Existence and Separation of Three Forms of Cytochrome P-450 from Rat Liver Microsomes*

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SUMMARY

Rat liver microsomes contain three spectrally distinguishable forms of cytochrome P-450. Each form is identified qualitatively and quantitatively by visible spectral changes that occur when the cytochromes P-450 combine with various ligands, such as cyanide. In addition to cytochrome P-450, rat liver microsomes also contain cytochrome b5, which may be removed from the microsomes by digestion with Subtilisin VII. The relative amounts and spectral properties of the three forms of cytochrome P-450 remaining in treated particles are not altered by the partial proteolytic digestion.

With difference spectral assays, methods have been developed for the chromatographic separation of the three forms of cytochrome P-450 in Subtilisin-treated particles. The particles are first treated with deoxycholate, and the clarified suspension is chromatographed on diethylaminoethylcellulose; a discontinuous gradient of KCl is used. Analysis of the affinities of the three separated forms (Forms I, II, and III) for various ligands yields the following binding constants for cyanide: Form I, K = 0.5 mM; Form II, K = 1.5 mM; and for octylamine: Form I, K1 = 0.025 mM and K2 = 0.12 mM; Form II, K1 = 0.016 mM and K2 = 0.056 mM; Form III, K = 0.30 mM. The binding constants are essentially identical with those observed for the three forms of cytochrome P-450 in microsomes and Subtilisin-treated particles. Thus, Subtilisin treatment, solubilization, or chromatography does not alter either the relative amounts of the three forms or their difference spectral characteristics.

Assays of the three forms after various pretreatments of rats have been undertaken. The relative amounts of the three forms of cytochrome P-450 are altered by pretreatment of the rats. Form III is increased by pretreatment with 3-methylcholanthrene; Form II is preferentially induced by phenobarbital; and dietary ethyl alcohol preferentially induces Form I, the form of cytochrome P-450 that exhibits the highest affinity for cyanide. Thus, this work has provided not only an assay for the various forms of cytochrome P-450 and a chromatographic separation for future work on recombination of the multienzyme oxidase, but a functional relationship for the three forms is suggested by the induction experiments. These are only spectrally identifiable forms that may be separated by these procedures, and, to date, there is no information on whether or not these forms differ structurally.

The principal components of the liver microsomal mixed function oxidase system, an electron transport pathway of microsomes that is responsible for the oxidation of steroids, fatty acids, drugs, and several xenobiotics, are NADPH-cytochrome c reductase, cytochrome P-450, and phospholipid. Reconstitution of the oxidase has been achieved by combining partially purified components that have been obtained from liver microsomes.

Evidence for a variety of forms of cytochrome P-450 has been based principally on visible spectrophotometric and electron paramagnetic spectroscopy, in addition to enzymic studies. For example, the visible spectral changes involved in the binding of ligands to cytochrome P-450 have been investigated extensively.

Characteristic binding of carbon monoxide, ethyl isocyanide, and other ligands to cytochrome P-450 of liver microsomes from normal and from phenobarbital- and 3-methylcholanthrene-treated animals led to the proposal that at least two forms of reduced cytochrome P-450 exist (2-6). These initial reports have been further substantiated in studies from several laboratories (7-12), the reports from which have suggested that oxidized cytochromes P-450 and P-448 (P-450) are two forms that are spectrally and enzymically distinct.

However, combined potentiometric and electron paramagnetic resonance titrations of cytochrome P-450 in liver microsomes from phenobarbital-treated rats led to evidence for two low spin hemoproteins that exhibit different midpoint potentials (13). These preliminary findings thus indicate that rat liver microsomes may contain an additional, third form of cytochrome P-450, that is, two low spin forms of cytochrome P-450 and a form that has been reported to be a high spin hemoprotein (5, 8). Thus, liver microsomes may contain a minimum of three forms of cytochrome P-450.

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1 David F. Wilson and Beverly Cohen, private communication.
Recently, the hydroxylation system of microsomes has been released from the isolated particles and resolved into fractions containing cytochrome P-450, an NADPH-dependent reductase, and phospholipid (14, 15). In reconstitution experiments, Lu et al. (16) found that the specificity of the hydroxylation system resides with the cytochrome P-450 fraction that is obtained from immature rats that had been treated with either phenobarbital or 3-methylcholanthrene. However, the elusiveness of these cytochromes to purification procedures, which result in either enrichment of heme content or separation of various forms, has made it difficult to determine cross-contamination of the various forms in different preparations of cytochrome P-450 precisely.

