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Partial Purification and Immunoreactivity of an 80000-Molecular-Weight Polypeptide Associated with Peroxisome Proliferation in Rat Liver

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Hypolipidaemic drugs and industrial plasticizers such as di-(2-ethylhexyl) phthalate, which cause proliferation of hepatic peroxisomes, also cause an increase in an 80000-mol.wt. polypeptide in the liver of rats and mice. This polypeptide has been designated as PPA-80 (PPA, for peroxisome-proliferation-associated; 80 for 80000 mol.wt.). The polypeptide PPA-80 was purified to over 90% purity from livers of rats treated with the peroxisome proliferators Wy-14,643, nafenopin, tibric acid and clofibrate by a single-step preparative sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic procedure. The antibodies raised against the PPA-80 polypeptide isolated from livers of rats treated with Wy-14,643 cross-reacted with polypeptide PPA-80 purified from the livers of rats treated with Wy-14,643, as well as from the livers of rats treated with nafenopin, tibric acid and clofibrate. The anti-(polypeptide PPA-80) antibodies did not cross-react with catalase, a marker enzyme for peroxisomes, or with NADPH–cytochrome *P*-450 reductase, which has the same approximate mol.wt., 80000. The intensity of immunoprecipitin bands formed with microsomal, large-particle and postnuclear fractions from livers of animals pretreated with peroxisome proliferators was significantly greater compared with equal amounts of protein from corresponding fractions obtained from control animals, suggesting that these agents all enhance the synthesis of the same 80000-mol.wt. polypeptide. Although the polypeptide PPA-80 was increased in the postnuclear, large-particle and microsomal fractions of livers of rats pretreated with peroxisome proliferators, the relative abundance of this peptide in the peroxisome-rich light-mitochondrial fraction and its lack in highly purified mitochondrial fractions suggest the localization of this polypeptide in peroxisomes and/or microsomal fraction. Additional studies are needed to establish unequivocally the subcellular localization of the polypeptide PPA-80 and to ascertain if this polypeptide is identical with the multi-functional protein displaying enoyl-CoA hydratase and β-hydroxyacyl-CoA dehydrogenase activities that was purified by Osumi & Hashimoto [(1979) *Biochem. Biophys. Res. Commun.* **89**, 580–584].

Administration of clofibrate [ethyl α-(*p*-chlorophenoxy)isobutyrate] and several other potent hypolipidaemic drugs results in a marked increase in the number of peroxisomes in liver cells of rats and mice (Hess *et al.*, 1965; Svoboda *et al.*, 1967; Reddy *et al.*, 1969, 1974, 1978; Reddy & Krishnakantha, 1975; Leighton *et al.*, 1975). The peroxisomes contain catalase, several H_2O_2-generating oxidases and carnitine acetyltransferase, as well as enzymes capable of catalysing the β-oxidation of long-chain fatty acids (de Duve & Baudhuin, 1966; Markwell *et al.*, 1973; Lazarow & de Duve, 1976; Masters & Holmes, 1977; Lazarow, 1978). The activities of these enzymes in liver are elevated in association with hypolipidaemic-drug-induced peroxisome proliferation (Reddy *et al.*, 1971; Krishnakantha & Kurup, 1972; Solberg *et al.*, 1972; Moody & Reddy, 1974, 1978*a*,*b*; Reddy & Krishnakantha, 1975; Kähönen, 1976; Lazarow, 1977; Osumi & Hashimoto, 1978*a*,*b*; Inestrosa *et al.*, 1979), suggesting that hepatic peroxisome proliferation and hypolipidaemia are interrelated effects of several structurally unrelated hypolipidaemic compounds (Reddy

Abbreviations used: SDS, sodium dodecyl sulphate; polypeptide PPA-80, peroxisome-proliferation-associated polypeptide of mol.wt. 80000.

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& Krishnakantha, 1975; Lazarow, 1977; Reddy *et al.*, 1978; Moody & Reddy, 1978*a*,*b*; Svoboda, 1978). In addition, these drugs exert a variety of effects on other subcellular organelles; of particular interest are an increase in mitochondrial protein content (Krishnakantha & Kurup, 1972) and the proliferation of smooth endoplasmic reticulum (Moody & Reddy, 1976; Kolde *et al.*, 1976; Anthony *et al.*, 1978).

Since phenobarbital and other xenobiotics that induce microsomal mono-oxygenases (Conney, 1967) are known to increase the amounts of the microsomal-membrane polypeptides in liver (Alvares & Siekevitz, 1973; Sharma *et al.*, 1979), we used SDS/polyacrylamide-gel electrophoresis to examine the polypeptide composition of postnuclear, large-particle and microsomal membranes obtained from livers of rats treated with the hypolipidaemic drugs clofibrate, nafenopin {2-methyl-2-[*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid}, Wy-14,643 {[4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid}, tibric acid (2-chloro-5-(3,5-dimethylpiperidinosulphonyl)benzoic acid) and BR-931 {[4-chloro-6-(2,3-xylidino)-2-pyrimidinyl]thio(*N*-β-hydroxyethyl)acetamide} in order to ascertain if these hepatic peroxisome proliferators induce specific polypeptide(s). In an earlier study, we reported the enhanced synthesis of a polypeptide with a molecular weight of approx. 80000 in all three subcellular fractions (Reddy & Kumar, 1977). Whether this polypeptide is specifically associated with drug-induced hepatic peroxisomes and/or microsomal fraction, or is induced in the membranes of all subcellular organelles, could not be ascertained. In the present studies, we have partially purified the 80000-mol.wt. polypeptide from livers of rats treated with peroxisome proliferators Wy-14,643, nafenopin, clofibrate and tibric acid and then made antibodies in rabbits to the partially purified polypeptide isolated from Wy-14,643-treated rat livers. These antibodies cross-reacted with the 80000-mol.wt. polypeptide isolated from the livers of rats treated with other hypolipidaemic compounds. Studies using the Ouchterlony double-diffusion technique (Ouchterlony, 1967) indicate that the 80000-mol.wt. polypeptide induced by the peroxisome proliferators is not the microsomal NADPH–cytochrome *P*-450 reductase, which has the same approximate molecular weight of 80000.

