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Cytochrome P-450$_{C\text{-}M/F}$, a New Constitutive Form of Microsomal Cytochrome P-450 in Male and Female Rat Liver with Estrogen 2- and 16α-Hydroxylase Activity†

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ABSTRACT: A new cytochrome P-450 isozyme, P-450$_{C\text{-}M/F}$, has been purified from untreated rat liver microsomes. The purified preparation was electrophoretically homogeneous and contained 12-15 nmol of P450/mg of protein and had a minimum molecular weight of 48 500. The NH_2-terminal amino acid sequence of P-450$_{C\text{-}M/F}$ was different from that of other P-450's. Immunoblot analysis of microsomes demonstrated that P-450$_{C\text{-}M/F}$ was present in the liver of untreated male as well as female rats. Treatment of rats with phenobarbital, 3-methylcholanthrene, or β-naphthoflavone did not induce P-450$_{C\text{-}M/F}$. Cytochrome P-450$_{C\text{-}M/F}$ exhibited little activities of 7-ethoxycoumarin and 7-ethoxyresorufin O-deethylation or hydroxylation of arylhydrocarbon, testosterone, androstenedione, and progesterone. In contrast, it was highly active in N-demethylation of ethylmorphine and benzphetamine and in 2- and 16α-hydroxylation of estrogens, particularly that of estradiol. These studies establish that cytochrome P-450$_{C\text{-}M/F}$ is constitutively present in both male and female rats and suggest that it may be involved in the oxidative metabolism of estradiol, particularly in the formation of estriol, the uterotropic metabolite of estradiol.

Cytochrome P-450 is a collective term for a group of microsomal hemeproteins that serve as the terminal oxidases in the mixed-function oxidase system (Cooper et al., 1965). This multicomponent system plays a vital role in the oxidation of a variety of foreign chemicals such as drugs (Conney, 1967; Lu et al., 1970), carcinogens (Thorgeirsson et al., 1973), and hydrocarbons (Orrenius & Ernster, 1974) as well as diverse endogenous substances such as fatty acids (Wada et al., 1968; Bjorkhem & Danielson, 1970), steroids (Kuntzman et al., 1964; Lu & Levin, 1974) and vitamin D (Hansson et al., 1981). A number of studies have been carried out to elucidate the multiplicity of this cytochrome species. These studies, most of which have been indirect in nature, include spectral characteristics, catalytic activity, substrate specificity, modulation of enzyme activity by specific inhibitors, and electrophoretic mobility. Comparisons have also been made between untreated control and experimental animals in sex, age, genetic background, species, maintenance conditions or treatment of animals with chemicals known to specifically induce or decrease cytochrome P-450 content in the liver [see review in Conney (1967)].

Recently, more definitive studies have been carried out by purifying cytochrome P-450 isozymes from microsomes and by studying their physicochemical properties; these have provided clear evidence for a multiplicity of this cytochrome species. Most of these studies, however, have been confined largely to the major forms of cytochrome P-450 that are induced in the liver of animals treated with xenobiotics such as phenobarbital (Guengerich et al., 1982) or 3-methylcholanthrene (Wood et al., 1983). To date, only a small number of cytochrome P-450 isozymes have been purified from livers of untreated animals (Schenkman et al., 1982; Kamataki et al., 1983; Ryan et al., 1984). These native forms of cytochrome P-450 are of great interest in view of the fact that they may be involved in the metabolism of endogenous substrates such as vitamin D (Hansson et al., 1981), testosterone (Ryan et al., 1982b; Cheng & Schenkman, 1983; Harada & Negishi, 1984), progesterone (Johnson et al., 1983; Cheng & Schenkman, 1984), estradiol (Johnson et al., 1983; Cheng & Schenkman, 1984), prostaglandins (Okita et al., 1981; Vatsis et al., 1982), fatty acids (Gibson et al., 1980; Tamburini et al., 1984), and cholesterol (Waxman, 1986). In this manner, such P-450 isozymes may play a significant role in regulation of the metabolism of a variety of natural compounds important to normal cellular metabolism. Some of these native P-450 isozymes are also known to be inducible by treatment of animals with xenobiotics.

