Cytochrome *P*-450 induction by clofibrate

Purification and properties of a hepatic cytochrome *P*-450 relatively specific for the 12- and 11-hydroxylation of dodecanoic acid (lauric acid)

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Hypolipidaemic drugs induce peroxisomal proliferation in the liver and many induce the formation of the hepatic endoplasmic reticulum in general and the formation of cytochrome *P*-450 in particular. We have induced the formation of rat liver microsomal cytochrome *P*-450 by the administration of the hypolipidaemic drug clofibrate, isolated the endoplasmic reticulum, solubilized the cytochrome *P*-450 from these membranes and subdivided the cytochrome *P*-450 into four fractions by the use of hydrophobic, anionic, cationic and adsorption chromatography. One of these fractions (cytochrome *P*-450 fraction 1) was highly purified to a specific content of 17 nmol of cytochrome *P*-450/mg of protein and the protein was active in a reconstituted enzyme system towards the 12- and 11-hydroxylation of the fatty acid, dodecanoic (lauric) acid, with preferential activity towards the 12-hydroxy metabolite. This reconstituted activity was absolutely dependent on NADPH, NADPH-cytochrome *P*-450 reductase and cytochrome *P*-450, indicating the role of the mixed-function oxidase system in the metabolism of lauric acid. Another fraction of the haemoprotein (cytochrome *P*-450 fraction 2) preferentially formed 11-hydroxylauric acid, whereas a third fraction (cytochrome *P*-450 fraction 3) exhibited only trace laurate oxidase activity and was similar to the phenobarbitone form of the haemoprotein in that these last two cytochromes rapidly turned-over the drug benzphetamine. The molecular weights and spectral properties of these cytochrome *P*-450 fractions are reported, along with the phenobarbitone-induced form of the enzyme and the nature of the cytochrome(s) induced by clofibrate pretreatment are discussed in the terms of possible haemoprotein heterogeneity.

Clofibrate [ethyl 2-(4-chlorophenoxy)-2-methyl-propanoate] is a widely used and potent hypolipidaemic drug used for the primary prevention of ischaemic heart disease (Heady, 1973). Chronic pretreatment of rats and other experimental animals with clofibrate results in massive hepatomegaly (Reddy *et al.*, 1980), characteristically associated with peroxisomal proliferation (Hess *et al.*, 1965), proliferation of the endoplasmic reticulum (Azarnoff *et al.*, 1975) and induction of cytochrome *P*-450 (Parker & Orton, 1980), the terminal haemoprotein component of the hepatic endoplasmic reticulum mixed-function oxidase system responsible for xenobiotic metabolism (Cooper *et al.*, 1965).

This increase in liver microsomal cytochrome *P*-450 after clofibrate pretreatment was not accompanied by an increase in the metabolism of model drug substrates such as aminopyrine, pentobarbitone or benzo[*a*]pyrene (Salvador *et al.*, 1970; Orton & Higgins, 1979), as may be expected, but resulted in a significant and selective increase in the liver microsomal 12- and 11-hydroxylation of fatty acids such as lauric (dodecanoic) acid, with preferential induction of the 12-hydroxylauric acid metabolite (Parker & Orton, 1980). These observations then suggested to us that clofibrate was acting as a relatively specific inducer of the mixed-function oxidase system responsible for fatty acid oxidation in direct contrast with the classical, broad-spectrum cytochrome *P*-450 inducer phenobarbitone, which is well known to stimulate the metabolism of many structurally diverse drugs and xenobiotics (Conney, 1967).

Accordingly, it is the objective of this report to

show (1) the absolute involvement of the cyto-chrome P-450 mixed-function oxidase system in fatty acid metabolism by solubilizing and purifying the haemoprotein from the membrane of the hepatic endoplasmic reticulum from rats pretreated with clofibrate and subsequently reconstituting the fatty acid 12- and 11-hydroxylase activity and (2) to compare the purified cytochrome P-450 types isolated from both phenobarbitone- and clofibrate-treated rats with respect to their abilities to catalyse the 12- and 11-hydroxylation of lauric acid and the metabolism of the model drug substrate benzphet-amine.