Experimental

Materials

The hypolipidaemic drugs used in the present study were generously given by the following sources: clofibrate, Ayerst Laboratories, New York, NY, U.S.A.; Wy-14,643, Wyeth Laboratories, Radnor, PA, U.S.A.; nafenopin, Ciba–Giegy Corp., Summit, NJ, U.S.A.; tibric acid, Pfizer Pharmaceuticals, Groton, CT, U.S.A. Purified acrylamide and *NN*-methylenebisacrylamide were obtained from Eastman Kodak, Rochester, NY, U.S.A. SDS and Coomassie Brilliant Blue R were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Standard proteins for SDS/polyacrylamide-gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. All other chemicals were obtained from commercial sources and were of the highest purity available.

Antibodies to NADPH–cytochrome *P*-450 reductase (purified from rat liver) were provided generously by Dr. Henry W. Strobel, Department of Biochemistry and Molecular Biology, University of Texas Medical School, Houston, TX, U.S.A. Highly purified NADPH–cytochrome *P*-450 reductase from rabbit liver was a generous gift from Dr. K. P. Vatsis, Department of Pharmacology, Northwestern University Medical School, Chicago, IL, U.S.A.; partially purified NADPH–cytochrome *P*-450 reductase from rat liver was provided by Dr. John J. Mieyal, Department of Pharmacology, Case Western Reserve University, Cleveland, OH, U.S.A.

Treatment of animals

Inbred male F344 rats weighing 120–150g were obtained from A. R. Schmidt/Sprague–Dawley, Madison, WI, U.S.A. For studies on the induction of hepatic peroxisome proliferation, the hypolipidaemic drugs were administered in powdered rat chow diet for 4 weeks at the concentrations indicated (w/w): Wy-14,643, 0.1%: clofibrate, 0.25%; nafenopin, 0.1%; tibric acid, 0.2%. For induction of the microsomal enzymes, a group of rats was injected daily with phenobarbital (80mg/kg body wt.) intraperitoneally for 1 week. At the end of the treatment periods the animals were killed under light ether anaesthesia and their livers were perfused with cold normal saline (0.9% NaCl) to eliminate erythrocytes from the hepatic sinusoids.

Subcellular fractionation

The perfused and minced livers were homogenized [10% (w/v) homogenate] in 0.25M-sucrose in a Potter–Elvehjem homogenizer. The unbroken cells and nuclei were removed by centrifugation at 700*g* for 10min in a Beckman J-21C centrifuge. The postnuclear supernatant was fractionated into postnuclear, large-particle and microsomal pellets as described by Kurup *et al.* (1970). The postnuclear supernatant was centrifuged at 105000*g* for 60min in a Beckman L5-65 ultracentrifuge to obtain the postnuclear pellet (consisting of all postnuclear membranes) or at 8300*g* for 10min in a Beckman J-21C centrifuge to obtain the large-particle pellet (consisting mostly of mitochondria, peroxisomes

and lysosomes). The supernatant collected after sedimentation of the large-particle pellet was centrifuged at 105000*g* for 60min in a Beckman L5-65 ultracentrifuge to obtain the microsomal pellet. All pellets were washed by resuspension in 0.25M-sucrose containing 0.03M-KCl and resedimented at the appropriate centrifugal force.

The postnuclear supernatant from livers of control and Wy-14,643-treated rats was also fractionated as described by de Duve *et al.* (1955) into heavy-mitochondrial, light-mitochondrial and microsomal fractions. This was done with the hope that the separation of the two mitochondrial fractions would provide some clue about the subcellular localization of the 80000-mol.wt. polypeptide, because peroxisomes appear to sediment with the light-mitochondrial fraction.

Pure liver mitochondrial fractions were isolated by the method of Schneider (1948); the purity was assessed by electron microscopy. The postnuclear supernatant obtained from a 10% homogenate in 0.33M-sucrose containing 10mM-Tris/HCl, pH7.4, and 1mM-EDTA was centrifuged at 8600*g* for 10min in a Beckman J-21C centrifuge to sediment the mitochondria. This pellet was resuspended in the homogenization medium and washed twice by sedimentation at 8300*g* for 10min to obtain pure mitochondria.