We report in this paper a new cytochrome P-450 species isolated from untreated male and female rat liver that is not inducible by treatment of animals with phenobarbital, 3-methylcholanthrene, or β-naphthoflavone. Since this newly identified P-450 isozyme is present constitutively in hepatic microsomes of both sexes, we propose the term "cytochrome P-450$_{C\text{-}M/F}$" for this molecule.

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Materials and Methods

Animals

Male and female Sprague-Dawley rats, 8–11 weeks of age, were purchased from Charles River Breeding Laboratories (Wilmington, MA). Animals were either uninduced or induced with phenobarbital (Merck, Rahway, NJ) by three daily intraperitoneal injections in physiological saline at a dose of 80 mg/kg of body weight, 3-methylcholanthrene (Sigma Chemical Co., St. Louis, MO; three daily intraperitoneal injections in corn oil at a dose of 30 mg/kg of body weight), or β-naphthoflavone (Sigma; three daily intraperitoneal injections in corn oil at a dose of 80 mg/kg of body weight). Liver microsomes were prepared according to standard methods (van der Hoeven & Coon, 1974) except that the final wash was made with 0.1 M KP_i[1] (pH 7.4) containing 20% glycerol (v/v) and 1 mM EDTA.

Purification of Cytochrome P-450$_{C\text{-}M/F}$

P-450$_{C\text{-}M/F}$ was purified from uninduced male and/or female rat liver microsomes by cholate solubilization, poly(ethylene glycol) (PEG) precipitation, (diethylamino)ethylcellulose (DEAE) anion-exchange chromatography, and hydroxylapatite chromatography (Table I).

Cholate and PEG Precipitation. Liver microsomes (3.0 g of protein), prepared from 30–40 untreated rats that had been starved for 16 h prior to sacrifice, were solubilized on ice in 300 mL of 100 mM KP_i (pH 7.4), 30% glycerol (v/v), 3% sodium cholate (w/v), 1 mM EDTA, and 1 mM dithiothreitol (DTT). A 50% solution (w/v) of poly(ethylene glycol) 8000 (J. T. Baker, Phillipsburg, NJ) was added to give a final concentration of 10% (w/v). After stirring for 30 min, the mixture was centrifuged at 37000*g* for 45 min. The PEG solution was added to the above supernatant to give a final concentration of 16% (w/v), and the mixture was stirred for 30 min at 4 °C. The mixture was centrifuged at 105000*g* for 1 h. Pellets ("10–16% PEG fraction") were collected that contained approximately 50% of the microsomal cytochrome P-450 content.

DEAE Anion-Exchange Chromatography. The 10–16% PEG fraction was dissolved in 50 mL of buffer A [10 mM KP_i (pH 7.4), 20% glycerol (v/v), 0.1% sodium cholate (w/v), 1 mM EDTA, 1 mM DTT, and 0.2% Emulgen 911 (w/v) (Kao-Atlas, Tokyo)] and applied onto a column of DE52 (Whatman, Clifton, NJ) (3.2 × 50 cm). The column was washed with 1 column volume of buffer A. Cytochrome P-450 was eluted from the column by using a linear gradient from 0 to 0.3 M NaCl in buffer A. At first, the protein concentration, total cytochrome P-450 content, and electrophoretic profiles of P-450 were determined in fractions eluted from the column, and the fractions enriched for a molecular weight corresponding to 48 500 were then collected. This molecular species eluted as the third peak after the two major cytochrome peaks (Figure 1). In more recent purification sequences, the location of P-450$_{C\text{-}M/F}$ was also identified by using a specific antiserum raised against the purified cytochrome.

Hydroxylapatite Chromatography. The pooled fraction was dialyzed against 10 mM KP_i (pH 7.4), 20% glycerol (v/v), 1 mM DTT, and 0.2% Emulgen 911 (w/v) (buffer B) for 16 h at 4 °C. The pH of the dialyzed fraction was adjusted to 6.8 by the addition of 5% acetic acid (v/v) and then applied to a hydroxylapatite column (2.5 × 6 cm) equilibrated with buffer C (the same as buffer B except that the pH of buffer C was 6.8) at room temperature. After the column was washed with 1 column volume of buffer C, cytochrome P-450 was eluted in a stepwise fashion by using 10, 30, and 100 mM KP_i (pH 7.4) containing 20% glycerol (v/v), 0.2% Emulgen (w/v), and 1 mM DTT. Elution of cytochrome P-450 was examined by protein concentration, total P-450 content, electrophoretic profile, and antisera against the cytochrome. P-450$_{C\text{-}M/F}$ was eluted by 30 mM KP_i.

