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Separation and Purification of Two Forms of Hepatic Cytochrome P-450 from Untreated Rabbits

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SUMMARY

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Two forms of hepatic cytochrome P-450 have been separated and partially purified from untreated rabbits. The carbon monoxide-reduced minus reduced difference spectrum of one form (A) had a peak of absorption at 450.2 nm while the same spectrum of the other form (B) gave a peak at 448 nm. Differences in the spectral properties of the two forms of the cytochrome were also seen in the Soret region of the spectra of oxidized preparations, in the Soret and visible regions of the spectra of reduced preparations complexed with carbon monoxide, and in the difference spectra formed by the addition of type I and type II compounds. The cytochromes were purified to 6.5 and 14.3 nmoles/mg of protein for forms A and B, respectively. The partially purified preparations were free of cytochrome b_5 and had barely detectable concentrations of NADPH-cytochrome *c* reductase, epoxide hydrase, and cytochrome P-420. The cytochrome in fraction A was about twice as active as the cytochrome in fraction B in support of benzpyrene hydroxylase, benzphetamine *N*-demethylase, and 7-ethoxycoumarin *O*-deethylase activities when combined with NADPH-cytochrome *c* reductase and lipid fractions. Monomeric molecular weights for both forms of the cytochrome were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be near 51,500.

INTRODUCTION

The hepatic microsomal mixed-function oxidase system is capable of metabolizing various endogenous compounds (i.e., steroids and fatty acids) and a multitude of exogenous chemicals, including a variety of drugs, pesticides, solvents, carcinogens, and mutagens. The versatility of the system appears to be associated with cytochrome P-450, the terminal oxidase. A number of studies have demonstrated that multiple forms of cytochrome P-450 exist in the livers of several species. Three forms of rat hepatic cytochrome P-450 have been described by Comai and Gaylor (1), using spectral techniques, and by Welton and Aust (2) and Alvares and Siekevitz (3), using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Mailman *et al.* (4) have isolated spectrally different forms of cytochrome P-450 in subfractions of mouse hepatic microsomes. Recently Ryan *et al.* (5) and Haugen *et al.* (6) have separated and partially purified multiple forms of hepatic cytochrome P-450 from phenobarbital- and 3-methylcholanthrene-treated rats and rabbits, respectively. The results of many other studies of spectral interactions and selective inhibition in hepatic microsomes are best explained by the presence of multiple forms of cytochrome

P-450 (e.g., refs. 7–11). The existence of multiple forms of P-450 and the concentration of each form may be responsible for both the versatility and specificity of mixed-function oxidase systems from different sources. Lu and co-workers (12) have shown that changes in the substrate specificity of the hepatic mixed-function oxidase system following treatment of rats with 3-methylcholanthrene are due to the induction of a form of cytochrome P-450 called P_1-450 (13) or P-448 (14).

Whether or not different forms of cytochrome P-450 represent different proteins or modifications of the same protein remains to be determined. Separation and purification of each form, followed by structural analysis, may be required to answer this question. Several forms of hepatic cytochrome P-450 have recently been purified. Imai and Sato (15) and van der Hoeven *et al.* (16) have purified P-450 from the livers of phenobarbital-treated rabbits, and Ryan *et al.* (17) have purified P-450 and P-448 from the livers of phenobarbital- and 3-methylcholanthrene-treated rats, respectively. As yet, however, little success has been reported in the purification of different forms of cytochrome P-450 from untreated animals. We have recently reported on the separation of two forms of hepatic cytochrome P-450 from untreated rabbits (18). The present paper details the methods for purification and compares the properties of these cytochromes.

METHODS AND MATERIALS

Preparation of microsomes. Adult male Dutch Belt rabbits (1200–1500 g) were used throughout this study. The rabbits were

