively to the cytoplasm of proximal-convoluted-tubular epithelium. These proteins appear to be absent in the renal medulla and glomeruli, which lack peroxisomes.

In summary, the present evidence indicates that, in response to hypolipidaemic-drug administration, there is a substantial induction of the liver and kidney peroxisomal fatty-acid-oxidation system in mice. The ability of the kidney to oxidize long-chain fatty acids has been recognized for over 30 years (Weinhouse *et al.*, 1950), but until now this function has been attributed to mitochondria. The hypolipidaemic effect of drugs had previously been attributed to hepatic peroxisome proliferation (Reddy, 1973; Reddy & Krishnakantha, 1975) and induction of the liver peroxisomal β-oxidation system (Lazarow, 1977). It would therefore be important to investigate the relative role of various organs in the catabolism of fatty acids under normal as well as pathophysiological states.

This work was supported by National Institutes of Health grant no. GM-23750. The excellent technical assistance of Saeed A. Qureshi and June P. Tow is gratefully acknowledged.

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