On the other hand, Gunsalus and co-workers (17, 18) have been able to purify a soluble, inducible form of cytochrome P-450 from *Pseudomonas putida*; the hemoprotein is involved in the methylenedioxyxylolation of camphor. From electron paramagnetic resonance measurements, approximately 60% of the induced forms in different preparations of cytochrome P-450 are able to purify a soluble, inducible form of cytochrome P-450. However, the elusiveness of these cytochromes to purification procedures, which result in either enrichment of heme content or separation of various forms, has made it difficult to determine cross-contamination of the various forms in different preparations of cytochrome P-450 precisely. In this report, evidence is presented for three forms of microsomal cytochrome P-450 in adult rat liver. A chromatographic method for separation of the three spectrally identifiable forms of cytochrome P-450 is given, and spectral characterization is described. With the visible difference spectral assay, the relative amounts of the forms of cytochrome P-450 have been found to be altered by pretreatment of animals with phenobarbital, 3-methylcholanthrene, and ethyl alcohol.

**Experimental Procedure**

**Preparation of Microsomes and Subtilisin-treated Microsomes—** Male adult rats of the Sprague Dawley strain, weighing 175 to 225 g, were fed Agway Laboratory animal chow *ad libitum*, except during the ethanol diet experiment. The rats were killed by decapitation, and each liver was perfused in situ with 75 ml of cold 0.25 M sucrose. The livers were excised, weighed, minced, and homogenized in a TenBroeck homogenizer; 3 volumes of 0.1 M potassium phosphate buffer (pH 7.4 and containing 10 mM nicotinamide and 2 mM glutathione) were used (20). The homogenate was centrifuged at 10,000 × g for 20 min. Microsomes were harvested by centrifugation of the resulting supernatant fraction for 1 hour at 105,000 × g. The pellet, whole microsomes, was used immediately; all procedures were performed at 4°C except as noted.

Whole microsomes were suspended in the homogenizing buffer, to which 20% (v/v) glycerol was added; the final concentration of protein was 10 mg per ml of suspension. Subtilisin VII (10 µg per mg of protein) was added, and the suspension was incubated aerobically at 4°C, with agitation, for various lengths of time. The Subtilisin-treated microsomes were collected by centrifugation at 105,000 × g for 1 hour. Each pellet was washed by suspending the residue in 1 volume of fresh buffer (without added glycerol) and centrifuging the resulting suspension again at 105,000 × g for 1 hour.

**Solubilization Procedure and Chromatography—** Subtilisin treated microsomes were suspended in cold 0.25 M sucrose to yield a protein concentration of 25 mg per ml. The solubilization procedure of Lu and Coon (14) was employed with slight modifications as follows. Glycerol was added to a final concentration of 30%, potassium citrate (pH 7.4) and potassium chloride were added to final concentrations of 0.1 M each, and the final concentration of dithiothreitol was 0.1 M. Sodium deoxycholate was added, 0.25 mg per mg of protein, and the suspension was homogenized gently for 10 min. It was diluted 2-fold with cold glass-distilled water and placed immediately on a diethylaminoethylcellulose column (2.2 × 20 cm) which had been washed with the eluting buffer (0.1 M Tris-HCl, pH 7.6 at 25°C, 0.05% deoxycholate, and 0.1 M dithiothreitol). Approximately 200 mg of protein were transferred to the column; the column was developed with a stepwise gradient of potassium chloride in the Tris buffer as described under “Results.” Fractions of 10 ml each were collected.

Diethylaminoethylcellulose, obtained from Sigma Chemical Co., was equilibrated between acid and base washes prior to equilibration in buffer (21). Resin (DE-52) obtained from Whatman was prepared according to the manufacturer’s directions. Each resin gave consistent elution profiles, but chromatographic behavior of the cytochromes P-450 was different on preparations of DEAE-cellulose from each source. Fractions from the diethylaminoethylcellulose column were desalted without delay by filtration through columns of Sephadex G-25 (2.2 × 15 cm) which had been equilibrated with 0.1 M potassium phosphate buffer, pH 7.4. This procedure removed salt and no more than 88% of the residual detergent, which was determined by measurement of 14C-labeled deoxycholate. Aliquots of the solubilized and desalted materials containing 14C deoxycholate were counted in Bray’s solution (22) in a Packard Tri-Carb liquid scintillation spectrometer (model 3375).

Spectrophotometry—Initially, spectrophotometric titrations and difference spectra measurements were performed on a Cary model 14 spectrophotometer equipped with a high intensity light source and a 0.1 optical density slide wire. More recently, spectral data have been obtained with a Perkin-Elmer recording spectrophotometer (model 356).

