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PURIFICATION AND PROPERTIES OF CYTOCHROME P-450 AND NADPH–CYTOCHROME *c* (P-450) REDUCTASE FROM HUMAN LIVER MICROSOMES

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Abstract—Cytochrome P-450 and NADPH–cytochrome *c* (P-450) reductase were purified to 10.6 nmoles per mg of protein and 19.9 units per mg of protein, respectively, from human liver microsomes. The purified cytochrome was assumed to be in a low spin state as judged by the absolute spectrum. *n*-Octylamine and aniline produced type II difference spectra and SKF 525-A and benzphetamine type I spectra when bound to the purified cytochrome P-450. The purified human cytochrome P-450 catalyzed laurate oxidation as determined by NADPH oxidation but not aniline hydroxylation, benzphetamine *N*-demethylation and 7-ethoxycoumarin *O*-deethylation when reconstituted with the reductases purified from human and rat liver microsomes. The human cytochrome P-450, however, catalyzed drug oxidations when cumene hydroperoxide was used as the oxygen source. The purified human NADPH–cytochrome *c* (P-450) reductase contained FAD and FMN at a ratio of 1:0.76. The reductase was capable of supporting 7-ethoxycoumarin *O*-deethylation activity of cytochrome P-448 purified from 3-methylcholanthrene-treated rat liver microsomes.

The liver microsomes have generally been known to contain cytochrome P-450 which catalyzes oxidative drug metabolism utilizing molecular oxygen and electrons derived from either or both NADPH and NADH. Current studies on the properties of cytochrome P-450 have shown that more than seven species of cytochrome P-450 exist in microsomes of rats and rabbits [1, 2]. Recent efforts made by several laboratories have realized the almost complete purification of more than two species of cytochrome P-450 from microsomes of the experimental animals [1–9], which were distinguishable in their catalytic properties, in molecular weight, in physical properties during purification and in immunological cross reactivity between each species of purified cytochrome P-450 and the antibody. In addition, NADPH–cytochrome *c* (P-450) reductase which transfers reducing equivalents from NADPH to cytochrome P-450 has also been purified [10–12].

Despite these extensive studies on cytochrome P-450 and NADPH–cytochrome *c* (P-450) reductase in experimental animals, little is known about these enzymes in human liver microsomes. The presence of cytochrome P-450 and NADPH–cytochrome *c* (P-450) reductase in human liver microsomes has been reported by several laboratories as reviewed by Pelkonen *et al.* [13]; however, only one paper concerning the partial purification of cytochrome P-450 from human liver microsomes was reported. To examine whether or not phospholipids are required for the drug oxidation activity, Kaschnitz and Coon [14] partially purified cytochrome P-450 of autopsy human liver microsomes from 0.23 nmole per mg of protein to 0.48 nmole per mg of protein by means of ammonium sulfate fractionation. To our knowledge, no reports have appeared on the purification of NADPH–cytochrome *c* (P-450) reductase of human liver microsomes.

Thus, this paper will describe some properties of cytochrome P-450 and NADPH–cytochrome *c* (P-450) reductase partially purified from human liver microsomes.

MATERIALS AND METHODS

Purification of cytochrome P-450 from human liver microsomes. Three of twenty-five human livers isolated from the patients by pathological examinations within approximately 12 hr after death were employed for the purification of cytochrome P-450. Other livers could not be utilized since in some cases, probably due to the autolysis after death, only very small amounts of microsomes were recovered and in the other cases only a trace or no detectable cytochrome P-450 remained in microsomes when handed over to us. The liver microsomes from two of three livers were divided and used for preliminary experiments and those from the third for the final purification. The patient (84 yr-old male) whose liver was subjected to the final purification died by apoplexy about five hr after being admitted to hospital. The liver was homogenized 10 hr after death with 1.15% potassium chloride and the washed microsomes were prepared by a method ordinarily employed for the experimental animals [15]. The washed microsomes were solubilized by the method described previously [16], and the solubilized microsomes were applied on a column of ω-amino-*n*-octyl Sepharose 4B (2.5 × 27 cm) which was developed for purification of cytochrome P-450 from phenobarbital- and 3-methylcholanthrene-treated rabbit liver microsomes by Imai