Materials and methods

Materials

DEAE-Sephacel, Sepharose 4B and CM-Sepha-dex were obtained from Pharmacia, Uppsala, Sweden; hydroxyapatite was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A.; Emulgen 911 was obtained from the Kao-Atlas Company, Tokyo, Japan, and sodium cholate, NADPH, lauric acid (unlabelled) and dilauroylglycerophospho-choline were purchased from Sigma Chemical Co., Poole, Dorset, U.K.; benzphetamine hydrochloride was a gift from Shell Research, Sittingbourne, Kent, U.K.; [^{14}C]lauric acid was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and clofibric acid (sodium salt) was obtained from ICI Pharmaceuticals, Macclesfield, U.K.; all other chemicals and reagents were purchased as the best grade available.

Animals and their pretreatment

Male 200 g rats (Wistar strain; University of Surrey Breeders; 20 animals) were injected intra-peritoneally for 3 consecutive days with sodium clofibrate (400 mg/kg body wt.) and killed on day 4. Livers were removed and immediately perfused with cold 0.9% (w/v) saline to remove contaminating haemoglobin. Microsomes (endoplasmic-reticulum fragments) were prepared by differential ultracentri-fugation from 0.25 M-sucrose as described pre-viously (Remmer et al., 1966) and stored at −80°C until required.

Solubilization and purification of mixed-function oxidase proteins

NADPH-cytochrome c (P-450) reductase (EC 1.6.2.4) was prepared from either phenobarbitone- or clofibrate-pretreated rat liver microsomes essen-tially by the method of Yasukochi & Masters (1976). The flavoprotein was totally uncontamin-ated by cytochrome P-450 and was judged homo-geneous by the appearance of one major band after analysis by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (specific content 21.3 μmol of cytochrome c reduced/min per mg of protein at 20°C). Cytochrome P-450 from the liver microsomes of phenobarbital-treated rats was purified to electrophoretic homogeneity (specific content 18 nmol of cytochrome P-450/mg of pro-tein), essentially as described by Guengerich (1978).