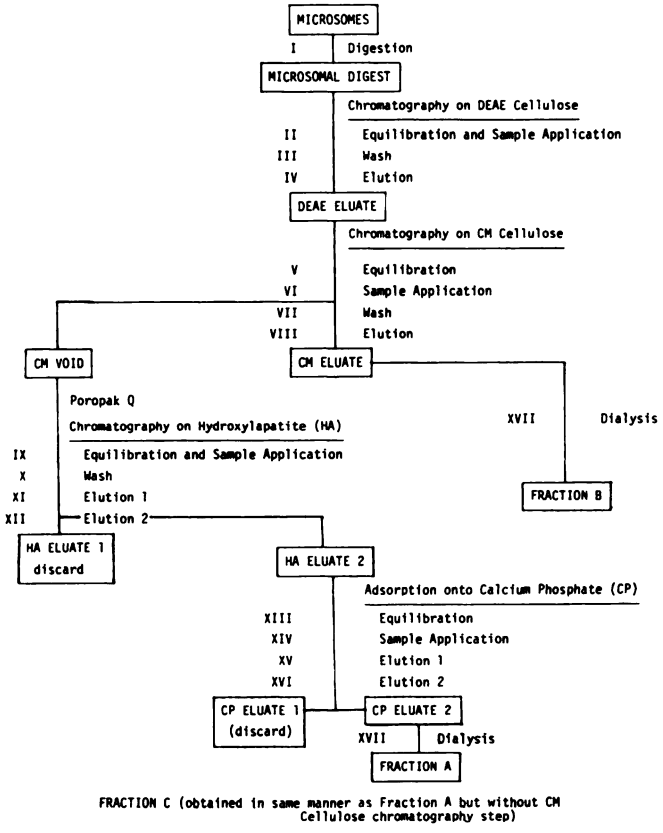

FIG. 1. *Procedure for separation and purification of two forms of hepatic cytochrome P-450 from untreated rabbits*

Roman numerals (I-XVII) refer to buffer solutions used at various steps of the procedure. The contents of the buffer solutions are shown in Table 1.

housed individually and allowed free access to food (NIH feed A) and water. Microsomes were prepared from rabbit livers and stored according to Lu *et al.* (12).

Preparation of cytochrome P-450 fractions. The procedures used to separate and partially purify cytochrome P-450 fractions from rabbit liver are outlined in Fig. 1. Buffer solutions used for each step are listed in Table 1. Phosphate buffer, pH 7.7, containing 20% glycerol, was used unless otherwise noted. Microsomes (1000–3000 mg of protein) were suspended in 0.1 M buffer containing 1 mM dithiothreitol and 1 mM EDTA to yield a final protein concentration of 10 mg/ml. Sodium cholate was then added to a final concentration of 1% (w/v), and the suspension was stirred for 20 min at 4°. The digested suspension was diluted with buffer I without cholate to lower the cholate concentration to 0.4% and applied to a column of DEAE-cellulose (2.5 × 50 cm) equilibrated with buffer II which contained 0.4% cholate. This amount of DEAE-cellulose was sufficient for preparations containing 1000 mg of protein; for larger preparations additional columns were run simultaneously. After application of the sample, the DEAE-cellulose was washed with 1 liter of buffer III which contained 0.25% cholate, and the fractions containing cytochrome P-450 were then eluted with buffer IV which contained 0.25% cholate and 0.15% Emulgen 913. These fractions were combined, diluted 5-fold with water containing 20% glycerol and 0.2% Emulgen 913, and applied to a column of CM-cellulose (1.5 × 30 cm) equilibrated with 0.02 M buffer–0.2% Emulgen 913. The column was washed with 100 ml of 0.1 M buffer–0.2% Emulgen 913. Some cytochrome P-450 was collected with the sample material which did not bind to the CM-cellulose (CM void fraction). A second cytochrome fraction (B) was eluted with the 0.5 M buffer. Fraction B was collected in small volumes (5–10 ml) and was dialyzed against 0.02 M buffer for 3 days with two or three changes of the dialysis solution. After dialysis, fraction B

TABLE 1

Buffer solutions for procedure used to separate and purify two forms of hepatic cytochrome P-450 from untreated rabbits

Roman numerals refer to steps detailed in Fig. 1.

Buffer	Phosphate buffer	Glycerol	Cholate	Emulgen 913	Dithiothreitol	EDTA
	M	%	%	%	*mM*	*mM*
I	0.100	20.0	1.00		1.00	1.00
II	0.100	20.0	0.40		1.00	1.00
III	0.100	20.0	0.25		1.00	1.00
IV	0.100	20.0	0.25	0.15	1.00	1.00
V	0.020	20.0		0.20		
VI	0.020	20.0	0.05	0.20	0.02	0.02
VII	0.100	20.0		0.20		
VIII	0.500	20.0		0.20		
IX	0.033	20.0				
X	0.033	20.0		0.05		
XI	0.080	20.0		0.20		
XII	0.150	20.0		0.20		
XIII	0.020					
XIV	0.020	2.7		0.25		
XV	0.300					
XVI	1.000					
XVII	0.020	20.0				