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was performed on slab or cylindrical polyacrylamide gels by the method of Laemmli (1970). Gels (7.5 or 10% polyacrylamide separating gels; 8cm long) were prepared from a stock of 30% acrylamide and 0.8% *NN*-methylenebisacrylamide. The separating gel contained 0.375M-Tris/HCl, pH8.8, 0.1% SDS, 0.024% tetramethylethylenediamine and 0.1% ammonium persulphate. The stacking gel was either 3% or 4.5% acrylamide with 0.125M-Tris/HCl, pH6.8, 0.1% SDS, 0.024% tetramethylethylenediamine and 0.1% ammonium persulphate. The electrode buffer used was 0.025M-Tris/glycine, pH8.1–8.3, with 0.1% SDS. The samples were solubilized in a solution containing 20% glycerol, 2% SDS, 5% mercaptoethanol and 0.001% Bromophenol Blue in 0.0625M-Tris/HCl, pH6.8, by heating for 3min in boiling water (Laemmli, 1970). Between 20 and 100μg of dissolved protein was loaded on the cylindrical gels and electrophoresed with a current of 4mA/tube until the tracking dye had reached the bottom of the gel. The slab gels were electrophoresed with a current of 20mA/slab. After the completion of the run, the proteins were fixed by immersing the gels in 10% (w/v) trichloroacetic acid for 30min and then stained in 0.1% Coomassie Blue in 50% (v/v) methanol/10% (v/v) acetic acid. The cylindrical gels were stained overnight and the slab gels for 2h. The gels were destained in 50% methanol/10% acetic acid and stored in 7.5% acetic acid/5% methanol.

The cylindrical gels were scanned at 540nm in a Beckman spectrophotometer (model 25). The area of the individual peaks was measured with a planimeter or by the area/weight method (M. K. Reddy *et al.*, 1975).

Purification of 80000-mol.wt. polypeptide from rats treated with hypolipidaemic drugs

The peroxisomal protein was purified by using a Bio-Rad vertical-preparative-slab-electrophoresis apparatus model no. 220 fitted with an eluting canal. A plug gel of 30% polyacrylamide with 0.05% *NN*-methylenebisacrylamide was used at the bottom; this was followed by 2ml of 50% glycerol in 0.375M-Tris/HCl, pH8.8, that served as the eluting canal. A separating gel of 7.5% polyacrylamide (30ml) was made above the eluting canal. After polymerization, a stacking gel (3%) was prepared. Postnuclear fractions obtained from livers of rats given hypolipidaemic drugs (Wy-14,643, nafenopin, clofibrate and tibric acid) were used for the purification of the 80000-mol.wt. polypeptide. The protein sample (5mg/ml; 2ml) was prepared by heating in the solubilizing sample buffer as described above and layered on the stacking gel. The electrophoresis was carried out with a current of 40mA/slab at a constant voltage. When the tracking dye reached the eluting canal, fractions of volume 3ml were collected and the A_{280} values measured. The fractions were analysed on SDS/polyacrylamide slab or cylindrical gel systems, and those containing the 80000-mol.wt. polypeptide were pooled, concentrated by freeze-drying and dialysed extensively against 0.02M-Tris/HCl, pH7.0, to remove SDS and lower the concentration of Tris/HCl.

Production of antibodies and characterization by Ouchterlony double-diffusion analysis

Five New Zealand white male rabbits weighing 2–3kg were immunized with the partially purified 80000-mol.wt. polypeptide isolated from livers of rats treated with 0.1% Wy-14,643. Approx. 700–900μg of polypeptide/ml was emulsified in equal volume of Freund's adjuvant (Difco, Detroit, MI, U.S.A.) and injected subcutaneously at multiple sites, at weekly intervals for 4 weeks. Then 1 week after the last injection, a booster injection of the polypeptide (without the adjuvant) was given intravenously and the rabbits were bled 5 days later. The γ-globulins were prepared from the antisera by $(NH_4)_2SO_4$ fractionation (Kabat & Mayer, 1961) and dialysed against 0.9% NaCl and then against deionized distilled water. The euglobulins that precipitated during dialysis were removed by cen-

trifugation and the clear γ-globulin fractions were kept frozen at −20°C until used.

Ouchterlony plates were prepared by using 1% agarose in 0.1M-Tris/barbiturate buffer, pH 8.6, containing 1μM-NaN_3 and 0.9% NaCl. The cross-reactivity of partially purified 80000-mol.wt. polypeptides, the solubilized subcellular fractions from livers, the purified NADPH–cytochrome *P*-450 reductase or catalase with the antibody prepared against the 80000-mol.wt. polypeptide was analysed by allowing the Ouchterlony plates to develop for 30–35h at room temperature (22±2°C) in a humidified chamber.

Enzyme assays

Catalase activity was assayed by the method of Lück (1963). Carnitine acetyltransferase was measured by the method of Markwell *et al.* (1973). Succinate dehydrogenase activity was determined by the method of Buchmann *et al.* (1966). Cathepsin D was measured by the method of Anson (1938). NADPH–cytochrome *P*-450 reductase was assayed spectrophotometrically by the procedure of Masters *et al.* (1967), except that 0.3M-potassium phosphate buffer was used.

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Subcellular localization of polypeptide PPA-80

We have previously shown that compounds that cause the proliferation of peroxisomes in liver cells selectively induce a polypeptide of apparent mol.wt. 80000 (Reddy & Kumar, 1977). SDS/polyacrylamide-gel-electrophoretic analysis in the present study of the polypeptide composition of large-particle, microsomal and postnuclear pellets obtained from the livers of rats treated with clofibrate, Wy-14,643, tibric acid and nafenopin confirmed the increase of this 80000-mol.wt. polypeptide (Fig. 1). This polypeptide will be referred to as PPA-80 (PPA for peroxisome-proliferation-associated; 80 for 80000 mol.wt.), since it is increased in the livers with drug-induced peroxisome proliferation and not in the livers of rats treated with phenobarbital (Fig. 2) and other microsomal enzyme inducers (Reddy & Kumar, 1977; Kumar & Reddy, 1977).