Routinely, for difference spectral assays of reduced cytochrome P-450 in the presence of carbon monoxide, the buffer was first saturated with carbon monoxide prior to addition of dithionite. When difference spectra resulting from carbon monoxide titrations were recorded, however, aliquots of a carbon monoxide-saturated buffer solution were added by a microliter syringe to stopped 3-ml cuvettes which contained the microsomal suspension to which a few grains of solid sodium dithionite had been added. The concentration of carbon monoxide in a solution saturated with carbon monoxide at atmospheric pressure in 0.1 M potassium phosphate, pH 7.4, was taken to be 0.92 mM at 20°C.

Cytochrome c and octylamine titrations were performed with freshly prepared solutions which were adjusted to neutral pH (11, 23). Cytochrome c tautomers were performed over the range of 0.1 to 20 mM or to the concentration of cytochrome c at which an increase in absorbance was no longer detectable. Protein concentrations in spectral assays were usually adjusted to about 1 mg per ml. 

Electron paramagnetic resonance spectroscopy at liquid helium temperatures was kindly carried out by Dr. Helmut Beinert of the Institute for Enzyme Research, University of Wisconsin.

**Enzymic and Hemoprotein Assays—** Microsomal NADPH-cytochrome c reductase was determined spectrophotometrically at 25°C. Protein was added to 0.1 M phosphate buffer (pH 7.4) which contained 0.23 µmol of cytochrome c and 1 µmol of NADPH. The final volume was 1.0 ml. Changes in absorbance at 550 nm were recorded with time, and a difference spectral extinction coefficient of 19.6 mm⁻¹ cm⁻¹ for ferrous cytochrome c was used (24). NADH-ferricyanide reductase was measured under the above conditions except that 2 µmoles of ferricyanide were substituted for cytochrome c, and the activity was calculated from the absorbance change at 420 nm using 1.02 mm⁻¹ cm⁻¹ as the extinction coefficient (25).
Cytochromes P-450, P-420, and b_5_ were determined by the methods described by Omura and Sato (26, 27). For cytochrome P-450, the sample was first saturated with carbon monoxide and then reduced, along with the reference sample, by the addition of a few grains of solid sodium dithionite. This procedure was used consistently to minimize the possible differences in lability and auto-oxidizability of the different forms of cytochrome P-450 (15). The extinction coefficient of 91 \text{mm}^{-1} \text{cm}^{-1} was used for the difference in absorbance between 450 and 400 nm. Cytochrome P-420 was measured similarly using an extinction coefficient of 110 \text{mm}^{-1} \text{cm}^{-1} for the difference in absorbance between 420 and 490 nm. Cytochrome b_5_ was determined from the difference spectrum of the NADPH-reduced minus oxidized form using 185 \text{mm}^{-1} \text{cm}^{-1} as the extinction coefficient for the difference in absorbance between 424 and 409 nm.

The heme content was determined from the dithionite-reduced minus ferricyanide-oxidized difference spectrum after formation of the pyridine hemochrome. An extinction coefficient of 34.7 \text{mm}^{-1} \text{cm}^{-1} was used for the calculation of heme content at 557 nm, as reported by Paul et al. (28).

Protein was determined by the method of Lowry et al. (29), using crystalline bovine serum albumin as the standard.

Other Treatments—For the phospholipase A treatment, microsomal protein was suspended in 0.1 M Tris-HCl (pH 7.4 at 37°C) to a final concentration of about 25 mg per ml. For every 5 mg of protein, 1 \mu mole of CaCl_2 was added. Phospholipase A (1 \mu g per mg of microsomal protein) was added, and the mixture was incubated at 37°C for 10 min. The control samples contained only Ca^2+ and buffer. The reaction was terminated by the addition of 1 \mu mole of EDTA per mg of protein. The reaction mixtures were homogenized with 3 additional volumes of buffer, and the resulting suspensions were centrifuged at 165,000 × g for 1 hour. The yield of protein in the resulting pellets was about 30%; the yield from controls (no phospholipase A) was about 50%. Lipids were extracted from the collected pellets with chloroform-methanol (2:1, v/v) (30). The solvent solutions were washed with water and evaporated. Residues were taken up in methanol and transferred to thin layer plates (250 \mu m thickness) coated with Silica Gel HF-254. The chromatograms were developed in chloroform-methanol-acetic acid-water (25:15:4:2, v/v) (31). The solvent front was followed by exposure to ultraviolet light and compared with standard samples.

When indicated, sodium phenobarbital was dissolved in water and injected intraperitoneally (80 mg per kg of body weight) daily for 3 days. 3-Methylcholanthrene was dissolved in corn oil, and a single intraperitoneal injection of 40 mg per kg of body weight was given 3 days prior to killing. After a period of adjustment, rats were fed ethyl alcohol in a liquid diet for 16 days as described by Lieber and DeCarli (31). Approximately 36% of the dietary calories were in the form of ethyl alcohol. In all experiments, control animals received equal volumes of the drug vehicle, and, in the dietary ethanol experiment, carbohydrate was substituted isosmotically and isovolumetrically for the ethyl alcohol in control diets.