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and co-workers [8, 9, 17]. Cytochrome P-450 was eluted by washing the column with the buffer described by Hashimoto and Imai [9] for elution of cytochrome P-448 species of 3-methylcholanthrene-treated rabbit liver microsomes. The fractions containing cytochrome P-450 were combined and were diluted 3-fold with 20% glycerol. The diluted sample was applied on a column of hydroxylapatite (3.5 × 5 cm). The column was equilibrated and washed with the buffers as described by Imai and Sato [8], followed by successive washing with 100 mM potassium phosphate (pH 7.25) containing 0.2% Emulgen 913 and 20% glycerol and with 150 mM potassium phosphate (pH 7.25) containing 0.2% Emulgen 913, 0.1% deoxycholate and 20% glycerol. The cytochrome P-450 fractions eluted with the latter buffer were combined and concentrated to about 4 ml using a Diaflo apparatus equipped with a Toyo UP-20 membrane. The concentrated sample was applied on a column of Sephadex LH-20 (2.5 × 40 cm) which had been equilibrated with 50 mM potassium phosphate (pH 7.25) and 50% glycerol [18]. The eluate of Sephadex LH-20 column was used as a final preparation.

Purification of cytochrome P-450 from phenobarbital or 3-methylcholanthrene-treated rat liver microsomes. Cytochrome P-450 and cytochrome P-448 were purified from phenobarbital and 3-methylcholanthrene-treated rat liver microsomes, respectively, by the methods previously described [16, 19]. The specific content of cytochrome P-450 was 10.5 nmoles per mg of protein and that of cytochrome P-448 17.6 nmoles per mg of protein.

Purification of NADPH–cytochrome c *(P-450) reductase from human liver microsomes.* NADPH–cytochrome *c* (P-450) reductase was purified by a modification of the method described for purification from pig liver microsomes by Yasukochi and Masters [12]. Human liver microsomes which had a considerable activity of NADPH–cytochrome *c* (P-450) reductase, but had only a trace amount of cytochrome P-450, were used for this experiment. The liver from a 48-yr-old male patient who died from brain cancer was obtained 5 hr after death and the washed microsomes (2 mg/ml) were treated with 1% Emulgen 913 containing 50 mM Tris–HCl (pH 7.7), 0.1 mM dithiothreitol and 20% glycerol. The treated microsomes were centrifuged at 105,000 *g* for 1 hr. Solid potassium chloride was added to the soluble microsomes to give 75 mM. The sample was subsequently applied to a column of DEAE–cellulose (DE-23) (2.5 × 32 cm) which had been equilibrated with 50 mM Tris–HCl (pH 7.7) containing 1% Emulgen 913, 0.1 mM dithiothreitol, 75 mM potassium chloride and 20% glycerol. After washing the column with a column volume of the same buffer, NADPH–cytochrome *c* (P-450) reductase was eluted by the linear gradient from 75 mM to 1 M in terms of the potassium chloride concentration. The reductase activity was eluted at about 300 to 400 mM of the potassium chloride concentration. The fractions having activity greater than 1.0 unit per ml were combined and applied on a column of 2′,5′-ADP–Sepharose 4B (0.9 × 11.0 cm) which had been equilibrated with 10 mM potassium phosphate (pH 7.7) containing 0.2% Emulgen 913 and 20% glycerol. The column was washed with a column volume of 200 mM potassium phosphate (pH 7.7) containing 0.2% Emulgen 913 and 20% glycerol, then with 10 mM potassium phosphate (pH 7.7) containing 20% glycerol. NADPH–cytochrome *c* (P-450) reductase was eluted with 10 mM potassium phosphate (pH 7.7) containing 0.7 mM NADP and 20% glycerol.

Purification of NADPH–cytochrome c *(P-450) reductase from phenobarbital-treated rat liver microsomes.* NADPH–cytochrome *c* (P-450) reductase of phenobarbital treated rat liver microsomes was purified by a minor modification of the method of Yasukochi and Masters [12]. The specific activity of the purified reductase was greater than 42 units per mg of protein.