was stored in 0.25-ml aliquots under nitrogen at −30°. The CM void fraction was passed through a column of Porapak Q (2.5 × 10 cm) to remove free Emulgen and then applied to a column of hydroxylapatite (2.5 × 20 cm) equilibrated with 0.02 M buffer. The hydroxylapatite was washed with 200 ml of 0.033 M buffer–0.05% Emulgen 913, and fractions containing cytochrome P-450 were eluted with 80 mM buffer–0.2% Emulgen 913 and 150 mM buffer–0.2% Emulgen 913. The fraction eluted with 80 mM buffer was discarded because of its low specific cytochrome content and significant contamination with cytochrome b_5. The second fraction was diluted 7.5-fold with water and added to calcium phosphate gel (160 mg/1000 mg of starting microsomal protein) suspended in 0.02 M buffer without glycerol and stirred for 15 min at 4°. The calcium phosphate gel was then collected by centrifugation at 10,000 × *g* for 10 min, suspended in 0.3 M buffer without glycerol, stirred for 10 min at 4°, and centrifuged. Some cytochrome P-450 of low specific content was recovered in the supernatant fraction and was discarded. The calcium phosphate gel was then suspended in 1 M buffer without glycerol, and the cytochrome (fraction A) was recovered in the supernatant fraction following centrifugation. Fraction A was then dialyzed and stored as described above for fraction B. Fraction C, containing both fractions A and B, was obtained by omitting the CM-cellulose chromatography step and following the procedure used for obtaining fraction A. The cytochrome preparations used for hydroxylase activity determinations were passed through Porapak Q or adsorbed to and eluted from calcium phosphate gel in order to remove free Emulgen. The procedures removed essentially all of the Emulgen from 0.2% Emulgen solutions having at least 10 times the volume of the samples.

Preparation of NADPH–cytochrome c reductase. For preparation of NADPH–cytochrome *c* reductase, rabbit hepatic microsomes (3000 mg of protein) were suspended in 0.1 M phosphate buffer containing 1 mM dithiothreitol and 1 mM EDTA to yield a protein concentration of 13 mg/ml. The suspension was sonicated and digested with sodium deoxycholate by the methods of Lu *et al.* (12). The digested suspension was fractionated by precipitation using ammonium sulfate. The precipitate forming between 30% and 45% saturation of the solution with ammonium sulfate was found to contain the highest specific NADPH–cytochrome *c* reductase activity. This precipitate was dissolved in a few milliliters of 0.1 M phosphate buffer, pH 7.7, containing 20% glycerol, 0.1 mM dithiothreitol, and 0.1 mM EDTA, and dialyzed against the same buffer solution. The dialyzed sample was applied to a column of DEAE-cellulose (2.5 × 60 cm) and eluted with a linear gradient of KCl (0–0.6 M) in the buffer solution used for dialysis but without glycerol. Fractions containing NADPH–cytochrome *c* reductase activity, which was eluted between 0.3 and 0.4 M KCl, were pooled, concentrated by ultrafiltration (Amicon, PM-30 membrane), and dialyzed as above. The final preparations contained 1000–2000 units of cytochrome *c* reductase per milligram of protein. (One unit is defined as the amount of enzyme which catalyzes the reduction of 1 nmole of cytochrome *c* per minute.)

Analytical procedures. Cytochrome *c* reductase activity was determined by the procedure of Masters *et al.* (19) as described by Lu *et al.* (12). Benzpyrene hydroxylase activity was determined by the method of Wattenberg *et al.* (20) under conditions described by Philpot and Bend (21) for assays using reconstituted systems. 7-Ethoxycoumarin *O*-deethylation was measured by the method of Ullrich and Weber (22), using incubation systems described by Philpot *et al.* (23). Benzphetamine *N*-demethylation was measured according to Lu *et al.* (12) by following the substrate-dependent rate of NADPH oxidation. Epoxide hydrase activity was determined by the method of Oesch *et al.* (24).

Cytochrome P-450 concentrations were determined by the method of Omura and Sato (25), using a millimolar extinction coefficient of 91. Cytochrome b_5 levels were determined as described previously (26). Type I and type II substrate difference

spectra were recorded as described by Remmer *et al.* (27), using benzphetamine and pyridine as ligands. All spectra were recorded on an Aminco DW-2 spectrophotometer.

Monomeric molecular weights were estimated by SDS[1]-polyacrylamide gel electrophoresis (28). SDS-polypeptide complexes were formed by incubating samples in 0.01 M sodium phosphate buffer, pH 7.3, containing 1% SDS and 1 mM EDTA. Inclusion of 1 mM dithiothreitol in the incubation did not alter the final results. Electrophoresis was carried out as described by Weber and Osborn (29), except that 1 mM EDTA was included in the electrophoresis buffer and the gels were subjected to electrophoresis for 40 min at 20° prior to application of the samples.