Microsomal cytochrome P-450 derived from clofibrate-induced rat liver was solubilized from the endoplasmic-reticulum membrane in the presence of sodium cholate and subsequently chromatographed on 8-amino-octyl-Sepharose 4B essentially as des-cribed by Guengerich (1978). The 8-amino-octyl-Sepharose 4B eluate containing the partially puri-fied cytochrome P-450 was dialysed against 2 × 3.5 litres of 20 mM-potassium phosphate buffer (pH 7.25), containing 20% (v/v) glycerol and 0.2% (v/v) Emulgen 911, and loaded on a hydroxyapatite column (2.6 cm × 15 cm), previously equilibrated with the above dialysis buffer, whereupon a dark-red band corresponding to cytochrome P-450 tightly adhered to the top of the column. This column was subsequently eluted by increasing (step-wise) the concentration of potassium phosphate to 50, 90 and 150 mM in the dialysis buffer, whereupon three separate pools of cytochrome P-450 were obtained, which were then dialysed against 2 × 4 litres of 5 mM-potassium phosphate buffer (pH 7.7) contain-ing 20% glycerol and 0.2% Emulgen 911. The three dialysed cytochrome P-450 pools were separately loaded on DEAE-Sephacel columns (2.6 cm × 10 cm) previously equilibrated with the last 5 mM dialysis buffer. Under these conditions, all of the cytochrome P-450 fractions deriving from the hydroxyapatite column were eluted from the DEAE-Sephacel column with 5 mM dialysis buffer as one pool with the exception of the 90 mM-hydroxy-apatite pool, which yielded a 5 mM and a 35 mM pool, all containing cytochrome P-450. All of these four fractions were now further purified in an identical manner by loading on a CM-Sephadex column (2.6 cm × 10 cm) previously equilibrated with 35 mM-potassium phosphate buffer, pH 7.5, con-taining 20% glycerol. All of the cytochrome P-450 fractions adsorbed on the top of the column, which was further washed with the above 35 mM buffer until the A_{280} of the eluate had decreased to less than 0.02, indicating the removal of the non-ionic detergent Emulgen 911. The cytochromes were then eluted from the CM-Sephadex column by increasing the ionic strength of the buffer to 300 mM. The eluted cytochromes were then stored in portions at −80°C and were designated as cytochrome P-450 fractions 1, 2, 3 and 4, as indicated in the purification flow chart (Fig. 1). It should be emphasized at this stage that these cytochrome designations do not signify structural heterogeneity, but rather serve as an aid to describe the different chromatographic profiles of the haemoprotein(s).
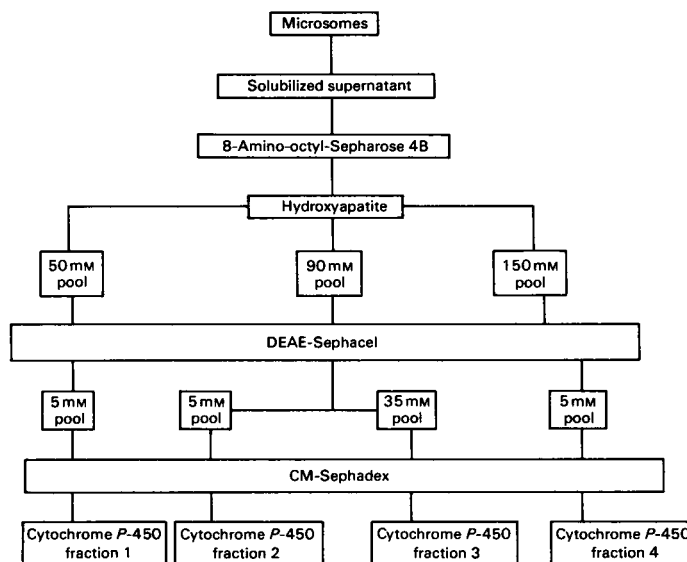
Fig. 1. *Flow-chart for the purification of four fractions of cytochrome P-450 isolated from the endoplasmic reticulum of clofibrate-treated rats*

Spectrophotometry

Quantitative spectrophotometric analysis of cytochrome *P*-450 was carried out on a Cary 219 split-beam recording spectrophotometer (Varian Instruments), using an absorption coefficient of $91\,\mathrm{mM}^{-1}\cdot\mathrm{cm}^{-1}$ for the absorbance increment between 450 and 490 nm of the ferrous–CO versus ferrous complex of the haemoprotein at 20°C (Omura & Sato, 1964). Accurate determination of the absorption maxima of the CO complexes were determined by slowly scanning the peak over an expanded wavelength scale. The absolute spectra of the ferric cytochrome *P*-450 preparations were determined by diluting the haemoprotein(s) in 50 mM-potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol (sample compartment) and recording the absorbance versus buffer only in the reference compartment over the wavelength range 350–700 nm. The Cary 219 spectrophotometer was wavelength-calibrated using a holmium oxide filter.

Spectral interactions of lauric acid with cytochrome *P*-450 were determined by difference spectroscopy as described by Schenkman *et al.* (1967).

Assay procedures

For the reconstitution of cytochrome *P*-450-dependent mixed-function oxidase activities, the general procedure of Haugen *et al.* (1975) was used in the presence of sonicated dilauroylglycerophos-

phocholine and NADPH as a source of reducing equivalents, at a cytochrome *P*-450/reductase ratio of 1:3 (nmol of cytochrome *P*-450/unit of reductase).

The metabolism of [^{14}C]lauric acid to 12- and 11-hydroxylated products was evaluated by both t.l.c. and high-pressure liquid chromatography essentially as described by Parker & Orton (1980). After a 5 min incubation period at 37°C, the incubation mixture was extracted into diethyl ether and the unmetabolized lauric acid and hydroxylated products were separated on silica gel GF $250\,\mu\mathrm{m}$ plates developed in hexane/diethyl ether/acetic acid (60:19:1, by vol.). The radioactive areas, co-chromatographing with authentic 12- and 11-hydroxylauric acid, were located by autoradiography and quantified by scanning of the plate on a Berthold LB 277-22 Scanner II. This t.l.c. method gives the total 12- and 11-hydroxy-fatty acid and these metabolites were further separated and quantified by h.p.l.c. on a Spherisorb ODS (C_{18}) $5\,\mu\mathrm{m}$ column, which was eluted with methanol/water/acetic acid (115:84:1, by vol.) and subsequently with methanol to elute the lauric acid. The lauric acid and its hydroxy-metabolites were detected with an L.C. Refractive Index Detector and quantified with a Berthold LB 503 h.p.l.c. Radioactivity Monitor.