Second Hydroxylapatite Chromatography. The fractions enriched in P-450$_{C\text{-}M/F}$ were pooled and diluted 3-fold with 20% glycerol (v/v) containing 1 mM DTT. After the pH of the solution was adjusted to 6.5 with 5% acetic acid (v/v), the P-450$_{C\text{-}M/F}$ solution was applied to a hydroxylapatite column (1.6 × 6 cm) that was equilibrated with buffer D at 4 °C (the same as buffer B except the pH of buffer D was 6.5). After the column was washed with 1 column volume of 10 mM KP_i (pH 7.2) containing 20% glycerol (v/v), 0.1 mM DTT, and 0.2% Emulgen (w/v), Emulgen 911 was removed from the column with 10 mM KP_i (pH 7.2) containing 20% glycerol (v/v). Cytochrome P-450 was eluted from the column with 0.5 M KP_i (pH 7.4) containing 20% glycerol (v/v). Eluates were dialyzed against 0.1 M KP_i (pH 7.4) containing 20% glycerol (v/v) and stored in liquid nitrogen.

Purification of Other Cytochrome P-450's and Cytochrome P-450 Reductase

P-450$_b$ (Haniu et al., 1984) was purified from liver microsomes of phenobarbital-treated rats according to the procedure of Waxman et al. (1985), who described this P-450 as P-450$_{PB4}$. P-450$_{BNF\text{-}B}$ (Guengerich et al., 1982), also termed P-450$_c$ (Ryan et al., 1982b), was purified from liver microsomes of β-naphthoflavone-treated rats by similarly using the procedure of Guengerich et al. (1982). P-450$_h$ (Ryan et al., 1984) and P-450$_i$ (Haniu et al., 1984) were purified according to the published method of Kamataki et al. (1983). All of these P-450 preparations were homogeneous on SDS–polyacrylamide gel electrophoresis and carried out characteristic hydroxylation reactions. Moreover, specific antibodies prepared against each P-450 recognized its own antigen but not other P-450's. NADPH–cytochrome P-450 reductase was purified from the liver of rats treated with phenobarbital (80 mg/kg of body weight, three daily subcutaneous injections) according to the method of Yasukochi and Masters (1976) to a specific activity of 40.2 μmol of cytochrome *c* reduction min^{-1} (mg of protein)$^{-1}$.

Preparation of Antibodies to P-450$_{C\text{-}M/F}$

Twenty micrograms of the homogeneously purified P-450 was mixed with Freund's complete adjuvant and injected subcutaneously into the backs of three Rhode Island Red hens. The same amount of the antigen with Freund's incomplete adjuvant was injected 2 weeks after the first immunization. At first, a specific immune titer against P-450$_{C\text{-}M/F}$ was examined in sera obtained from hen. After there was a significant increase in the titer, eggs were collected and stored at 4 °C until use. An IgY fraction was isolated by using PEG cuts as determined previously (Polson et al., 1980). Antibodies against P-450$_b$, P-450$_c$, P-450$_h$, and P-450$_i$ were prepared in rabbits. The anti-P-450$_h$ and the anti-P-450$_i$ IgG fractions

[1] Abbreviations: AD, androst-4-ene-3,17-dione; CM, carboxymethyl; DEAE, diethylaminoethyl; DTT, dithiothreitol; E_1, 3-hydroxyestra-1,3,5(10)-trien-17-one (estrone); E_2, 3,17β-dihydroxyestra-1,3,5(10)-triene (estradiol); E_3, 3,16α,17β-trihydroxyestra-1,3,5(10)-triene (estriol); E_4, 3,15α,16α,17β-tetrahydroxyestra-1,3,5(10)-triene (estetrol); EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Ig, immunoglobulin; KP_i, potassium phosphate buffer; P, pregn-4-ene-3,20-dione (progesterone); PEG, poly(ethylene glycol); SDS, sodium dodecyl sulfate; T, 17β-hydroxyandrost-4-en-3-one (testosterone); TLC, thin-layer chromatography.

were treated with microsomes of livers from female and male animals, respectively, to remove possible contaminants as described previously (Kamataki et al., 1983).