Materials—Common chemicals were of the highest purity of commercially available reagents. Subtilisin VII (Lot 60C-1150-1), phospholipase A (from Vipera russeli; Lot 120C-2501), NADPH, NADH, deoxycholic acid (sodium salt), and diethylaminoethylcellulose were purchased from Sigma Chemical Co. Cytochrome c (equine heart, salt-free) and diithiothreitol were obtained from Eastman Organic Chemicals. Carbon monoxide (C.P. grade) was obtained from Matheson Gas Products Division, Will Ross, Inc, diethyldimethylenamine (DE-52) from Whatman, and Sephadex G-25 from Pharmacia Fine Chemicals. Silica Gel HF-254 was prepared by E. Merck (Darmstadt). [14C]Deoxycholic acid was obtained from International Chemical and Nuclear Corp.

RESULTS—Effect of Subtilisin VII Treatment on Whole Microsomes—Prior to ion exchange chromatography of detergent-treated preparations (see below), cytochrome b_5_ was first removed from whole microsomes by digestion with Subtilisin VII. As others have reported, incubation of microsomal membranes with certain proteases preferentially elicits removal of NADPH-cytochrome c reductase and cytochrome b_5_ (32, 33). The time dependence of the Subtilisin digestion process on residual rat liver microsomal cytochrome c is shown in Fig. 1. After 18-hour incubation, removal of NADPH-cytochrome c reductase was 98% complete, and removal of cytochrome b_5_ was 95% complete. No conversion of cytochrome P-450 to cytochrome P-420 was observed; cytochrome P-420 was almost undetectable (<10% of total hemoprotein) at each time point. The use of 10 \mu g of Subtilisin VII per mg of microsomal protein removed from 50 to 60% of the original protein by the end of the 13-hour incubation. However, the specific content of cytochrome P-450 and NADH-ferricyanide reductase of the digested, washed microsomes remained unchanged after an initial small increase in specific activity (Fig. 1). In addition, the ability of the cytochrome P-450 in Subtilisin-treated microsomes to combine with cyanide remained unchanged. In Fig. 1, cyanide binding is shown for the high affinity activity form of cytochrome P-450 (form I, \alpha_{CN} = 0.5 \text{ mm} M^{-1}); see below). Concentrations of Subtilisin VII greater than 10 \mu g per mg of microsomal protein resulted in drastic solubilization of protein with loss of selectivity, conversion of cytochrome P-450 to cytochrome P-420, and other undesirable and generally irreversible changes.

A close comparison of the cyanide titrations of whole and Subtilisin-treated microsomes revealed little difference in either

![Fig. 1. The effect of Subtilisin VII on microsomal components. The zero time aliquot contained 1.15 nmoles of cytochrome P 450 (○—○) and 0.69 nmoles of cytochrome b_5_ (□—□) per mg of microsomal protein. NADPH-cytochrome c reductase (☆—☆) and NADH-ferricyanide reductase (●—●) had specific activities of 82.5 nmoles per min per mg of microsomal protein and 4.5 μmoles per min per mg of microsomal protein, respectively. Form I of cytochrome P-450 (□—□) had a specific content of 0.48 nmole per mg of protein (23).](http://www.jbc.org/)
specific content or binding constants of the three forms of cytochrome P-450 in the two preparations. A partial spectrophotometric titration of whole microsomes with cyanide is given in Fig. 2A. Upon addition of cyanide, a difference spectrum, with $\lambda_{\text{max}}$ at 442 nm and $\lambda_{\text{min}}$ at 405 nm, was generated. A Hofstee plot of the data (Fig. 3A) revealed triphasic curves which correspond to three spectrally distinguishable forms of cytochrome P-450, each of which exhibited distinct cyanide affinity constants. Corresponding data for Subtilisin-treated microsomes are given in Figs. 2B and 3B, respectively.

There were slight, reproducible losses of all forms of P-450 after Subtilisin treatment. The specific contents were calculated with an extinction coefficient for cyanide as determined for the separated forms, which are described below. The contributions of the higher affinity forms (called Form I and Form II in this report) to the absorbance of the lower affinity forms (Form II and Form III) were subtracted appropriately before calculation of the specific content in whole and Subtilisin-treated microsomes. The specific content calculated from the data given in Fig. 3 revealed that, in whole microsomes, Form I ($K_{CN}=0.5$ mM), Form II ($K_{CN}=0.8$ mM), and Form III ($K_{CN}=4.9$ mM) had concentrations of 0.57, 0.38, and 0.48 nmole per mg of microsomal protein, respectively. Subtilisin-treated microsomes contained Forms I, II, and III in concentrations of 0.45, 0.32, and 0.47 nmole per mg of microsomal protein, respectively. Thus, only subtle changes in the binding constants and specific contents of the cyanide-binding components of microsomes resulted during Subtilisin digestion.