Protein was determined by the method of Lowry *et al.* (1951) and benzphetamine *N*-demethylase activity determined by the colorimetric procedure of Nash (1953).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Electrophoresis of the purified cytochrome P-450 fractions was carried out by the method of Laemmli (1970) on 10% polyacrylamide slab gels and stained for protein with Coomassie Brilliant Blue.

Results

Purification of cytochrome P-450 from clofibrate-treated rats

A typical purification of cytochrome P-450 is shown in Table 1 and it can be seen that the cytochrome P-450 fractions have been purified to various extents using the procedures described in the Materials and methods section. For example, the cytochrome P-450 fraction 1 appeared to be the purest haemoprotein exhibiting a specific content of 17.2 nmol of cytochrome P-450/mg of protein, which is close to the theoretical value of 20 (assuming a monomeric molecular weight of approx. 50000). In contrast the other three cytochrome P-450 fractions exhibited a lower purity as judged by their lower specific contents.

This purity analysis agrees with a sodium dodecyl sulphate/polyacrylamide-gel electrophoretic analysis of the above cytochrome P-450 fractions as shown in Fig. 2.

Of the four cytochrome P-450 fractions isolated from the endoplasmic reticulum of clofibrate-treated

rats (tracks 3–6, Fig. 2), clofibrate cytochrome P-450 fraction 1 appeared to be the purest (track 5, corresponding to a monomeric mol.wt. of 52000) with only minor contamination by other bands, thus agreeing with the above high specific content observed for this protein. Clofibrate cytochrome P-450 fraction 2 was less pure (track 3) and was contaminated by a higher-molecular-weight species corresponding to a mol.wt. of approx. 77000. It should be noted that the major bands in tracks (3) and (5) (corresponding to the major molecular species in the microsomal cytochrome P-450 region) exhibit approximately the same molecular weight. The cytochrome P-450 isolated from phenobarbitone-treated rats is shown in comparison with the highly-purified clofibrate cytochrome P-450 fraction 1 in Fig. 2 (tracks 7 and 8 respectively) and it should be noted that the former protein consistently runs behind the latter, indicating a small, but consistent difference in their respective mol.wts. (53000 versus 52000 respectively).

Spectral properties of cytochrome P-450 fractions

The spectral properties of the various cytochrome P-450 fractions are summarized in Table 2 along with the phenobarbitone-induced form of the haemoprotein for comparison. The cytochrome P-450 fraction 1 appears to be distinctly different in spectral properties from the other fractions, including the phenobarbitone form. This is seen more

Table 1. *Purification of cytochrome P-450 from the hepatic endoplasmic reticulum of rats pretreated with clofibrate*
Abbreviation: N.D., not determined.

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Cytochrome P-450 (nmol/ml)	Total cytochrome P-450 (nmol)	Cytochrome P-450 specific content (nmol/mg)	Yield (%)
Microsomes	170	26.8	4556	40.7	6919	1.52	100
Solubilized supernatant	1650	2.11	3490	3.63	5990	1.72	87
8-Amino-octyl-Sepharose 4B	500	1.21	605	4.4	2200	3.6	32
Hydroxyapatite (50 mM pool)	63	1.61	101.4	4.4	277	2.7	4
DEAE-Sephacel (5 mM pool) and CM-Sephadex (300 mM pool) (fraction 1)	7	0.62	4.3	10.6	74	17.2	1.1
Hydroxyapatite (90 mM pool)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
DEAE-Sephacel (5 mM pool) and CM-Sephadex (300 mM pool) (fraction 2)	8.2	1.39	11.4	10.8	88.6	7.8	1.3
Hydroxyapatite (90 mM pool)	8.2	1.39	11.4	10.8	88.6	7.8	1.3
DEAE-Sephacel (35 mM pool) and CM-Sephadex (300 mM pool) (fraction 3)	5	0.52	2.6	4.13	20.7	8.0	0.3
Hydroxyapatite (150 mM pool)	45	1.91	86	4.34	195	2.3	2.8
DEAE-Sephacel (5 mM pool) and CM-Sephadex (300 mM pool) (fraction 4)	6.5	1.34	8.7	4.1	20	3.1	0.3