The distribution of polypeptide PPA-80 in the crude postnuclear, large-particle and microsomal fractions prepared from livers of rats treated with 0.1% (w/w) Wy-14,643 for 4 weeks was determined by SDS/polyacrylamide-gel electrophoresis. As shown in Table 1, polypeptide PPA-80 accounts for approx. 16, 20 and 35% of the protein content respectively of postnuclear, large-particle and microsomal fractions obtained from the livers of rats pretreated with Wy-14,643. In the livers of treated

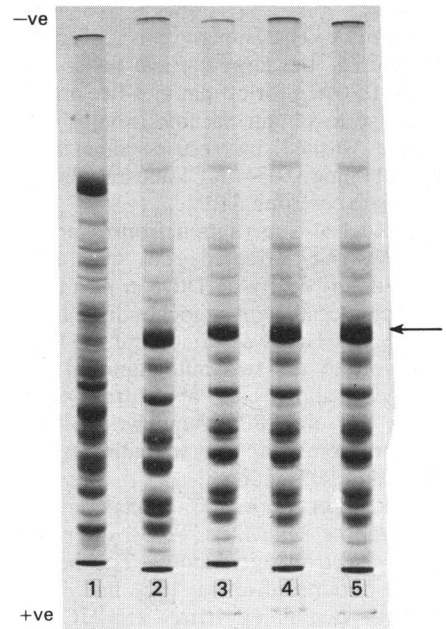

Fig. 1. *SDS/polyacrylamide-gel-electrophoresis profile of large-particle pellets of livers from (1) normal, (2) 0.1% Wy-14,643-treated, (3) 0.25% clofibrate-treated, (4) 0.1% nafenopin-treated and (5) 0.2% tibric acid-treated rats*

The large-particle fractions were prepared by differential centrifugation as described in the Experimental section. Approx. 100μg of protein/gel was applied on 7.5%-acrylamide separating cylindrical gels with 3% stacking gel. Arrow indicates the position of the polypeptide PPA-80. The intensity of staining of the top band in treated livers is decreased when compared with the control.

animals approx. 70% of the polypeptide PPA-80 present in the post-nuclear fractions is recoverable in the large-particle pellet, and the remainder in the microsomal pellets (results not shown).

SDS/polyacrylamide-gel-electrophoretic analysis of heavy-mitochondrial, light-mitochondrial, microsomal and postmicrosomal-supernatant fractions

The electrophoretic patterns of the postnuclear, heavy-mitochondrial, light-mitochondrial, microsomal and postmicrosomal-supernatant fractions of liver from control and Wy-14,643-treated rats are shown in Fig. 3. In general, the polypeptide PPA-80 increased in amount in all fractions obtained from the livers of rats treated with Wy-14,643 (Fig. 3, slots 2, 4, 6, 8 and 10). Of particular interest, however, is the relative abundance of this polypeptide in the light-mitochondrial (Fig. 3, slot 6) and

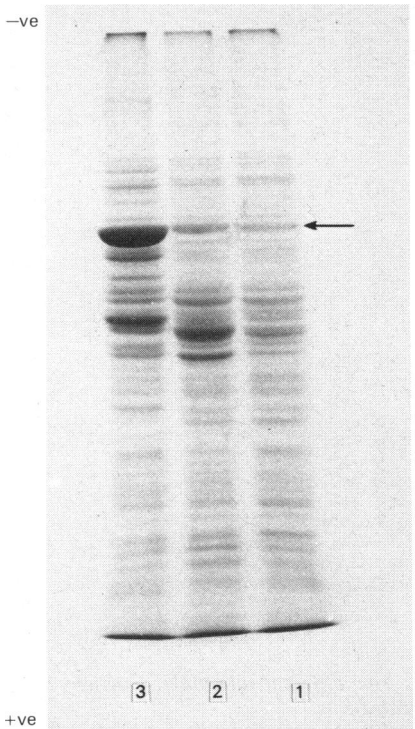

Fig. 2. *SDS/polyacrylamide-gel-electrophoresis pattern of microsomal fractions of livers from (1) normal, (2) phenobarbital-treated and (3) 0.1% Wy-14,643-treated rats*

The microsomal fractions were prepared by differential centrifugation as described in the Experimental section. Approx. 15 μg of protein was applied in each slot on a 10%-acrylamide gel. Arrow indicates the position of 80000-mol.wt. polypeptide (PPA-80).

Table 1. *Localization of polypeptide PPA-80 in the post-nuclear, large-particle and microsomal fractions of normal and Wy-14,643-treated rat livers*

Wy-14,643 was administered in powdered chow at a dietary concentration of 0.1% for 4 weeks. The fractions were prepared from control and treated rats by differential centrifugation as described in the Experimental section. The relative area under the peak corresponding to polypeptide PPA-80 on the densitometric tracings of the SDS/polyacrylamide-gel-electrophoresis cylindrical gels was determined by planimetry. The quantity of polypeptide PPA-80 in the subcellular fractions is presented in terms of the area under the peak (as percentage of total area), ± S.D.; the numbers in parentheses indicate the numbers of animals used for the analysis.

Subcellular fraction	Quantity of polypeptide PPA-80 (% of the fraction)	
	Control	Wy-14,643-treated
Postnuclear	4.0 ± 0.1 (6)	15.8 ± 1.3 (6)
Large-particle	7.7 ± 1.1 (10)	20.1 ± 1.0 (10)
Microsomal	6.5 ± 0.8 (10)	34.8 ± 1.5 (10)

the microsomal fraction (Fig. 3, slot 8) after the administration of Wy-14,643. As expected, the relative specific activities of catalase and cathepsin D in the light-mitochondrial fraction were higher than those observed in the heavy-mitochondrial fraction (results not shown). The NADPH–cytochrome *P*-450 reductase activity was considerably higher in the microsomal and light-mitochondrial fraction than in the heavy-mitochondrial fraction.