Subtilisin VII, at a concentration of 10 $\mu$g per mg of microsomal protein, thus yielded almost complete removal of cytochrome $b_5$ and NADPH-cytochrome $c$ reductase, with no significant alteration of cytochromes P-450 during the incubation at 4°C.

**Fig. 2.** Difference spectra resulting from the titration of liver microsomes with cyanide. Protein concentrations were 1 mg per ml. Three-milliliter cuvettes were used. A, whole microsomes; B, Subtilisin-treated microsomes.

**Fig. 3.** Hofstee plots of the cyanide titration data given in Fig. 2. A, whole microsomes; B, Subtilisin-treated microsomes.

**Diethylaminoethylcellulose Chromatography—**Subtilisin-treated microsomes were clarified with deoxycholate as described under "Experimental Procedure," and the three forms of cytochrome P-450 were separated by ion exchange chromatography on diethylaminoethylcellulose. A stepwise gradient of potassium chloride was used. A typical elution profile from Whatman (DE-52) diethylaminoethylcellulose is shown in Fig. 4A. Protein emerged in three main parts of the chromatogram, and a smaller amount was eluted by 0.5 mM potassium chloride, along with phospholipid and traces of cytochrome $b_5$ that were not removed previously during the Subtilisin digestion. Each of the three main protein peaks contained cytochrome P-450, but the cytochrome, as eluted, was extremely unstable. Passage of the combined, eluted materials immediately through Sephadex G-25 greatly enhanced stability. Thus, immediately after separation on diethylaminoethylcellulose, the solutions were desalted on Sephadex G-25 as described under "Experimental Procedure," and fractions from each protein peak that contained high concentrations of cytochrome P-450 were usually combined. The stabilized materials thus obtained were colored and somewhat turbid; protein containing cytochrome P-450 could be collected by sedimentation at 105,000 x g for 2 hours. After centrifugation at 105,000 x g for 2 hours, the resulting pellet contained 91% of the cytochrome P-450 and 65% of the remaining $^{14}$C-labeled deoxycholate, which was not removed by Sephadex chromatography.

The nature of the stabilization is not clear. However, it should be noted, emphatically, that salt from the elution gradient is
removed along with some detergent. High salt concentrations were reported by Imai and Sato (34) to solubilize cytochrome P-450. The use of [14C]deoxycholate for solubilization revealed that no more than 88% of the added detergent was removed by passage through Sephadex G-25.

Cytochrome P-450 assays were performed on the separated, stabilized fractions (e.g., obtained as shown in Fig. 4B). Approximately 170 mg of Subtilisin-treated, solubilized microsomes were chromatographed on diethylaminoethylcellulose (Sigma Chemical Co.) and desalted. The amount of cytochrome P-450 in the fractions was low (<10%). However, cytochrome P-420 was labile, even after desalting on Sephadex G-25, and the cytochrome P-420 carbon monoxide spectrum disappeared in about 24 hours; upon standing for 24 hours, there was little decrease in the carbon monoxide-bound reduced cytochrome P-450. Based on pyridine hemochrome assays, the carbon monoxide extinction coefficients determined for each of the three separated forms varied from 88 to 95 mm⁻¹ cm⁻¹, in agreement with values reported by Omura and Sato (26) and Lu et al. (16). There was no enrichment of cytochrome P-450 per mg of protein.

Spectrophotometric Studies—Extensive investigations of binding of ligands to cytochrome P-450 have been reported. Imai and Sato (7) and Jefcoate et al. (11) have shown that oxidized cytochrome P-450 combines with primary amines and cyanide to give characteristic difference spectra. In microsomes, octylamine combines with cytochrome P-450 to produce a type II difference spectrum (λmax 430 nm, λmin 390 to 410 nm) in which two binding constants, 0.015 and 0.2 mm, are obtained. Cyanide ions produce a difference spectrum (λmax 445 nm; λmin 405 nm) with binding constants that vary depending on pretreatment of experimental animals and microsomes. Jefcoate et al. (11) reported for cytochrome P-450 a cyanide binding constant of 6 to 8 mm from phenobarbital-induced rabbit liver microsomes, and 15 mm for 3-methylcholanthrene-induced rabbit liver microsomes. Accordingly, the three forms of cytochrome P-450 separated chromatographically (Fig. 4, A and B) and stabilized by desalting on Sephadex G-25 were titrated with octylamine and cyanide, in addition to titration of reduced hemoprotein with carbon monoxide. These titrations resulted in difference spectra and corresponding affinity constants for ligands which were similar to those constants observed for cytochrome P-450 contained in whole and Subtilisin-treated microsomes. Each protein peak revealed a single binding species for both cyanide and carbon monoxide.