Cytochrome *c*, α-chymotrypsin, trypsin, alcohol dehydrogenase, catalase, and bovine serum albumin were used as standards for molecular weight estimations.

The gels were fixed and stained for protein simultaneously in a solution containing 0.25% Coomassie blue in 50% methanol and 7% acetic acid for 1 hr at room temperature as described by Alvares and Siekevitz (3). Excess dye was removed by extensive washing with 30% methanol for 48 hr.

Peroxidase activity was ascertained by the method of Clausen (30) as described for polyacrylamide gel systems by Welton and Aust (2).

Linear scans of SDS–polyacrylamide gels were recorded at 550 nm using a Gilford spectrophotometer, model 2400-2, equipped with a Gilford linear transport, model 2410S.

Protein concentrations were determined by the method of Lowry *et al.* (31).

The following chemicals and biochemicals were purchased from various companies as described below: NADPH, horse heart cytochrome *c* (type VII), trypsin (type XII), α-chymotrypsin (type IV), bovine serum albumin, and catalase, from Sigma Chemical Company; alcohol dehydrogenase, from Miles Laboratories; sodium cholate, sodium deoxycholate, and ammonium sulfate (ultrapure), from Schwarz/Mann; DEAE-cellulose (DE-52), from Whatman Biochemicals, Ltd.; hydroxylapatite and calcium phosphate gel, from Bio-Rad Laboratories; and Porapak Q, from Waters Associates, Milford, Mass. 7-Hydroxycoumarin was obtained from J. T. Baker Chemical Company and recrystallized three times from an ethanol–water solution (1:4). 7-Ethoxycoumarin was synthesized by Dr. Jack Bend according to the method of Ullrich and Weber (22). *d*-Benzphetamine hydrochloride was a gift from Dr. P. W. O'Connell of the Upjohn Company. Emulgen 913 was a gift from the Kao-Atlas Company, Tokyo. All other chemicals used were purchased from commercial sources.

[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

RESULTS

Cytochrome P-450 contained in detergent-digested hepatic microsomes from untreated rabbits was eluted from DEAE-cellulose as a single peak by a 0.15% Emulgen solution. (Details for all chromatography steps are described in Fig. 1 and Table 1.) Prior to elution with Emulgen, at least 1 ml of buffer solution per milligram of sample protein was passed through the column. This step was required in order to attain reproducible results.

The absolute spectrum of dithionite-reduced cytochrome P-450 preparations from DEAE-cellulose showed peaks of absorption at 421 nm and 555 nm, which suggested the presence of large amounts of cytochrome b_5. The carbon monoxide difference spectrum of the reduced samples showed that the cytochrome P-420 content was low (less than 5% of the P-450 level). Forty to sixty per cent of the microsomal cytochrome P-450 was recovered from the DEAE-cellulose at a concentration of 3–4.5 nmoles/mg of protein (Table 2). The majority of the recovered cytochrome did not bind when applied to a column of CM-cellulose. However, a second cytochrome-containing fraction was obtained when the eluent buffer concentration was raised to 0.5 M. [This fraction (B), which contained cytochrome at a concentration of 10–14.3 nmoles/mg of protein, will be discussed later.] The spectra of the nonbinding CM

TABLE 2

Purification and yield of cytochrome fractions from hepatic microsomes of untreated rabbits

A complete description of fractions A, B, and C is provided in the text.

Fraction	Cytochrome P-450	Cytochrome yield[a]
	nmoles/mg protein	%
Microsomes	0.8–1.0	100
DEAE-cellulose eluate	3.0–4.5	40–60
Fraction A	5.5–6.5	2–5
Fraction B	10.0–14.3	2–5
Fraction C	4.5–5.0	10–20

[a] These values are based on the total microsomal concentration of cytochrome P-450. The concentrations of the different forms of cytochrome P-450 in microsomes could not be determined.