Purification of the polypeptide PPA-80

Preparative slab-gel electrophoresis was used to isolate the polypeptide PPA-80 from the post-nuclear pellets of livers of rats treated with the peroxisome proliferators Wy-14,643, clofibrate, nafenopin and tibric acid. The SDS/polyacrylamide-gel profiles of the proteins in the fractions collected after separation on preparative slab-gel electrophoresis are illustrated in Fig. 4. When fractions 6 and 7 (Fig. 4) were pooled, dialysed and concentrated by freeze-drying, the resulting protein consisted predominantly of polypeptide PPA-80, as judged by the Coomassie Brilliant Blue staining of the SDS/polyacrylamide gel of this fraction (Fig. 5). Although several minor contaminants were detected when the gel was overloaded with 30 μg of protein, no attempts were made to purify this fraction further. Densitometric scanning of gels loaded with 5, 10 or 20 μg of protein/gel and further analysis of the area of the individual bands gave an estimate of over 90% purity for the polypeptide PPA-80. The band migrated with a R_m value (relative to the migration of the tracking dye) of 0.56 ± 0.05. The purification, elution and freeze-drying did not change the mobility of this polypeptide (Fig. 5). This polypeptide can be stored frozen for at least 3 months. Frequent freezing and thawing appeared to degrade the molecules into low-molecular-weight fragments.

Immunochemical relationships between the polypeptide PPA-80 induced by different peroxisome proliferators

The purified polypeptide PPA-80 isolated from the livers of rats treated with Wy-14,643 was injected into rabbits in order to produce antibodies. Since the antisera produced against this polypeptide exhibited relatively low titres of precipitating antibodies, the antibodies were concentrated by preparing the γ-globulins. When used in the immuno-

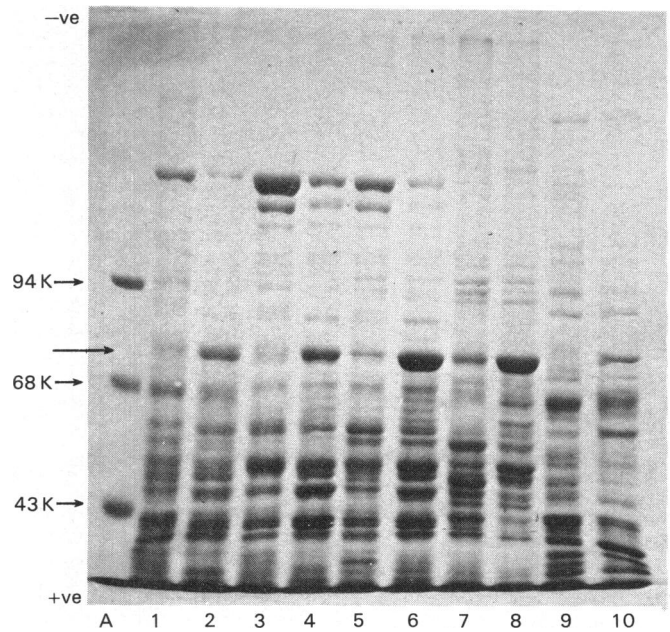

Fig. 3. *SDS/polyacrylamide-slab-gel electrophoretic profiles of proteins from different subcellular fractions of liver from normal and 0.1% Wy-14,643-treated rats*

Subcellular fractions were prepared as described by de Duve *et al.* (1955), and electrophoresed as described in the text. Slots: A, molecular-weight standards (94K denotes mol.wt. 94000, etc.); 1 and 2, postnuclear supernatant; 3 and 4, heavy-mitochondrial fraction; 5 and 6, light-mitochondrial fraction; 7 and 8, microsomal fraction; 9 and 10, soluble fractions (post-microsomal). Odd numbers represent the fractions from normal rats and the even numbers represent fractions from 0.1%-Wy-14,643-treated-rats. Protein concentration per slot was 20μg. Unlabelled arrow indicates PPA-80 polypeptide.

diffusion assays, the antibodies gave a single precipitin line with the polypeptide PPA-80 antigen isolated from Wy-14,643-treated rat livers (Fig. 6*a*).

Since the antibodies were raised against polypeptide PPA-80 purified from Wy-14,643-treated rat livers, it was important to ascertain whether they would also react with the polypeptide PPA-80 isolated from the livers of rats treated with other hepatic peroxisome proliferators. As shown in Fig. 6(*a*), the polypeptide PPA-80 from the livers of rats treated with different peroxisome proliferators reacted with the antibodies prepared against the polypeptide PPA-80 of Wy-14,643-treated rats and the precipitin lines were confluent, indicating immunological identity of the antigens.

The antibodies raised against the polypeptide PPA-80 from the rats treated with Wy-14,643 cross-reacted with the microsomal fractions prepared from rats treated with other peroxisome proliferators to give single precipitin lines of identity. However, purified rat liver catalase (Reddy & Kumar, 1979) did not cross-react with the antibodies against polypeptide PPA-80 (Fig. 6*b*, well 8).

Semi-quantitative Ouchterlony double-diffusion procedures using various concentrations of protein from microsomal, large-particle and postnuclear fractions demonstrated a marked increase in the amount of polypeptide PPA-80 in the fractions obtained from the pretreated animals in comparison with control animals.