Chemicals

Methyl cholate, dilauroyl-L-3-phosphatidylcholine, benzo[*a*]pyrene, 3-methylcholanthrene, β-naphthoflavone, and 16α-HOAD were obtained from Sigma. Phenobarbital and ethylmorphine were obtained from Merck. Benzphetamine was obtained from Upjohn Co. (Kalamazoo, MI), 7-ethoxycoumarin from Aldrich Chemical Co. (Milwaukee, WI), NADPH from Boehringer Mannheim GmbH, and *N*-(2-hydroxyethyl)piperadine-*N*′-2-ethanesulfonic acid (HEPES) from GIBCO (Grand Island, NY). DE52 and CM52 were obtained from Whatman (Clifton, NJ) and DEAE-Sephadex A-50 and 2′,5′-ADP Sepharose 4B, from Pharmacia (Piscataway, NJ). Hydroxylapatite (Bio-Rad HTP; Bio-Rad, Richmond, CA) was washed prior to use according to the specification provided by the manufacturer. Horseradish peroxidase coupled rabbit IgG vs chicken IgY was obtained from Miles (Naperville, IL). 7-Ethoxyresorufin was a generous gift from Dr. Arleen B. Rifkind. Cholic acid methyl ester was purified by silica gel chromatography using a mixture of chloroform/ethyl acetate (1:10 v/v) as an eluting solvent and subsequently recrystallized from ethyl acetate/methanol. Cholic acid was prepared by hydrolysis of the methyl ester. Sodium cholate was crystallized from an ethanolic solution of cholic acid by using a concentrated sodium hydroxide solution.

[2-^{3}H]E_2 (specific activity (SA) 22.1 Ci/mmol), [4-^{14}C]E_2 (SA 56.4 mCi/mmol), [4-^{14}C]E_1 (SA 56.4 mCi/mmol), and [4-^{14}C]AD (SA 52.0 mCi/mmol) were obtained from New England Nuclear (Boston, MA). [7-^{3}H]Cholesterol (SA 9.6 Ci/mmol), [4-^{14}C]T (SA 56.9 mCi/mmol), and [4-^{14}C]P (SA 56 mCi/mmol) were obtained from Amersham (Arlington Heights, IL). [16α-^{3}H]E_2 and [19-$^3H_3$]AD were prepared by a method described previously (Fishman et al., 1980; Miyairi & Fishman, 1985). 15α-HOE_1 and 6β-HOE_2 were gifts of Dr. M. Levitz of New York University School of Medicine, and 15α-HOE_2 was prepared from 15α-HOE_1 by reduction with $NaBH_4$. 6α-HOE_1 was a gift of Dr. T. Nambara of Tohoku University, Japan. P, AD, T, E_2, E_1, E_3, 6-oxoE_2, 6α-HOE_2, 2-HOE_3, 15α-HOE_3 (E_4), 6β-HOP, 16α-HOP, 6β-HOAD, 6-oxoAD, 6β-HOT, and 7α-HOT were obtained from Steraloids, Inc. (Wilton, NH). 2α-HOP, 2α-HOAD, 2α-HOT, 16α-HOT, 2-HOE_1, 4-HOE_1, 16α-HOE_1, 2-HOE_2, 4-HOE_2, and 6-oxoE_1 were synthesized according to conventional methods in our laboratory.

Enzyme Assays

By use of a reconstituted system (Coon, 1978), the following P-450-dependent enzyme activities were assayed: ethylmorphine *N*-demethylase (Nash, 1953); benzphetamine *N*-demethylase (Nash, 1953); 7-ethoxycoumarin deethylase (Waxman et al., 1982); 7-ethoxyresorufin deethylase (Rifkind et al., 1984); arylhydrocarbon hydroxylase (Nebert & Bausserman, 1970); AD 19-hydroxylase; E_2 2- and 16α-hydroxylases; and cholesterol 7α-hydroxylase. The system consisted of, in a final volume of 500 μL, 0.1 nmol of purified cytochrome P-$450_{C\text{-}M/F}$, 0.5 unit of NADPH–P450 reductase, 30 μg of dilauroyl-L-3-phosphatidylcholine, 25 μmol of HEPES (pH 7.4), 0.4 μmol of NADPH, and the substrate. Each mixture was first incubated without NADPH at 37 °C for 2 min, and the reaction was then initiated by addition of NADPH.