clearly when the absolute ferric absorption spectra of cytochrome *P*-450 fractions 1 and 2 are compared as shown in Fig. 3. Clearly, the cytochrome *P*-450 fraction 2 is primarily a low-spin haemoprotein as

characterized by the absorbance maximum at 416 nm (Kumaki *et al.*, 1978) and lack of a charge transfer band (Brill & Williams, 1961) at 650 nm (Fig. 3*b*). In contrast, cytochrome *P*-450 fraction 1 is a mixture of both high-spin and low-spin haemoproteins characterized by the shoulder at 396 nm (corresponding to the high-spin form) and
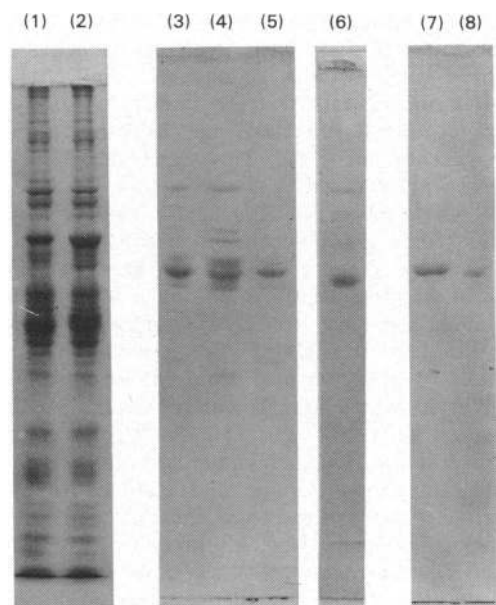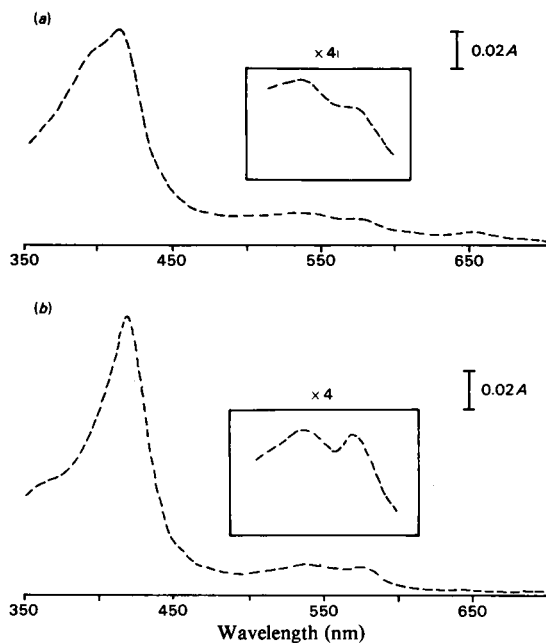

Fig. 2. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of cytochrome P-450 fractions isolated from the endoplasmic reticulum of rats pretreated with clofibrate*

Electrophoresis was from top to bottom of the gel, with the anode at the bottom. (1) Control rat liver microsomes (15 μg); (2) clofibrate-induced rat liver microsomes (15 μg); (3) clofibrate cytochrome *P*-450 fraction 2 (1.4 μg); (4) clofibrate cytochrome *P*-450 fraction 4 (4 μg); (5) clofibrate cytochrome *P*-450 fraction 1 (0.6 μg); (6) clofibrate cytochrome *P*-450 fraction 3 (0.5 μg); (7) phenobarbitone-induced cytochrome *P*-450 (0.6 μg); (8) clofibrate cytochrome *P*-450 fraction 1 (0.31 μg).