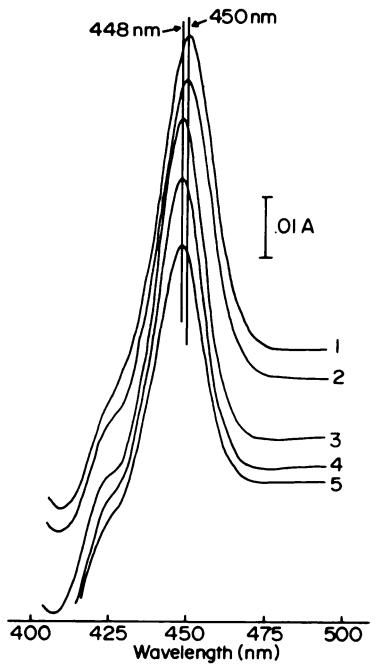

FIG. 2. *Carbon monoxide-reduced minus reduced difference spectra of hepatic cytochrome fractions from untreated rabbits*

1, fraction A (0.57 nmole of cytochrome per milliliter, 6.3 nmoles of cytochrome per milligram of protein; *2*, fraction C (0.55 nmole of cytochrome per milliliter, 5 nmoles/mg of protein); *3*, fraction B (0.58 nmoles of cytochrome per milliliter, 10.8 nmoles/mg of protein); *4*, fraction B (0.50 nmole of cytochrome per milliliter, 10.0 nmoles/mg of protein); *5*, fraction B (0.44 nmoles of cytochrome per milliliter, 13.6 nmoles/mg of protein). All spectra are those of samples diluted with 0.1 M phosphate buffer, pH 7.7, containing 20% glycerol. Absorption maxima are 450.2 nm for *1*, 450 nm for *2*, and 448 nm for *3*, *4*, and *5*.

void fraction were similar to those obtained from the samples from the DEAE-cellulose procedure. Two cytochrome-containing fractions were obtained from the CM void fraction by chromatography on hydroxylapatite. The first fraction, eluted with 80 mM buffer–0.2% Emulgen, was contaminated with cytochrome b_5 and had a low specific content of cytochrome P-450. This fraction was discarded. The second fraction, eluted with 150 mM buffer–0.2% Emulgen, contained about 5 nmoles of cytochrome P-450 per milligram of protein. This fraction was adsorbed onto calcium phosphate gel and two cytochrome-containing fractions were obtained again, one with 0.3 M buffer and a second with 1 M buffer. The first fraction was similar to the first fraction obtained by chromatography on hydroxylapatite and was discarded. The second fraction (A) contained cytochrome P-450 at a concentration of 5.5–6.5 nmoles/mg of protein. The recovery of microsomal cytochrome in fraction A plus fraction B was 4–10%. A significant increase in recovery was obtained by eliminating the CM-cellulose chromatography step. The cytochrome obtained in this manner (fraction C) was equal to 10–20% of the microsomal cytochrome and had a concentration of 4.5–5 nmoles/mg of protein.

Difference spectra formed by the addition of carbon monoxide to dithionite-reduced samples demonstrated that A and B contained different forms of cytochrome (Fig. 2); the peak of absorption for fraction A occurred at 450.2 nm, and for fraction B, at 448 nm. Fraction C produced the same peak of absorption observed in microsomes (450 nm), suggesting the presence of both forms of the cytochrome. Additional differences were noted when the absolute spectra of the cytochrome fractions were examined (Fig. 3A, B, and C). The spectrum of oxidized fraction B indicated the presence of substantial amounts of cytochrome in

the high-spin state. This is seen from the peak of absorption near 390 nm (Fig. 3A). The amount of high-spin cytochrome in preparations of fraction B varied; however, fraction B was always distinguishable from fraction A on this basis. The spectrum of the CO complex of dithionite-reduced cytochrome from fraction A showed a peak of absorption at 449.5 nm, while the peak for fraction B was at 447.5 nm. The peak for fraction C appeared at 449.2 nm, again suggesting that it contained both A and B. A difference in the spectra of the CO complexes was also seen in the visible area; fraction A absorbed maximally at 554 nm, and fraction B, at 550 nm. This wavelength difference was reproducible. The spectra of dithionite-reduced preparations were essentially identical for all three fractions (Fig. 3B). The absolute spectra shown (Fig. 3A, B, and C) are all consistent with preparations having no detectable cytochrome b_5 and little cytochrome P-420 (26). NADPH-cytochrome *c* reductase and epoxide hydrase activities in these preparations were also very low or absent (Table 3).