Analytical procedure. Cytochrome P-450 was determined by the method of Omura and Sato [20], and the cytochrome content was calculated using a molar extinction coefficient of 91 $mM^{-1}cm^{-1}$. To stabilize the cytochrome, the determinations were carried out in the presence of 0.2% Emulgen 913 and 20% glycerol as reported previously [21]. NADPH–cytochrome *c* (P-450) reductase activity was measured at room temperature (25°) using cytochrome *c* as an electron acceptor by the method of Phillips and Langdon [22]. The activity was defined as a unit which reduces one μmole of cytochrome *c* per min. FAD and FMN were determined using snake venom phosphodiesterase as described by Iyanagi and Mason [23]. The contents of the flavins were calculated by the equation reported by Bessey *et al.* [24]. The incubation mixture for 7-ethoxycoumarin *O*-deethylation and laurate oxidation activities consisted of 0.2 nmole per ml of human cytochrome P-450 or 0.02 nmole per ml of rat cytochrome P-448, 0.5 unit per ml of NADPH–cytochrome *c* (P-450) reductase, 4μg per ml of dilauroyl L-3-phosphatidylcholine, 50 mM HEPES (pH 7.4), 15 mM $MgCl_2$ and 0.2 mM lauric acid or 0.5 mM 7-ethoxycoumarin. The final volumes of the assay mixture were 3.0 ml for 7-ethoxycoumarin *O*-deethylation and 1.0 ml for laurate oxidation assay, respectively. The *O*-deethylation activity for 7-ethoxycoumarin was measured by fluorescence increase as described by Ullrich and Weber [25]. Laurate oxidation was determined by measuring NADPH-oxidation [26]. The reaction was started by addition of NADPH, and was carried out in a cuvette under an atmosphere of air at 25° (room temperature). The incubation mixture for cumene hydroperoxide-dependent drug oxidations consisted of 0.5 nmole of cytochrome P-450 purified from human liver microsomes and phenobarbital-treated rat liver microsomes, 50 μg of dilauroyl L-3-phosphatidylcholine, 0.1 M potassium sodium phosphate (pH 7.4), 3.3 mM cumene hydroperoxide and a substrate (benzphetamine, 1.0 mM; *N*-monomethylaniline and *N,N*-dimethylaniline, 3.0 mM; aniline, 10.0 mM) in a final volume of 1.0 ml. The reaction was started by addition of cumene hydroperoxide and the mixture was incubated aerobically at 37° for 15 min. Aniline hydroxylation activity was determined by measuring *p*-aminophenol by the method of Imai *et al.* [27], and the *N*-demethylation activities of the other substrates were estimated by measuring formaldehyde according to the method of Nash [28]. Protein was determined by the method of Lowry *et al.* [29], using bovine serum albumin as the standard.

Materials. ω-Amino-*n*-octyl Sepharose 4B was synthesized from cyanogen bromide-activated Sepharose 4B and 1,8-diaminooctane by the method described by

Cuatrecasas [30]. Sephadex LH-20, Sepharose 4B and 2′,5′-ADP-Sepharose 4B were purchased from Pharmacia Fine Chemicals, Sweden, DEAE–cellulose (DE 23) from Whatman, U.S.A., and hydroxylapatite (Bio Gel HT) from Bio Rad, U.S.A. Horse heart cytochrome *c* was a product of Boehringer Mannheim, and dilauroyl L-3-phosphatidylcholine a product of Serdary Res. Lab., Canada. NADPH was purchased from Oriental Yeast Co., Japan; dithiothreitol, HEPES (*N*-2-hydroxyethylpiperazine N′-2-ethane sulfonic acid) and snake venom phosphodiesterase (type I) from Sigma, U.S.A.; and cumene hydroperoxide from Nakarai Chemicals, Japan. Emulgen 913 was kindly supplied by Kao-Atlas Co., Japan; benzphetamine by Upjohn Co., U.S.A., SKF 525-A by Smith-Klein and French Lab., U.S.A.; and 7-ethoxycoumarin by Dr. Y. Okada, Kyusyu University, Japan. Other chemical reagents are of the highest grade commercially available.