Fig. 3. *Absorption spectra of two fractions of cytochrome P-450 (ferric forms) isolated from the endoplasmic reticulum of clofibrate-treated rats*

The haemoproteins were diluted to a concentration of 1 μM with 50 mM-potassium phosphate buffer, pH 7.25, containing 20% glycerol; absorbance maxima were determined as described in the text. (*a*) Cytochrome *P*-450 fraction 1; (*b*) cytochrome *P*-450 fraction 2.

Table 2. *Spectral properties of cytochrome P-450 isolated from the hepatic endoplasmic reticulum of both clofibrate- and phenobarbitone-treated rats*

All cytochromes were assayed at 20°C at a concentration of 1 μM in 50 mM-potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol; absorbance maxima were determined as described in the text.

		Absorbance maxima of ferric form (nm)		
Cytochrome *P*-450	Absorbance maxima of ferrous–CO complex (nm)	α	β	Soret
Clofibrate fraction 1	451.8	570	540	415*
Clofibrate fraction 2	450.8	570	537	416
Clofibrate fraction 3	450.5	570	536	418
Clofibrate fraction 4	450.5	570	540	418
Phenobarbitone-induced form	450.3	570	535	417

* Shoulder at 396 nm.

the presence of the charge-transfer band at 650nm (Fig. 3a). In addition, the position of the ferrous–CO complex of cytochrome P-450 fraction 1 is shifted to a longer wavelength by approx. 1 nm compared with the other cytochrome P-450 fractions, and in this respect is similar to the absorbance wavelength maximum of the cytochrome P-450 in the native environment of the microsomal membrane of clofibrate-treated rats (results not shown).

Because of the substantial differences in the spin states of cytochrome P-450 fractions 1 and 2 (Fig. 3) we determined the spectral interaction of lauric acid with these two cytochrome P-450 pools and these data are shown in Fig. 4. Lauric acid gave a classical type I spectral change (Schenkman et al., 1967) in difference spectrum with both cytochrome P-450 pools, reflecting a change in the spin state of the haemoproteins from the low- to the high-spin configuration. Differences in the kinetic binding constants were noted between these cytochrome P-450 pools and are shown in the inset to Fig. 4.

Catalytic activity of the purified cytochrome P-450 fractions

As indicated in the introduction, we were anxious to determine if the 12- and 11-hydroxylation of lauric acid was mediated by the cytochrome P-450-dependent mixed-function oxidase system. Although the induction of both these activities and cytochrome P-450 formation by clofibrate pre-

treatment and the inhibition of fatty acid oxidation by an atmosphere of CO (O_2/CO, 1:4) were indicative of haemoprotein involvement (Parker & Orton, 1980), we decided to clearly delineate the role of cytochrome P-450 in fatty acid oxidation by reconstituting the enzymic activity from the soluble purified components described above. As shown in Table 3, the fatty acid hydroxylase activity exhibited an absolute requirement for NADPH, cytochrome P-450 (fraction 1) and NADPH-cytochrome P-450 reductase and exhibited a partial dependence on lipid. This behaviour is clearly consistent with the hypothesis that the 12- and 11-hydroxylation of lauric acid is mediated by the cytochrome P-450-dependent mixed-function oxidase system. When a similar experiment was carried out in the presence of cytochrome P-450 fraction 2, a similar pattern was observed (results now shown).