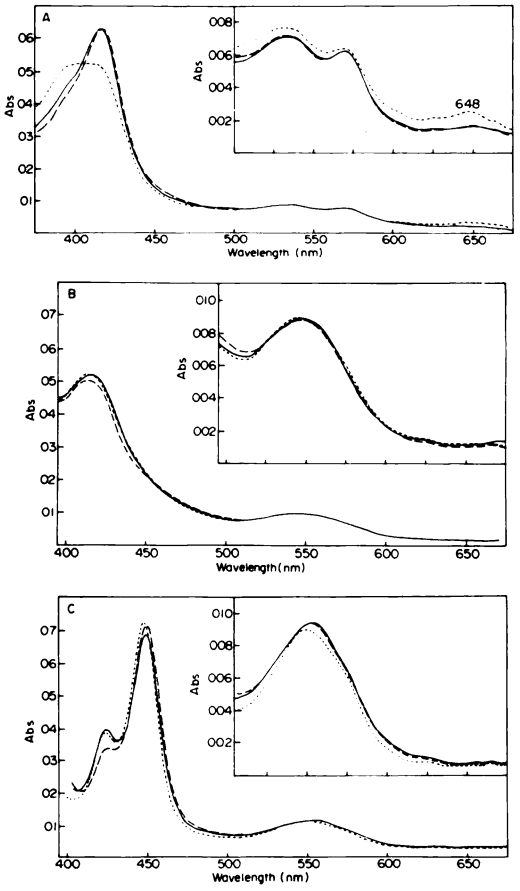

FIG. 3. *Absolute spectra of hepatic cytochrome P-450 preparations obtained from untreated rabbits*

A. Oxidized spectra. B. Reduced spectra. C. Carbon monoxide-reduced spectra. - - -, fraction A (0.57 nmole of cytochrome per milliliter, 6.3 nmoles/mg of protein; -----, fraction B (0.50 nmole of cytochrome per milliliter, 10.8 nmoles/mg of protein); ——, fraction C (0.55 nmole of cytochrome per milliliter, 5 nmoles/mg of protein). All spectra were recorded with samples diluted with 0.1 M phosphate buffer, pH 7.7, containing 20% glycerol. Absorption maxima are listed in Table 4.

Type II substrate difference spectra formed with cytochrome fractions A and B upon the addition of pyridine (Fig. 4). However, two differences were noted between the spectra formed: first, the magnitude of the spectrum formed with fraction B was greater than that formed with fraction A; second, the spectrum formed with fraction A showed a broad minimum between 390 and 410 nm while the spectrum from B showed a minimum at 393 nm. As shown in Fig. 4, the difference between the type II spectra formed with fractions A and B closely resembled a type I substrate difference spectrum with a trough in the area of 420 nm and peak near 390 nm. The addition of benzphetamine to fraction A resulted in the formation of a type I substrate difference spectrum (Fig. 4). No similar interaction was observed when benzphetamine was added to fraction B. The absorption maxima and minima for all spectra recorded are summarized in Table 4.

Fraction A and B cytochrome preparations were active in the metabolism of benzpyrene, 7-ethoxycoumarin, and benzphetamine (Table 5). In all cases the activity supported by fraction A was about twice as great as that supported by fraction B. No activity was detected when either reductase or cytochrome was absent from the incubation mixtures. With both cytochrome fractions some benzpyrene hydroxylase activity was noted without the addition of the lipid fraction, but 7-ethoxy-

TABLE 3

Concentrations of some contaminating proteins in purified cytochrome P-450 preparations from untreated rabbits

Results shown are for the highest concentrations measured from several preparations. See METHODS AND MATERIALS for description of fractions.

Cytochrome fraction	NADPH-cytochrome *c* reductase		Epoxide hydrase	Cytochromes	
				b_5	P-420
	units/mg protein	*units/nmole cytochrome*	*units/mg protein*	*% heme*	
A	<7	<1.5	<1	ND[a]	<5%
B	<4	<0.4	ND	ND	<5%

[a] ND = not detected.

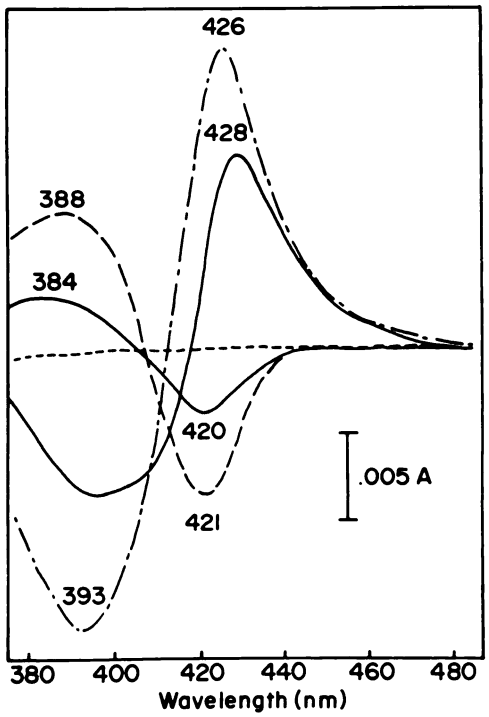

FIG. 4. *Difference spectra formed with hepatic cytochrome P-450 in fractions A and B from untreated rabbits*