Hofstee plots of the cyanide titrations within a single comparable experiment are shown in Fig. 5, A to C. A summary of the results from several experiments is presented in Table I. Affinities for cyanide binding to the oxidized forms and carbon monoxide binding to the reduced forms are given as the averages and standard deviations from a total of 10 and 4 separate experiments, respectively. A total range of binding constants is given for the somewhat more variable octylamine data. Titrations with carbon monoxide produced hyperbolic curves for Forms I and II. Consequently, the relative affinity constants could be determined only for these two forms. Octylamine titrations resulted in two affinity constants, which were routinely observed for Forms I and II; similar pairs of constants had been reported earlier for rabbit liver microsomal cytochrome P-450 (30). Form III yielded only a single affinity constant for octylamine.

The cyanide extinction coefficients for each of the separated forms of cytochrome P-450 were calculated from the extrapolated maximal changes in absorbance at 442 and 405 nm and the heme content, as measured by pyridine hemochrome assay. From four separate experiments, nine values for the extinction coefficients were obtained. The values for the three forms were similar, ranging from 69.6 to 82.6 mm⁻¹ cm⁻¹, with an average of 76.1 mm⁻¹ cm⁻¹, S.D. 4.0. A value of 75 mm⁻¹ cm⁻¹, which was obtained from a calculation that was based on the P-450 content, was determined from the carbon monoxide difference spectrum and the maximal change in absorbance at 442 and 405 nm. Thus, internally consistent data were obtained.

The combination of cyanide with Form I P-450 is apparently reversible. After titration with cyanide, the material was centrifuged at 105,000 × g for 2 hours; the pellet was suspended in fresh phosphate buffer, and the resulting suspension was titrated again with cyanide. There was a recovery of 72% of the heme protein and a 50% increase in specific content of Form I, which was due mostly to non-cytochrome-containing protein remaining in the supernatant fraction. No alterations in spectral characteristics were produced by pretreatment with cyanide.

Variability, when observed in these experiments, seemed to be related to salt concentrations (34), elution time from diethyl-
FIG. 5. Hofstee plots of the cyanide titrations of the separated forms of cytochrome P-450. Separated fractions from a Sigma diethylaminoethylcellulose column (as in Fig. 4B) were stabilized by desalting on Sephadex G-25 which had been equilibrated with 0.1 M potassium phosphate buffer, pH 7.4. The protein concentration in some of the samples was adjusted to about 1 mg per ml with the phosphate buffer. The titrations were performed over the range of 0.1 to 20 mM or to the concentration of cyanide that no longer caused increases in absorbance. A, Form I cytochrome P-450 eluted at 0.3 M potassium chloride; protein concentration was 1.0 mg per ml. B, Form II cytochrome P-450 eluted at 0.2 M potassium chloride; protein concentration was 0.6 mg per ml. C, Form III cytochrome P-450 eluted at 0.1 M potassium chloride; protein concentration was 1.2 mg per ml.

**Table I**

**Binding constants of chromatographed forms**

The separated, stabilized forms of cytochrome P-450 were titrated with cyanide, carbon monoxide, and octylamine, then affinity constants were determined. The results of the cyanide and carbon monoxide titrations are the means of at least 10 and 4 experiments, respectively, with their standard deviations. A range of binding constants is given from three titrations performed with octylamine as the ligand.

<table>
<thead>
<tr>
<th>Cytochrome P-450</th>
<th>Cyanide K</th>
<th>Carbon monoxide K</th>
<th>Octylamine K</th>
<th>λ_{max}</th>
<th>λ_{min}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Form I</td>
<td>0.46 ± 0.13</td>
<td>1.25 ± 0.14</td>
<td>0.018-0.032</td>
<td>432</td>
<td>410</td>
</tr>
<tr>
<td>Form II</td>
<td>1.6 ± 0.3</td>
<td>0.74 ± 0.31</td>
<td>0.005-0.027</td>
<td>432</td>
<td>410</td>
</tr>
<tr>
<td>Form III</td>
<td>4.9 ± 2.1</td>
<td></td>
<td>0.29-0.32</td>
<td>427</td>
<td>392</td>
</tr>
</tbody>
</table>

The separated forms of cytochrome P-450 were titrated with cyanide, carbon monoxide, and octylamine, then affinity constants were determined. The results of the cyanide and carbon monoxide titrations are the means of at least 10 and 4 experiments, respectively, with their standard deviations. A range of binding constants is given from three titrations performed with octylamine as the ligand.