RESULTS

Partial purification and spectral properties of human cytochrome P-450. Human liver microsomes were solubilized with cholic acid. From the solubilized microsomes, cytochrome P-450 was purified using ω-amino-*n*-octyl Sepharose 4B, hydroxylapatite and Sephadex LH-20 columns. The results of the final purification experiment are shown in Table 1. Since considerable differences among human liver materials at the purification steps were observed, the results obtained by preliminary experiments will also be briefly described together with the results of the final purification experiment. Treatment of washed human liver microsomes with 0.8% cholic acid in the presence of 100 mM potassium phosphate (pH 7.25) resulted in higher than 90 per cent solubilization of cytochrome P-450. A considerable amount of dark brown pellet was precipitated after the centrifugation of the treated microsomes. Accordingly, the specific content of cytochrome P-450 increased about 1.5-fold by the solubilization and the centrifugation. A marked difference between liver materials was observed in the elution profile of cytochrome P-450 from ω-amino-*n*-octyl Sepharose 4B column. The major portion of cytochrome P-450 in microsomes from one of the two livers employed for preliminary experiments was eluted from the column when the buffer containing 0.08% Emulgen 913 [8] was used, while in the other one only about 11 per cent of the applied cytochrome was eluted by the buffer but about 52 per cent of the applied cytochrome P-450 was eluted by increasing Emulgen 913 concentration to 0.2 per cent.

Thus, in the final experiment, the column was washed stepwise with these buffers. The majority (78 per cent) was eluted by the buffer containing the higher Emulgen concentration. The eluate was diluted 3-fold with 20% glycerol then applied on a column of hydroxylapatite. After washing the column with 35 mM potassium phosphate (pH 7.25) containing 0.2% Emulgen 913 and 20% glycerol, cytochrome P-450 from phenobarbital-treated rabbit and rat liver microsomes has been eluted by increasing the phosphate concentration to 80 mM and 100 mM, respectively [8, 16]. However, in the preliminary experiments using human liver microsomes, less than 23 per cent of the applied cytochrome P-450 was recovered at 100 mM phosphate concentration. Only a small amount of the cytochrome P-450 was further eluted by increasing the phosphate concentration to 150 mM. Alternatively, the major portion of the cytochrome was eluted by addition of 0.1% cholic acid to 150 mM potassium phosphate (pH 7.25) containing 0.2% Emulgen 913 and 20% glycerol. The specific content of the cytochrome eluted by the latter buffer was 5.18 nmoles per mg of protein and the recovery was 23.6 per cent at this column. Since the total recovery was not high enough for further purification and for examination of the properties of cytochrome P-450, the step of washing with 150 mM potassium phosphate (pH 7.25) containing Emulgen 913 and 20% glycerol was omitted in the final experiment; the recovery at this column step was thereby increased to 48 per cent with an increase in the specific content from 1.4 nmoles per mg of protein to 3.9 nmoles per mg of protein. Cytochrome b_5 could not be separated from cytochrome P-450 fractions at the ω-amino-*n*-octyl Sepharose 4B column step, but was removed by washing the hydroxylapatite column with the 100 mM potassium phosphate buffer. A slightly turbid yellow material was eluted simultaneously with cytochrome b_5 by this washing step. The fractions containing larger amounts of cytochrome P-450 were combined and concentrated. The concentrated sample was then applied to the column of Sephadex LH-20 column to remove excess detergents [18]. The elution profile determined as optical density at 280 nm showed a broader tailing. It was assumed that the tailing was due to the presence of Emulgen 913 after the peak fractions; thus the latter fractions having an optical density at 417 nm lower than 0.064 were discarded. The specific content of cytochrome P-450 in this final preparation was 10.6 nmoles per mg of protein with the recovery of 31.3 per cent. This preparation was free of detectable cytochrome b_5 but contained 0.007 unit of NADPH–cytochrome *c* (P-450) reductase activity per nmole of cyto-

Table 1. Partial purification of cytochrome P-450 from human liver microsomes

	Protein (mg)	Cytochrome P-450 (nmole)	Recovery (%)	Specific content (nmoles/mg protein)
Microsomes	1334	170	100	0.12
Soluble microsomes	884	155	91.2	0.17
ω-Amino-*n*-octyl column eluate	97	133	78.2	1.37
Hydroxylapatite column eluate	16.3	64.2	37.8	3.9
Sephadex LH-20 column eluate	5.0	53.2	31.3	10.6