Radiometric analysis of the hydroxylations of steroids was performed by using site-specific ^{3}H-labeled substrates. After incubation, the reaction mixture was mixed with 500 μL of chilled 0.01 N hydrochloric acid containing 1% charcoal (w/v). After standing on ice for 20 min, the mixture was centrifuged at 2000 rpm for 10 min. The supernatant was then transferred to another tube and lyophilized. The amount of tritium recovered in an aliquot (500 μL) of the sublimed fraction was quantitated by using a liquid scintillation counter.

The qualitative identification of metabolites of P, AD, T, E_1, and E_2 was carried out by use of the reconstituted cytochrome P-450 system in a final volume of 2 mL with 4.44 nmol of [4-^{14}C]steroid as substrate. After 7 min of incubation, the reaction was terminated by the addition of 0.5 mL of 2% ascorbic acid solution (w/v) in chilled water, and products were extracted into 3 mL of ethyl acetate twice. The organic layer was dried over anhydrous sodium sulfate and concentrated under a nitrogen stream. The concentrated extract was examined by TLC with authentic hydroxylated steroid metabolites as standards. After chromatography, the TLC plate was exposed to a sheet of LKB Ultrofilm for 48 h, and the film was then developed. The nature of positive spots was determined by comparing their R_f's to those of the authentic standards. Plate areas corresponding to positive spots were scraped off and counted by using a Tricarb liquid scintillation counter.

N-Terminal Amino Acid Determination

Automatic Edman degradation (Hunkapiller et al., 1983) was performed on the purified P-$450_{C\text{-}M/F}$ by using a gas-phase sequencer (Model 470A, Applied Biosystems, Foster City, CA). PTH–amino acid derivatives were identified by an on-line HPLC system (Model 120A, Applied Biosystems).

Other Analytical Methods

SDS–polyacrylamide gel electrophoresis was performed in 10% acrylamide gel by using the procedure described by Maizels (1971). The proteins used for molecular weight standards were those in a prestained, high molecular weight range kit from Bethesda Research Laboratory (Gaithersburg, MD); these proteins were myosin (200 000), phosphorylase B (94 400), bovine serum albumin (68 000), ovoalbumin (43 000), α-chymotrypsinogen (25 700), and β-lactoglobulin (18 400). The total cytochrome P-450 content was determined according to the method of Omura and Sato (1964). Protein concentration was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as the standard protein with an appropriate buffer blank for each step of purification. Ouchterlony double immunodiffusion analysis was performed by using plates obtained from Cooperbiomedical (Malvern, PA). Five to seven microliters of antigens or antibodies was added to each well in the plate. Western blot analysis of cytochrome P-450 was performed according to the procedure of Guengerich et al. (1982b), using horseradish peroxidase coupled rabbit IgG against IgY as a detector enzyme. When microsomes were the source of antigen, 5 mg/mL of cholate-solubilized microsomes were used.

RESULTS

Purification of Cytochrome P-$450_{C\text{-}M/F}$. The elution profiles of total cytochrome P-450 content and localization of the immunoreactive material with an antiserum prepared against P-$450_{C\text{-}M/F}$ from DE52 chromatography are shown in Figure 1. A representative purification of P-$450_{C\text{-}M/F}$ from 30–40 male rats is also summarized in Table I. P-$450_{C\text{-}M/F}$ was eluted from the DE52 column, following two major peaks, as the third peak at NaCl concentrations from 0.15 to 0.22 M. Elution profiles were also similar for P-$450_{C\text{-}M/F}$ from liver

Table I: Purification of Cytochrome P-450$_{C-M/F}$ from Rat Liver Microsomes[a]

fraction	cytochrome P-450 (nmol)	protein (mg)	specific content of P-450 (nmol/mg)	yield (%)	purification (x-fold)
microsomes	2755	3060	0.90	100	1
cholate solubilized	2617	2810	0.93	95.3	1.03
DE52 cellulose	432.5	59.2	7.30	15.7	8.1
first hydroxylapatite	151.4	12.5	12.1	5.4	13.4
second hydroxylapatite	125.6	9.3	13.5	4.6	15.0

[a] Cytochrome P-450$_{C-M/F}$ was purified from untreated male rat livers as described under Materials and Methods.