We then investigated the purified cytochrome P-450 fractions isolated from the microsomes of clofibrate-treated animals with respect to their ability to catalyse the 12- and 11-hydroxylation of lauric acid and the N-demethylation of the model drug substrate benzphetamine and included the cytochrome P-450 isolated from phenobarbitone-treated animals for comparison. As shown in Table 4, there was a wide range of catalytic activity in a reconstituted system, dependent entirely on the source of cytochrome P-450. For example, cytochrome P-450 fraction 1 preferentially hydroxylated lauric acid at the 12-position, whereas cytochrome P-450 fraction 2 catalysed the formation of more of the 11-hydroxylated product, as is clearly seen in the product ratios. Both cytochrome P-450 fractions 3 and 4 exhibited a low turnover with lauric acid as substrate and, interestingly, fraction 3 was active in the N-demethylation of benzphetamine. This highest
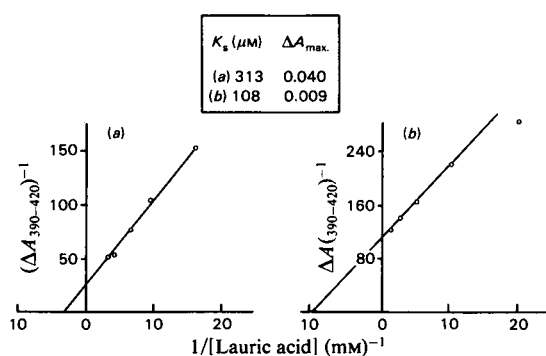

K_s (μM)	$\Delta A_{max.}$
(a) 313	0.040
(b) 108	0.009

Fig. 4. *Double-reciprocal plots for the spectral interaction of lauric acid with cytochrome P-450 isolated from the endoplasmic reticulum of clofibrate-pretreated rats*
Cytochrome P-450 was diluted to a concentration of 1 μM with 50 mM-potassium phosphate buffer, pH 7.25, containing 20% glycerol and titrated with lauric acid as described by Schenkman et al. (1967). The inset shows the apparent spectral dissociation constants (K_s) and maximum spectral interactions ($\Delta A_{max.(390-420)}$) in the presence of cytochrome P-450 fraction 1 (a) and cytochrome P-450 fraction 2 (b).

Table 3. *Requirement for the components of the cytochrome P-450-dependent mixed-function oxidase activity in the metabolism of lauric acid in a reconstituted enzyme system*

Component omitted	Activity (%)*
Complete system†	100
Minus cytochrome P-450	0
Minus NADPH-cytochrome P-450 reductase	0
Minus dilauroylglycerophosphocholine	34
Minus NADPH	0

* 100% activity is equivalent to 21 nmol of total 12- and 11-hydroxylauric acid formed/nmol of cytochrome P-450 per min.

† The complete reconstituted system was as described in the Materials and methods section in the presence of cytochrome P-450 fraction 1 isolated from clofibrate-treated rats.

Table 4. *The reconstitution of drug and fatty acid oxidative activities in the presence of purified cytochrome P-450 derived from either clofibrate- or phenobarbitone-treated rats*

The conditions for reconstitution of cytochrome *P*-450-dependent mixed-function oxidase activity were as described in the Materials and methods section.

Source of cytochrome P-450	Lauric acid hydroxylase (nmol of metabolite formed/ min per nmol of cytochrome P-450)		Ratio 12-/11- hydroxylated form	Benzphetamine N-demethylase (nmol of formaldehyde formed/ min per nmol of cytochrome P-450)
	12-Hydroxy	11-Hydroxy		
Clofibrate cytochrome P-450 fraction 1	20.0	4.9	4.1	27
Clofibrate cytochrome P-450 fraction 2	4.4	7.6	0.6	114
Clofibrate cytochrome P-450 fraction 3	1.3	1.8	0.7	245
Clofibrate cytochrome P-450 fraction 4	2.2	0.9	2.4	121
Phenobarbitone-induced cytochrome P-450	Trace	Trace	—	246

activity of fraction 3 in supporting drug metabolism was comparable with the activity observed in the presence of the phenobarbitone form of the haemo-protein (Table 4).

Discussion

We have successfully solubilized and purified cytochrome *P*-450 in active form from the hepatic endoplasmic reticulum of rats that had been pre-treated with the hypolipidaemic drug clofibrate. The cytochrome *P*-450 was fractionated into four pools and the resultant haemoproteins were purified to various extents. Cytochrome *P*-450 fraction 1 was judged to be highly purified on the basis of haemoprotein specific content and by analysis on sodium dodecyl sulphate/polyacrylamide-gel electro-phoresis, whereas the other three haemoprotein fractions were of lesser purity. In addition, these cytochrome *P*-450 fractions exhibited various ex-tents of catalytic activity towards both the 12- and 11-hydroxylation of lauric acid and the *N*-de-methylation of benzphetamine. Furthermore, we have substantiated the absolute requirement for the cytochrome *P*-450 mixed-function oxidase system in the hydroxylation of lauric acid.