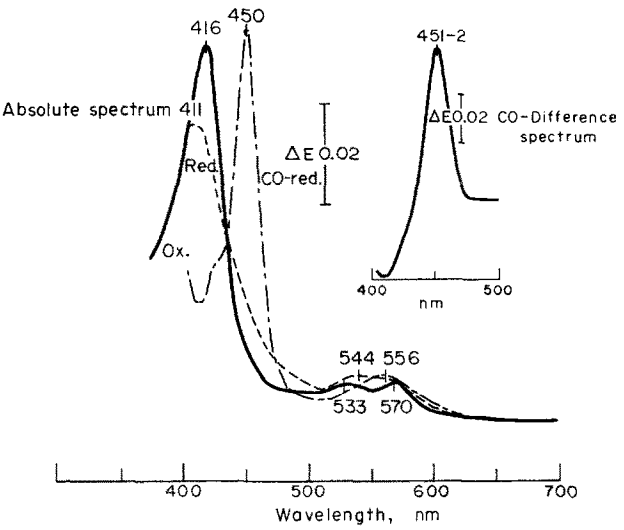

Fig. 1. Absolute spectra (left) and carbon monoxide difference spectrum (right) of cytochrome P-450 purified from human liver microsomes. The spectra were recorded in the presence of 0.66 nmole per ml of the purified cytochrome P-450, 20 mM potassium phosphate (pH 7.25) and 20% glycerol.

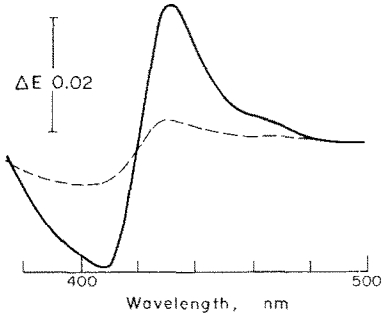

Fig. 2. Aniline and *n*-octylamine-induced difference spectra of cytochrome P-450 purified from human liver microsomes. The concentrations of aniline (– – –) and *n*-octylamine (——) were 5.0 and 0.6 mM, respectively. The spectra were recorded using 1.0 nmole per ml of cytochrome P-450 in potassium phosphate (0.1 M, pH 7.25).

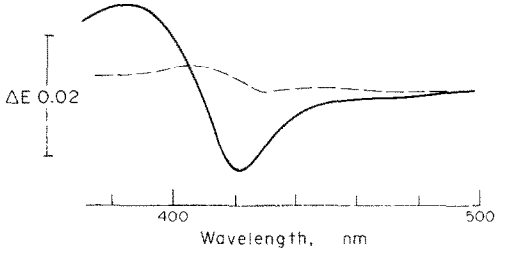

Fig. 3. SKF 525-A and benzphetamine-induced difference spectra of cytochrome P-450 purified from human liver microsomes. The concentrations of SKF 525-A (——) and benzphetamine (– – –) were 0.3 and 1.0 mM, respectively. The other experimental conditions are the same as for Fig. 2.

chrome P-450. A portion of the final sample (1 ml) was dialyzed against 1000 ml of 50 mM potassium phosphate (pH 7.25) containing 20% glycerol. The dialysis for 3 days resulted in the aggregation of the protein to form an insoluble pellet when centrifuged at 3000 rev./min for 10 min. The dialysis and the centrifugation, however, did not alter cytochrome P-450 to cytochrome P-420. Therefore, the combined eluate from the Sephadex LH-20 column was used without further treatment for examination of the properties of this cytochrome preparation.

Spectral properties of the purified cytochrome P-450 were examined. As shown in Fig. 1, the oxidized form showed peaks at 570, 533 and 416–7 nm, the reduced form at 544 and 411 nm and the carbon monoxide-bound reduced form at 556 and 450 nm, respectively. The presence of a peak at 416–7 nm in the oxidized spectrum suggests that the purified cytochrome P-450 is mostly in a low spin state. The carbon monoxide difference spectrum of the reduced cytochrome P-450 had the peak at 451–2 nm. These results indicate that the purified cytochrome P-450 has spectral properties similar to the purified cytochrome P-450 from phenobarbital-treated rabbits and rats. The addition of a substrate has been known to produce spectral changes

Table 2. Partial purification of NADPH–cytochrome *c* (P-450) reductase from human liver microsomes

	Protein (mg)	fp_T* (units)	Recovery (%)	fp_T* (units/mg protein)
Microsomes	1178	166	100	0.14
Soluble microsomes	528	149	89.8	0.28
DEAE–cellulose column eluate	113.4	123	74.1	1.08
ADP–Sepharose 4B column eluate	3.78	75.2	45.3	19.9

* fp_T: NADPH–cytochrome *c* (P-450) reductase.