Immunodiffusion with purified mitochondrial fractions

The extracts of purified mitochondria obtained from livers of rats treated with Wy-14,643 and phenobarbital were examined by the Ouchterlony double-diffusion method by using the antibodies for the polypeptide PPA-80. The mitochondrial fractions from control and phenobarbital-treated animals showed no precipitin lines. When the purified mitochondrial fractions obtained from the livers of Wy-14,643-treated animals were analysed by SDS/polyacrylamide-gel electrophoresis, no increase in the amount of polypeptide PPA-80 was observed. Occasionally, a single faint precipitin line was observed with the mitochondrial extracts pre-

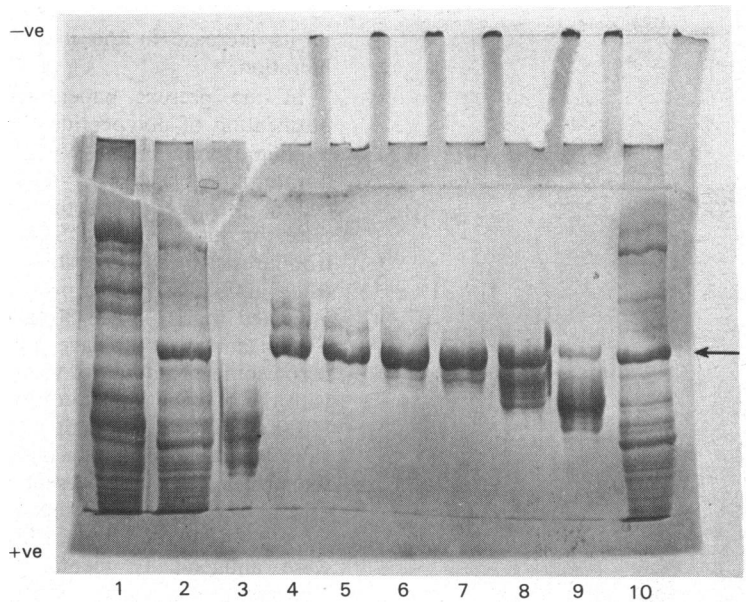

Fig. 4. *SDS/polyacrylamide-slab-gel profiles of the proteins in the fractions eluted from preparatory slab-gel electrophoresis*

The postnuclear fractions of livers from rats given 0.1% Wy-14,643 for 4 weeks were separated on 7.5% polyacrylamide gel with a 3% stacking gel. The conditions used for separation and elution are given in the Experimental section. Slots 1, 2 and 10 had approx. 150μg of protein each. The rest of the slots had approx. 30μg of protein each. The arrow indicates the position of the polypeptide PPA-80. Slots: 1, control postnuclear fraction; 2 and 10, postnuclear fraction from Wy-14,643-treated rats; 3, the protein eluted along with the tracking dye; 4–9, various fractions collected by elution of protein from the slab gel corresponding to the fractions from the top to the bottom.

pared from Wy-14,643-treated animals. This may have been the result of peroxisomal contamination. The microsomal and large-particle fractions from Wy-14,643-treated rat livers always formed clear precipitin lines with the same amount of protein.

Immunochemical relationship between polypeptide PPA-80 and NADPH–cytochrome P-450 reductase

Since the polypeptide PPA-80 and the liver microsomal NADPH–cytochrome *P*-450 reductase have approximately the same mol.wt. (80000) and migrate similarly on SDS/polyacrylamide gels (Fig. 7), it was necessary to investigate the possibility that the polypeptide PPA-80 induced by the hypolipidaemic peroxisome proliferators might be NADPH–cytochrome *P*-450 reductase. As shown in Fig. 8, rabbit anti-(polypeptide PPA-80) antibody formed a single precipitin band with the purified polypeptide PPA-80 or with microsomal preparation from liver of Wy-14,643-treated rats. The anti-(rat liver NADPH–cytochrome *P*-450 reductase) antibody did not cross-react with the purified polypeptide PPA-80; however, as expected, a single precipitin band was observed with microsomal preparation from the Wy-14,643-treated rats. Likewise, the purified rat liver NADPH–cytochrome *P*-450 reductase failed to react with the antibodies prepared against polypeptide PPA-80 (results not shown), suggesting that the polypeptide PPA-80 and NADPH–cytochrome *P*-450 reductase are antigenically dissimilar.

Discussion

The results of the SDS/polyacrylamide-gel-electrophoretic analysis of the polypeptide composition of the postnuclear, large-particle and microsomal membranes of rat liver reported in the present paper confirm a previous report from this laboratory (Reddy & Kumar, 1977) that compounds which cause proliferation of liver peroxisomes induce a polypeptide of apparent mol.wt. 80000. Phenobarbital, 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane and allylisopropylacetamide, which cause proliferation of smooth endoplasmic reticulum in hepatic parenchymal cells, did not appreciably increase this polypeptide (Reddy & Kumar, 1977; Kumar & Reddy, 1977). This polypeptide has been designated as PPA-80 because