Electron Paramagnetic Resonance Spectra—Electron paramagnetic resonance spectra of oxidized cytochrome P-450 in microsomes has revealed a low spin state of iron (36). Accordingly, spectra of the three separated forms were investigated. The spectra for all three forms, measured at 20° K, were indicative of the low spin heme iron of cytochrome P-450 with g values of 2.43, 2.26, and 1.92. The spectra obtained at 8° K showed large high spin heme signals at g = 6 and similar signals at g = 8 and g = 3 for each of the three forms. Thus, the electron paramagnetic resonance spectra of the three forms were essentially identical, and the apparent ratios of the high spin to low spin signals were similar. These results were somewhat surprising in light of the known electron paramagnetic resonance spectra of microsomal cytochrome P-450 (36) and the octylamine titration data, which had suggested that Forms I and II were low spin hemoproteins at room temperature. However, they are strikingly similar to the results recently reported by Nebert and Kon (37) in a thorough study of low temperature electron paramagnetic resonance spectra of liver microsomes to which acetone or commonly used detergents had been added. The appearance of a large high spin signal near g = 6, not native to microsomes, with concomitant losses of signals at g = 8 and g = 2.27 was observed in all microsomal preparations containing such detergents as cholate, deoxycholate, Lubrol, Triton X-100, or sodium dodecyl sulfate, and in preparations that had been aged (8, 37). Deoxycholate produced the greatest effect. In this study, deoxycholate was not totally removed from each of the forms of cytochrome P-450 by chromatography on Sephadex G-25, and samples were kept frozen in liquid nitrogen several days prior to running the electron paramagnetic resonance spectra. The appearance of small high spin signals at g = 6 at liquid helium temperatures seems, in part, to be related to a temperature-dependent conversion of spin state since the signal tends to be less observable at temperatures above 10° K (37). A temperature-dependent conversion of spin state also has been observed recently in visible spectral measurements (38). The multiple contributing factors...
resulting in the present electron paramagnetic resonance observations are not readily analyzable, and future work obviously must await complete removal of detergent from the resolved forms of cytochrome P-450.

**Phospholipase A Treatment**—Several lines of evidence suggest that phospholipid is required for the maintenance of spectral integrity of liver microsomal cytochrome P-450. For example, Mason *et al.* (36) found that the cytochrome P-450 structure was functionally altered by treatment of microsomes with deoxycholate or a combination of lipase and cobra venom. Although additional phospholipid was required for reconstitution experiments (14), the cytochrome P-450 obtained chromatographically contained phospholipid. Recently, Chaplin and Mannering (39) have used phospholipase C to investigate the effects of phospholipid digestion on microsomal drug metabolism. Also, the three forms of cytochrome P-450 were observed to be labile when intact microsomes were incubated with phospholipase A (40). Therefore, the lability of the separated forms was studied.

The three forms of cytochrome P-450 were collected and desalted; particles obtained by centrifugation were suspended in 0.1 M Tris-HCl, pH 7.4, at 37°. After phospholipase A treatment, the samples exhibited no visible spectral absorbance, that is, neither cytochrome P-450 nor cytochrome P-420 was detected after treatment with phospholipase A under these conditions. Control samples (i.e., incubated without phospholipase A) retained cytochrome P-450, which combined with cyanide in the spectrophotometric titration. In Table II are the data for Form I cytochrome P-450. The percentage of increase over control was based on titrations of whole microsomes with cyanide.

**Table II: Effect of phospholipase A on Form I of cytochrome P-450**

The percentage of increase over control was based on titrations of whole microsomes with cyanide.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Form III</th>
<th>Form II</th>
<th>Form I</th>
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<tbody>
<tr>
<td>3-Methylcholanthrene</td>
<td>185</td>
<td>53</td>
<td>27</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>130</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Ethanol diet</td>
<td>-13</td>
<td>47</td>
<td>81</td>
</tr>
</tbody>
</table>

**Discussion**

In this report, three forms of cytochrome P-450 in adult rat liver microsomes have been identified by their differing affinities for cyanide. Prior to intensive study of these forms, methods had to be developed for accurate assay (29), stabilization of the isolated forms (41), and removal of contaminating cytochrome b₅ (this report). Mild proteolytic digestion of microsomal protein, with the use of Subtilisin VII, was found to be a reproducible method for depleting microsomes of cytochrome b₅ (Fig. 1) without affecting the cyanide-binding components. Chromatographic separation of the particles containing the three cyanide-binding components was then achieved on diethylaminoethylenemellose by utilizing methods principally developed by others (14, 15) and a stepwise elution procedure. Chromatographic reproducibility was finally exhibited when a single lot of ion exchange resin was used, and the worker is cautioned that the elution profiles may vary somewhat with different lots. The separated material showed a greater stability, as determined by spectral characteristics, after passage through Sephadex G-25 (41).