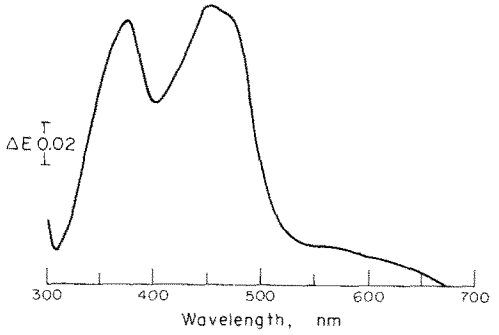

Fig. 4. Absolute spectrum of NADPH–cytochrome *c* (P-450) reductase purified from human liver microsomes. The spectrum of the final preparation of the purified NADPH–cytochrome *c* (P-450) reductase (19.9 units per mg of protein) was recorded using 10 mM potassium phosphate (pH 7.7) containing 0.7 mM NADP and 20% glycerol as a reference which was contained in the final preparation.

of cytochrome P-450 [31–33]. To examine the ability of substrates to produce spectral changes of human cytochrome P-450, *n*-octylamine and aniline were used as typical type II compounds and benzphetamine and SKF 525-A as type I compounds. As can be seen in Fig. 2, *n*-octylamine and aniline exhibited type II difference spectra. The peak in the *n*-octylamine-induced difference spectrum was at 432 nm and the trough at 408 nm. The shoulder near 391 nm was also seen, indicating that this purified cytochrome P-450 contains a small amount of a high spin species. The aniline-induced difference spectrum had a peak at 431 nm and a broad trough at 401 nm. Benzphetamine and SKF 525-A exhibited type I spectra (Fig. 3). However, the magnitude of the benzphetamine-induced type I spectrum was very low and the shape of the spectrum was somewhat different from the typical type I spectrum shown in the literature [33].

Partial purification and properties of human liver NADPH–cytochrome c *(P-450) reductase.* To examine whether human NADPH–cytochrome *c* (P-450) reductase supports cytochrome P-450-mediated drug oxidations, NADPH–cytochrome *c* (P-450) reductase was also partially purified from human liver microsomes (Table 2). Approximately 90 per cent of the activity was solubilized by 1% Emulgen 913 in the presence of 50 mM Tris–HCl (pH 7.7). The elution from DEAE–cellulose column with the linear gradient of potassium chloride resulted in 3.8-fold purification of the reductase activity. The reductase fractions did not contain cytochrome b_5 or cytochrome P-450. The combined sample was applied on a 2′,5′-ADP–Sepharose column which had been equilibrated with 10 mM potassium phosphate (pH 7.7) containing 20% glycerol. The column was washed with 200 mM potassium phosphate (pH 7.7) containing 0.2% Emulgen 913 and 20% glycerol, followed by 10 mM potassium phosphate (pH 7.7) containing 20% glycerol. The reductase was eluted with 0.7 mM NADP in 10 mM potassium phosphate (pH 7.7) and 20% glycerol. The recovery of the reductase activity was 45.3 per cent and the specific activity was 19.9 units per mg of protein, and this preparation was used without further treatments. The absolute spectrum of the final sample is shown in Fig. 4. The absolute spectrum was similar to that seen with the reductase purified from phenobarbital-treated rat liver microsomes by Vermilion and Coon [34], except that no shoulder was observed near 410 nm. This fact suggests that no heme is contained in the present preparation. Iyanagi and Mason [23] were the first to demonstrate that NADPH–cytochrome *c* (P-450) reductase purified from rabbit liver microsomes contained FAD and FMN at a ratio of 1:1. To discover whether the purified human NADPH–cytochrome *c* (P-450) reductase contains both FAD and FMN, the flavins were determined by the method previously described [23] using snake venom phosphodiesterase. The addition of the phosphodiesterase produced about 1.94-fold increase in the fluorescence which is due to the conversion of FAD to FMN. Thus, the purified reductase preparation was calculated to contain both FAD and FMN at a ratio of 1:0.76 [24].