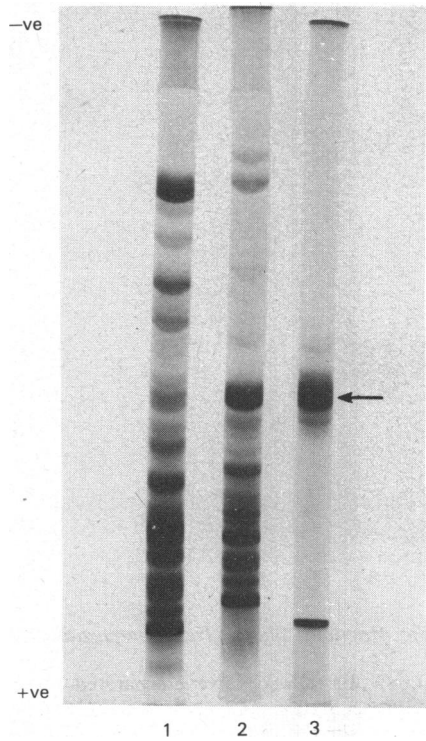

Fig. 5. *SDS/polyacrylamide cylindrical gels showing partially purified polypeptide PPA-80 from the livers of animals treated with 0.1% Wy-14,643*

Approx. 100μg of postnuclear fractions and 30μg of partially purified polypeptide were separated in 7.5% polyacrylamide gels with 3% stacking gel. Arrow indicates the position of 80000-mol.wt. polypeptide. Gels: (1) control postnuclear fraction; (2) postnuclear fraction from 0.1% Wy-14,643-treated rats; and (3) the partially purified polypeptide PPA-80 from Wy-14,643-treated rats.

of its increase in the liver with peroxisome proliferation.

In the present paper, we report the partial purification of polypeptide PPA-80 induced by the hypolipidaemic drugs Wy-14,643, nafenopin, clofibrate and tibric acid. The electrophoretic procedure produced a protein which was over 90% pure, as judged by SDS/polyacrylamide-gel electrophoresis, in a single step. The results of studies using antibodies to this protein produced in rabbits indicated that: (1) the 80000-mol.wt. peptide present in animals that have not been pretreated with peroxisome proliferators was antigenically identical with the polypeptide induced by Wy-14,643; (2) the 80000-mol.wt. polypeptides present in the three subcellular fractions were antigenically identical; (3) the protein was not present in purified mitochondria; and (4) the 80000-mol.wt. polypeptides induced by Wy-14,643, clofibrate, tibric acid and nafenopin were antigenically identical. Therefore all these hypolipidaemic agents induce the synthesis of the same 80000-mol.wt. polypeptide as determined by molecular weight and by antigenic properties.

Previous studies in our laboratory on the increases in the amounts of polypeptide PPA-80 in rats treated concurrently with Wy-14,643 and allylisopropylacetamide, an agent which inhibits catalase synthesis, suggested that this polypeptide was synthesized in the absence of the synthesis of enzymically active catalase (Reddy & Kumar, 1977). The results presented here, which demonstrate that antibodies to polypeptide PPA-80 do not cross-react with purified rat liver catalase, rule out the possibility that polypeptide PPA-80 might be an apoenzyme precursor form of catalase. Since there is no cross-reactivity between anti-(polypeptide PPA-80)antiserum and NADPH–cytochrome *P*-450

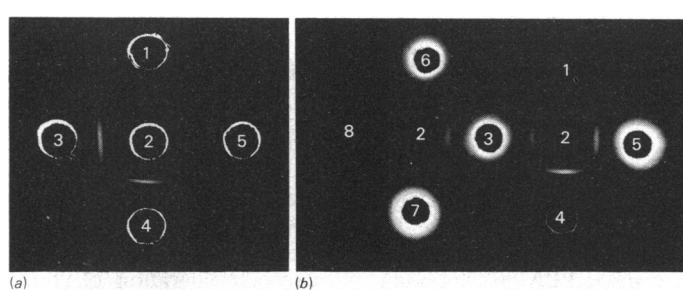

Fig. 6. *Ouchterlony double-diffusion analysis using rabbit anti-(polypeptide PPA-80) antiserum*

Immunodiffusion was carried out as described in the Experimental section. (*a*) Cross-reactivity of rabbit anti-(polypeptide PPA-80) serum (well 2) against partially purified polypeptide PPA-80 from the livers of rats treated with 0.1% Wy-14,643 (well 4), 0.1% nafenopin (well 3), or 0.25% clofibrate (well 5); non-immune serum in well 1. (*b*) Cross-reactivity of rabbit anti-(polypeptide PPA-80) serum (wells labelled 2) against microsomal fractions obtained from the livers of rats treated with the peroxisome proliferators 0.1% Wy-14,643 (well 3), tibric acid (well 5), nafenopin (well 6) or clofibrate (well 7). Well 4 contains partially purified polypeptide PPA-80 from the livers of Wy-14,643-treated rat. Well 8 contains 45μg of purified rat catalase. Well 1 contains non-immune rabbit serum.

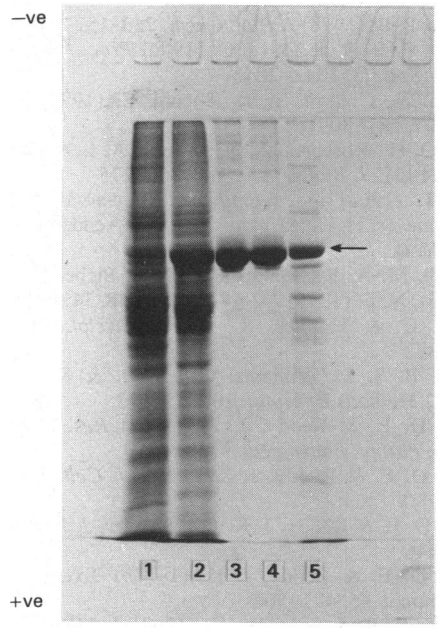

Fig. 7. *SDS/polyacrylamide-slab-gel-electrophoretic pattern of partially purified polypeptide PPA-80 and rabbit and rat liver NADPH–cytochrome P-450 reductase*

Samples (approx. 100μg) of microsomal fraction from normal and 0.1% Wy-14,643-treated animals were electrophoresed on 10% slab gels with 4.5% stacking gel. Gels: (1) normal rat; (2) 0.1% Wy-14,643-treated rat; (3) partially purified polypeptide PPA-80; (4) rabbit NADPH–cytochrome *P*-450 reductase; (5) partially purified rat NADPH–cytochrome *P*-450 reductase. Purified proteins were 10μg each.