Preliminary examination of the three separated forms showed them to have differing affinities for octylamine and carbon monoxide, in addition to the more diagnostic cyanide difference spectra. Extinction coefficients determined for the carbon monoxide complexes ranged from 88 to 95 mM⁻¹ cm⁻¹, in agreement with those reported by Omura and Sato (26) and Lu *et al.* (16), but at variance with the value of 38 mM⁻¹ cm⁻¹ reported by Mannering (42) for a “soluble” cytochrome P-450 from phenobarbital-treated rats. These spectral similarities and other observations, e.g., that phospholipase A treatment removed phospholipid and destroyed spectral characteristics of the separated materials, are consistent with the view that a number of forms...
Liver microsomes from rats treated with the indicated drug were titrated with cyanide, along with respective controls. The drug-induced microsomes were then separated chromatographically, stabilized, and analyzed for Forms I, II, and III of cytochrome P-450. Each experiment was performed with three rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Form I</th>
<th>Form II</th>
<th>Form III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cyanide K</td>
<td>Specific content</td>
<td>Cyanide K</td>
</tr>
<tr>
<td>3-MC* microsomes</td>
<td>0.12</td>
<td>0.47</td>
<td>0.68</td>
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<tr>
<td>Control microsomes</td>
<td>0.24</td>
<td>0.37</td>
<td>0.53</td>
</tr>
<tr>
<td>3-MC* separated forms</td>
<td>0.39</td>
<td>0.37</td>
<td>0.72</td>
</tr>
<tr>
<td>PW* microsomes</td>
<td>0.4</td>
<td>1.06</td>
<td>2.1</td>
</tr>
<tr>
<td>Control microsomes</td>
<td>0.22</td>
<td>0.83</td>
<td>1.1</td>
</tr>
<tr>
<td>PB* separated forms</td>
<td>0.48</td>
<td>1.1</td>
<td>2.4</td>
</tr>
<tr>
<td>Ethyl alcohol diet microsomes</td>
<td>0.52</td>
<td>0.54</td>
<td>1.4</td>
</tr>
<tr>
<td>Control diet microsomes</td>
<td>0.53</td>
<td>0.293</td>
<td>1.5</td>
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<tr>
<td>Ethyl alcohol diet separated forms</td>
<td>0.50</td>
<td>0.53</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* 3-Methylcholanthrene.

Phenobarbital and 3-methylcholanthrene exhibit different specificities as inducers of microsomal enzymes (45). Chronic administration of ethyl alcohol to rats causes a proliferation of smooth endoplasmic reticulum and an increase in a TPNH-dependent ethanol-oxidizing system (46, 47). In the present induction experiments, there was a marked increase in Form III of cytochrome P-450 when adult rats were pretreated with 3-methylcholanthrene. Others have reported induction of cytochrome P-448 (P-450) by treatment with 3-methylcholanthrene and little or no change in cytochrome P-450 (10, 16, 48). On the other hand, Hildebrandt and Estabrook (49) calculated that the cytochrome P-448 content in liver from 3-methylcholanthrene-treated rabbits was only 35% of the total carbon monoxide-binding pigment. Because of the inability to purify cytochrome P-448 or P-448 at that time, it was almost impossible to determine cross-contamination in these various preparations. Form III of cytochrome P-450, inducible by 3-methylcholanthrene, may be identical with cytochrome P-448, but some question remains as to whether or not P-448 is actually present in microsomes from untreated animals. The spectral maximum of Form III reduced with CO was at 450 nm. Form I of cytochrome P-448 is preferentially induced by ethyl alcohol, a phenomenon which was first observed by Joly et al. (50). Form I of cytochrome P-450 may also be the cyanide-sensitive factor of mixed function oxidations of stearyl coenzyme A (51), which had been proposed in earlier communications (23, 41).

Clearly, additional work on the many aspects of separate forms of mammalian liver cytochrome P-450 is needed. However, these procedures for assay, stabilization, and separation of the three forms, which appear to retain their native spectral characteristics, may be helpful in carrying out studies of further resolution, enzyme properties, physical properties, effects of inducers, etc., on the various forms. Hopefully, methods will soon be developed for complete removal of detergent and for purification of each form to homogeneity to allow independent investigations of similarities of primary structure, as well as those factors that seem to impart distinguishing spectral characteristics to the various forms.
REFERENCES

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Existence and Separation of Three Forms of Cytochrome P-450 from Rat Liver Microsomes
Karen Comai and James L. Gaylor


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