Reconstitution of drug oxidation activities. We have not detected any activities for aniline hydroxylation and benzphetamine *N*-demethylation, using human cytochrome P-450 and both reductases purified from human and phenobarbital-treated rat liver microsomes. The results shown in Table 3 reveal that the purified human cytochrome P-450 did not catalyze 7-ethoxycoumarin *O*-deethylation. To discover the ability of human reductase to support drug oxidations, cytochrome P-448 purified from 3-methylcholanthrene-treated rat liver microsomes was used as a reference.

Table 3. Reconstitution of 7-ethoxycoumarin *O*-deethylation using cytochrome P-450 and NADPH–cytochrome *c* (P-450) reductase partially purified from human and rat liver microsomes

	7-Ethoxycoumarin *O*-deethylation (nmoles 7-hydroxycoumarin formed/nmole P-450/min)	
	Human cytochrome P-450	Rat cytochrome P-448*
NADPH–cyt. *c.* (P450) reductase (fp_T, 1.5 unit)		
Human fp_T	0	13.5† (13.1, 13.8)
Rat fp_T‡	0	12.5 (12.0, 13.0)

* Cytochrome P-448 purified from 3-methylcholanthrene-treated rat liver microsomes.
† Means of duplicate determinations.
‡ NADPH–cytochrome *c* (P-450) reductase purified from phenobarbital-treated rat liver microsomes.

Table 4. Requirements for laurate oxidation as determined by NADPH oxidation

	Laurate oxidation rate (nmoles NADPH oxidized/nmole P-450/min)
Complete system	2.63* (2.78, 2.47)
$-fp_T$ (human) $+fp_T$(rat, 0.5 unit)†	2.13 (2.65, 1.61)
$-fp_T$	0.42 (0.62, 0.21)
− P-450	0.15 (0.31, − 0.01)

The complete system consisted of human cytochrome P-450 (0.2 nmole), human NADPH–cytochrome *c* (P-450) reductase (fp_T, 0.5 unit), dilauroyl L-3-phosphatidylcholine (4 μg) and other components as described in Materials and Methods.

* Means of duplicate determinations. The values are differences between the experimental (in the presence of laurate) and control (in the absence of laurate).

† The human reductase was omitted but 0.5 unit of the reductase purified from phenobarbital-treated rat liver microsomes was added.

The human reductase was able to support the cytochrome P-448-mediated *O*-deethylation activity to an extent similar to the reductase purified from rat liver microsomes, while in the case of human liver cytochrome P-450 no activity was detected. Actually, minus values were obtained probably due to NADPH oxidation. Since laurate is reported to be oxidized by liver microsomal cytochrome P-450 [35, 36], laurate oxidation activity was also measured by recording NADPH oxidation rate (Table 4). In the complete system, the synthetic phospholipids, dilauroyl L-3-phosphatidylcholine, was added in addition to human cytochrome P-450 and NADPH–cytochrome *c* (P-450) reductase, and the activities were corrected by the NADPH-oxidation rate in the absence of laurate. The highest activity for laurate oxidation was obtained in the presence of all components, and it was shown that rat liver NADPH–cytochrome *c* (P-450) reductase almost equally supported the oxidation when added in place of the human reductase. In the absence of either reductase or cytochrome P-450, only low activities were observed.

Using a purified preparation of cytochrome P-450 from phenobarbital-treated rabbit liver microsomes, Nordblom and Coon [37] reported that cytochrome P-450 oxidized various substrates in the presence of cumene hydroperoxide. As shown in Fig. 5, both human cytochrome P-450 and the cytochrome P-450 purified from phenobarbital-treated rat liver microsomes oxidized *N,N*-dimethylaniline, *N*-monomethylaniline, benzphetamine and aniline, in that order. This order of activity indicates that both of these cytochrome P-450 preparations oxidize these substrates in an order similar to phenobarbital-treated rabbit cytochrome P-450 in the presence of cumene hydroperoxide.