In the light of the existence of multiple forms of cytochrome *P*-450 (Omura, 1978; Yasunobu *et al.*, 1980), we compared the four cytochrome *P*-450 fractions (particularly the highly purified cyto-chrome *P*-450 fraction 1) described in the present paper with that form of the haemoprotein induced by phenobarbitone. Cytochrome *P*-450 fraction 1 ex-hibited unique spectral properties that were clearly distinct from the phenobarbitone-induced form. The former haemoprotein exists as a mixture of both low- and high-spin states and metabolically supported substantial laurate oxidation (preferentially the 12-hydroxy metabolite) and was relatively inactive with respect to benzphetamine metabolism (com-pared with the other cytochromes), in contrast with

the phenobarbitone-induced form of the haemo-protein, which exhibited the opposite relative rates of metabolism. In addition, differences in the mono-meric molecular weights of the cytochrome *P*-450 fraction 1 and the phenobarbitone-induced enzyme make it tempting to speculate that these two haemoproteins are distinct forms. However, it must be emphasized that the accepted criteria for protein heterogeneity between cytochrome *P*-450 types are not fully met by the experiments reported in the present paper and these proteins clearly need additional biochemical characterization. For ex-ample, most laboratories delineate cytochrome *P*-450 multiplicity on the basis of spectral properties, substrate specificity, monomeric molecular weight, limited proteolysis, immunological properties, amino acid composition and *N*-terminal analysis in the absence of the more definitive amino acid sequence data, which has not been reported for any mam-malian cytochrome *P*-450 to date.

Notwithstanding the fact that cytochrome *P*-450 fraction 2 is not homogeneous and only half as pure as cytochrome *P*-450 fraction 1, it is nevertheless of interest to compare these two haemoprotein frac-tions. These cytochromes have apparently the same molecular weight, yet their spectral properties and metabolic profiles are certainly different from each other. This suggests to us one of two possibilities. First, that they are distinct gene products with similar molecular weights not readily discernible by sodium dodecyl sulphate/polyacrylamide-gel electro-phoresis or secondly that they are the same enzyme but cytochrome *P*-450 fraction 1 has residual inducer (clofibrate) or one of its metabolites tightly or covalently bound to the enzyme, thus producing the observed high-spin state. It should be emphasized that subsequent binding of lauric acid to cyto-chrome *P*-450 fraction 1 producing more high-spin haemoprotein (Fig. 4) is readily explained by the fact that there is no known substrate of mammalian cytochrome *P*-450(s) that has the ability to convert

the haemoprotein exclusively (100%) into the high-spin form. Therefore the cytochrome *P*-450 fraction 1 described above exists as a mixture of both low- and high-spin forms, as evidenced by the presence of only a shoulder at 396 nm in the absolute spectrum (Fig. 3).

The high metabolic activity of cytochrome *P*-450 fraction 3 towards the *N*-demethylation of benzphetamine and the equally low activity towards the 12- and 11-hydroxylation of lauric acid is reminiscent of the phenobarbitone-induced form of the haemoprotein (Table 4). Again it would be hasty to conclude an identity between these two cytochromes, but it is nevertheless noteworthy that the chromatographic procedures used to purify the phenobarbitone-induced form of the enzyme are identical with those used to purify cytochrome *P*-450 fraction 3 obtained in our experiments.

In conclusion, the total hepatic microsomal cytochrome *P*-450 induced by clofibrate pretreatment in the rat has been purified and isolated into four separate fractions, one of which (fraction 1, described herein) has been shown to exhibit a relatively high specificity for the 12-hydroxylation of lauric acid in a reconstituted enzyme system. The significance of the induction of this unique form of cytochrome *P*-450 with respect to the biological properties of clofibrate remains to be established.

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