——, type I (absorption maximum at 384 nm) and type II (absorption maximum at 428 nm) substrate difference spectra formed by the addition of benzphetamine (2 mM) and pyridine (8 mM), respectively, to samples containing fraction A cytochrome (0.57 nmole/ml, 6.3 nmoles/mg of protein); -·-·-, type II substrate difference spectrum formed by the addition of pyridine (8 mM) to a sample containing fraction B cytochrome (0.58 nmole/ml, 10.8 nmoles/mg of protein); - - -, fraction A type II substrate difference spectrum minus fraction B type II substrate difference spectrum; -----, baseline of zero absorbance difference. Difference spectra were obtained by the addition of the ligand to the sample cuvette and an equal amount of solvent (water) to the reference cuvette subsequent to recording a baseline.

coumarin deethylase and benzphetamine demethylase activities were not detectable in the absence of added lipid fraction (Table 5).

In contrast to the spectral and activity differences between the forms of cytochrome P-450 in fractions A and B, their apparent monomeric molecular weights estimated by SDS-gel electrophoresis were essentially identical: 51,700 ± 750 for fraction A and 51,600 ± 700 for fraction B (N = 4).

Figure 5 shows that a single band was observed when gels containing fraction B were stained with Coomassie blue, while at least one additional minor contaminant was evident in gels containing fraction A. A single major band was also observed on gels containing fraction C or mixtures of fraction A plus fraction B (data not shown). The location of the cytochrome preparations on the gels corresponded to a band observed in gels containing rabbit hepatic microsomes (Fig. 5). Peroxidase activity was not detected in gels containing fractions A, B, or C.

DISCUSSION

Although significant progress has been made in the purification of hepatic cytochrome P-450 from animals treated with various inducers (15–17), the present report is the first to describe similar success using preparations from untreated animals. The results of this study show that

TABLE 4

Absorption maxima and minima for cytochrome P-450 fractions from rabbit liver

Absolute spectra	Fraction A		Fraction B		Fraction C	
	nm		*nm*		*nm*	
Oxidized	418, 534, 570		(395–415),[a] 534, 570		417, 534, 570	
Reduced	414, 545		414, 545		416, 545	
Reduced-CO	(423), 449.5, 554		(423), 447.5, 550		(423), 449.2, 554	
Difference spectra	Peak	Trough	Peak	Trough	Peak	Trough
		nm		*nm*		*nm*
CO-reduced minus reduced	450.2	409	448	406	450	409
Type I (benzphetamine)	(385)	421			(385)	420
Type II (pyridine)	(428)	(395–410)	426	493	428	(395–410)

[a] Wavelengths in parentheses indicate that the maximum or minimum appeared as a shoulder and was estimated.

TABLE 5

Mixed-function oxidase activities supported by purified cytochrome fractions from untreated rabbits in reconstituted systems

Results are typical of several preparations; incubation and analysis procedures are referenced in METHODS AND MATERIALS.

Fractions included in incubations			Benzpyrene hydroxylase	7-ethoxycoumarin *O*-deethylase	Benzphetamine *N*-demethylase
Reductase[a]	Lipid[b]	Cytochrome[c]			
			nmoles product/min/nmole cytochrome		
−	+	A	ND[d]	ND	ND
−	+	B	ND	ND	ND
+	−	A	0.067	ND	ND
+	−	B	0.095	ND	ND
+	+	A	0.533	0.80	3.4
+	+	B	0.225	0.45	1.7

[a] The concentration of reductase was 100 units/ml.

[b] The concentration of lipid was 0.1 mg/ml.

[c] The concentration of either cytochrome fraction was 0.2 nmole/ml.