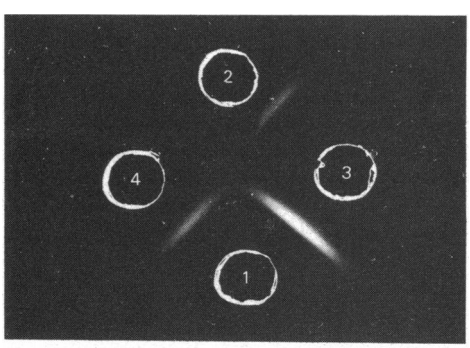

Fig. 8. *Ouchterlony double-diffusion patterns of the anti-(polypeptide PPA-80) and anti-(NADPH–cytochrome P-450 reductase) sera against microsomal extracts and purified polypeptide PPA-80*

Immunodiffusion was carried out as described in the Experimental section. Wells: (1) anti-(polypeptide PPA-80) serum; (2) anti-(cytochrome *P*-450 reductase) serum; (3) microsomal extract from Wy-14,643-treated rat liver; (4) polypeptide PPA-80.

reductase or between anti(NADPH–cytochrome *P*-450 reductase) serum and polypeptide PPA-80, the possibility that these two proteins are identical can be ruled out.

Although our studies indicate that polypeptide PPA-80 is induced by drugs which increase peroxisome proliferation, it is not certain if this polypeptide is localized exclusively in peroxisomes. The demonstration that polypeptide PPA-80 is present in all three subcellular fractions of the livers from rats pretreated with peroxisome proliferators may reflect the induction of this polypeptide in all cytoplasmic organelles or exclusively in the peroxisomes and/or the smooth endoplasmic reticulum. The relative increase of this polypeptide in the light-mitochondrial fraction when compared with the heavy-mitochondrial fraction suggests the localization of polypeptide PPA-80 in peroxisomes. The failure to obtain a clear-cut immunoprecipitin band with the highly purified mitochondria when reacted against anti-(polypeptide PPA-80) antibodies also suggests the localization of polypeptide PPA-80 in an organelle(s) other than mitochondria. Since polypeptide PPA-80 is also increased in microsomal fractions of livers of rats treated with peroxisome proliferators, it is possible that this may be due to: (1) concomitant increase of this polypeptide in the endoplasmic reticulum (microsomal fraction), (2) contaminant smaller-sized proliferated peroxisomes or their limiting membranes, which may co-sediment with microsomal fractions as a result of partial or complete extraction of their matrix proteins during homogenization (Reddy & Svoboda, 1971; Moody & Reddy, 1976; Leighton *et al.*, 1975); or (3) the existence of numerous continuities between peroxisome membrane and smooth endoplasmic reticulum (Novikoff & Shin, 1964; Reddy & Svoboda, 1971) that may share common membrane proteins. The possibility that this polypeptide is associated exclusively with peroxisomes is considered likely because polypeptide PPA-80 appears to be a major protein component (on SDS/polyacrylamide-gel electrophoresis) induced in livers by peroxisome proliferators and not by microsomal-enzyme inducers. Additional studies, however, are necessary to establish unequivocally the subcellular localization of this polypeptide. This may be accomplished either by further subfractionation of cytoplasmic organelles to obtain a highly purified peroxisome fraction or by localizing the polypeptide PPA-80 at the ultrastructural level by immunoferritin or immuno-peroxidase procedures. The availability of antibodies to polypeptide PPA-80 should facilitate such studies.

The exact nature of the polypeptide PPA-80, as well as its role, if any, in drug-induced lipid metabolism remain to be ascertained. On the basis of structure–biological-activity studies with several

hypolipidaemic compounds and industrial plasticizers (Reddy & Krishnakantha, 1975; Moody & Reddy, 1978*a*,*b*), we concluded that hepatic peroxisome proliferation and hypolipidaemia are interrelated effects of structurally unrelated chemicals. Since all these compounds increase hepatic peroxisome proliferation and induce polypeptide PPA-80 as well as the activity of the fatty acyl-CoA β-oxidation system (Lazarow, 1977; Krahling *et al.*, 1978; Osumi & Hashimoto, 1978*a*,*b*; Inestrosa *et al.*, 1979), we entertained the possibility that polypeptide PPA-80 may be involved in lipid metabolism (Reddy & Kumar, 1977). Osumi & Hashimoto (1979) purified the heat-labile peroxisomal enoyl-CoA hydratase from the livers of rats treated with the peroxisome proliferator di-(2-ethylhexyl) phthalate (Reddy *et al.*, 1976; Kumar & Reddy, 1977) and demonstrated on SDS/polyacrylamide-gel electrophoresis that this purified enzyme corresponds to the polypeptide PPA-80 identified by us. The positive identification of the polypeptide PPA-80 as the peroxisomal enoyl-CoA hydratase now requires investigation.

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