DISCUSSION

The existence of multiple species of cytochrome P-450 in microsomes of experimental animals has been confirmed. In these experiments we obtained different cytochrome P-450 fractions which were eluted by different buffers at the same column step. For example, some of the cytochrome P-450 applied on the ω-amino-*n*-octyl Sepharose 4B was eluted by washing the column with the buffer containing 0.08% Emulgen 913 while the remaining portion was eluted by the buffer containing 0.2% Emulgen 913. A similar difference in the elution profile was seen at the hydroxylapatite and CM-Sephadex (A-50) columns. Although not shown, the cytochrome P-450 preparations obtained in the preliminary experiments were applied on the CM-Sephadex column, applying the method of Imai and Sato [8].

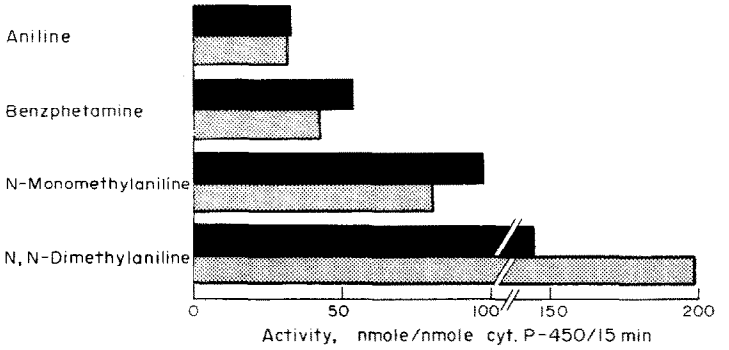

Fig. 5. The cumene hydroperoxide-dependent drug oxidation activities of cytochrome P-450 purified from human and phenobarbital-treated rat liver microsomes. The incubation mixture contained 0.5 nmole of cytochrome P-450 purified from either human (■) or phenobarbital-treated rat liver microsomes (▨) and other components as described in Materials and Methods.

However, the applied cytochrome P-450 was eluted quite broadly when the column was washed with a stepped increase in phosphate concentrations. In addition, marked differences in the elution profiles of cytochrome P-450 between human liver materials were also observed. These results probably suggest that there is more than one species of cytochrome P-450 in human liver microsomes.

In accordance with the finding by Iyanagi and Mason [23] using NADPH–cytochrome *c* (P-450) reductase purified from rabbit liver microsomes, we obtained evidence that human NADPH–cytochrome *c* (P-450) reductase also contains FAD and FMN. Our purified preparation of the reductase is assumed to be about 33 per cent as roughly calculated in comparison with the activity of the electrophoretically homogeneous reductase preparations (approximately 60 units per mg of protein) [12, 38]; thus it seems reasonable to assume that the pure human reductase also contains FAD and FMN in a ratio of 1:1.

Since we have observed considerable activity for 7-ethoxycoumarin *O*-deethylation using human liver microsomes (not shown), it is probable that we have purified a particular species of cytochrome P-450 which does not catalyze drug oxidations as far as the substrates employed in these experiments. If this cytochrome P-450 species is a particular one, then it may exist in larger amount than the other species since we purified the cytochrome with the recovery of about 31 per cent from microsomes in the final experiment. As regards the electron flow from NADPH to cytochrome P-450 in the reconstituted system, electrons are assumed to be transferred to cytochrome P-450 via NADPH–cytochrome *c* (P-450) reductase, since NADPH was oxidized at faster rate when cytochrome P-450 was present in addition to the reductase, and since carbon monoxide bound to cytochrome P-450 upon the addition of NADPH. Although the concentration of the detergent has not yet been determined, a small amount of the detergent may remain in the final preparation. Therefore, it is possible that the remaining detergent inhibits the drug oxidations. It is also possible to assume that the purified cytochrome P-450 and NADPH–cytochrome *c* (P-450) reductase was proteolytically modified during the period after death. However, the proteolytic digestions of liver microsomes have been known to result in alterations in the properties of cytochrome P-450 and NADPH–cytochrome *c* (P-450) reductase. Cytochrome P-450 is quite readily converted to the inactive form, cytochrome P-420 [39], and NADPH–cytochrome *c* (P-450) reductase to another form which is incapable of transferring electrons to cytochrome P-450, probably due to the lack of the hydrophobic peptides cleaved by the proteolytic digestions [40]. Therefore, the proteolytic modification of these enzymes during the period after death appears to be minimal.

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