[d] ND = not detectable.

at least two spectrally distinct forms of hepatic cytochrome P-450 exist in untreated rabbits. The two forms of the cytochrome can be separated from a partially purified preparation by chromatography on CM-cellulose. The form of the cytochrome which binds to the CM-cellulose is eluted in a highly purified fraction (B) which is essentially free of epoxide hydrase, cytochrome b_5, and NADPH-cytochrome *c* reductase. In contrast, the CM void fraction contains these enzymes in addition to cytochrome P-450. Chromatography of the CM void fraction on hydroxylapatite and calcium phosphate is effective in removing the contaminating proteins but does not markedly improve the apparent specific content of the cytochrome P-450 in the preparation. This may be due to loss of heme; calculations based on analysis of spectra indicate that the cytochrome P-450 in fraction A is about 30% pure although the results of the SDS–polyacrylamide gel electrophoresis experiments strongly suggest a much greater degree of purity.

The forms of cytochrome P-450 contained in fractions A and B are spectrally distinct. The peak of absorption in the CO-reduced minus reduced difference spectrum occurs at 450.2 nm for fraction A and at 448 nm for fraction B. The oxidized spectrum of fraction B indicates the presence of significant amounts of cytochrome in the

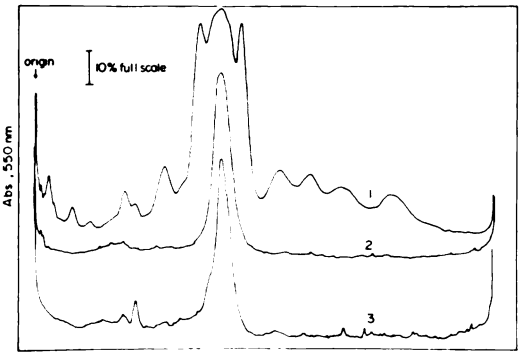

FIG. 5. *SDS-polyacrylamide gel electrophoresis of partially purified cytochromes and microsomes from untreated rabbits*

Gels were subjected to electrophoresis with samples, stained with Coomassie blue, and scanned at 550 nm; *1*, microsomes (56 μg of protein); *2*, fraction B (8 μg of protein, 13.6 mmoles of cytochrome per milligram of protein); *3*, fraction A (12 μg of protein, 6.3 nmoles of cytochrome per milligram of protein). Full-scale absorptions were 2 A for gel *1* and 1 A for gels *2* and *3*.

high-spin state, whereas the oxidized spectrum of fraction A does not. This apparent difference in spin state between fractions A and B is reflected in the type I and type II binding spectra which they form. The spectrum formed by the addition of pyridine to fraction A is a composite of type I and type II spectra. This demonstrates the availability of a type I binding site, as does the type I spectrum formed by the addition of benzphetamine to fraction A. On the other hand, fraction B exhibits no discernible type I binding and forms a large, symmetrical type II spectrum with pyridine. This is in agreement with the findings of Schenkman (32), who has reported that both symmetry and increased spectral magnitude result when type I characteristics are eliminated from the spectra formed by type II ligands. The spectral properties of the cytochrome in fraction B are similar to those of the rabbit hepatic cytochrome induced by 3-methylcholanthrene (21, 33–36). The possibility that the cytochrome in fraction B is induced by dietary factors and is the same as the cytochrome induced by 3-methylcholanthrene is being investigated.

The catalytic activities of fractions A and B are also different. Fraction A is about twice as active as fraction B in the *N*-demethylation of benzphetamine, the *O*-deethylation of 7-ethoxycoumarin, and the hydroxylation of benzpyrene. This may be due to greater concentrations of Emulgen in fraction B than in fraction A. However, the ratios of absorbance between 280 and 418 nm are similar for each preparation after treatment with Porapak Q. The differences in catalytic activity may also be due to the presence of an endogenous substrate or inhibitor in fraction B.

The relationship between the forms of cytochrome P-450 in fractions A and B and other forms of cytochrome P-450 purified from treated rabbits is not clear. Haugen *et al.* (6) have reported that two uninduced forms of hepatic cytochrome P-450 can be isolated from phenobarbital-treated rabbits. The monomeric molecular weights of 47,000 and 60,000 reported by Haugen *et al.* (6) for the uninduced cytochromes contrast significantly with the nearly identical monomeric molecular weights (about 51,500) reported here for two forms of cytochrome P-450 from untreated rabbits.

It is evident that untreated animals have several forms of hepatic cytochrome P-450 (1–4). We have shown that at least two forms of the cytochrome can be separated and purified from rabbits. Additional work is required to determine how many forms of hepatic cytochrome P-450 exist and what the catalytic function of each form is. It is also important that dietary and environmental factors be investigated in order to determine their effects on the relative concentrations of each form of the cytochrome. Clearly, variable concentrations of multiple forms of hepatic cytochrome P-450 may be responsible for the substrate specificities of different hepatic microsomal mixed-function oxidase systems.

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