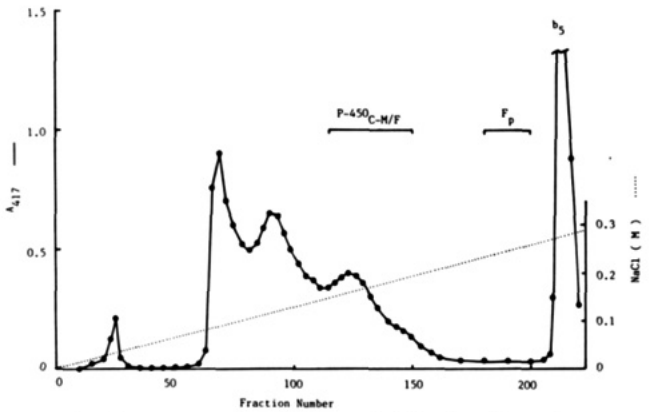

FIGURE 1: Chromatography of cholate-solubilized rat liver microsomes on a DE52 cellulose column. Approximately 3 g of solubilized microsomal protein from 30–40 male rats was applied to a DE52 cellulose column according to the method described under Materials and Methods. The linear gradient from 0 to 300 mM NaCl in 10 mM potassium phosphate buffer (pH 7.4) started at fraction 1 and ended at fraction 220. Each fraction contained 3.5 mL of eluate. Fractions from 115 to 150 contained P-450$_{C-M/F}$ as judged from the presence of immunoreactive material with an antiserum to the cytochrome.

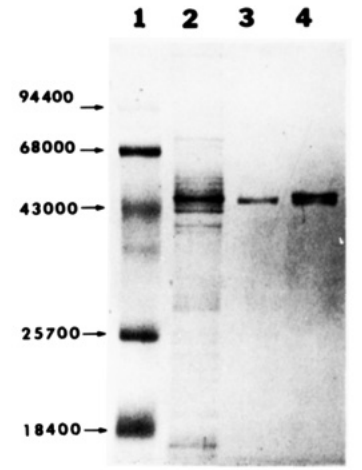

FIGURE 2: SDS–polyacrylamide gel electrophoresis of the purified cytochrome P-450$_{C-M/F}$. After electrophoresis, the 10% acrylamide gel was stained by Coomassie Brilliant Blue R-250 and destained by methanol/acetic acid. Electrophoresis was from the top to the bottom of the picture. (1) Protein molecular weight standards; (2) 15 μg of microsomes from livers of untreated male rats; (3) 1 μg of the purified P-450$_{C-M/F}$; (4) 1 μg of the purified P-450$_{C-M/F}$ and 2 μg of P-450$_b$. M_r of P-450$_{C-M/F}$ and P-450$_b$ corresponded to 48 500 and 52 000, respectively.

microsomes of untreated female rats and rats treated with 3-methylcholanthrene.

P-450$_{C-M/F}$ was eluted from hydroxylapatite at a very low ionic strength, i.e., 10–30 mM KP_i. This property is rather unusual for cytochrome P-450's, which are generally known to bind to hydroxylapatite quite well. The overall recovery of P-450$_{C-M/F}$ was 4.6%. On the basis of the enrichment of its specific activity, it was calculated that P-450$_{C-M/F}$ accounts for about 10–20% of the total cytochrome P-450 content in untreated male rat liver microsomes. P-450$_{C-M/F}$, purified from untreated male rat liver microsomes, exhibited a M_r of 48 500 and a mobility slightly faster than that of P-450$_b$ (M_r 52 000) when analyzed on SDS gel (Figure 2). The purified P-450$_{C-M/F}$ also gave rise to a single spot on two-dimensional SDS gel (data not shown). The purified cytochrome P-450$_{C-M/F}$ had a specific content of 12–15 nmol of heme protein/mg of protein (Table I) when an extinction coefficient of 91 mM^{-1} cm^{-1} for the reduced CO adduct was used (Omura & Sato, 1964) and with the Lowry protein assay (Lowry et al., 1951).

Spectral Studies. The absolute spectrum of the ferric form of P-450$_{C-M/F}$ determined at room temperature had an absorbance maximum at 417 nm and two minor peaks at 529 and 570 nm. The P-450$_{C-M/F}$ was predominantly in the low-spin state, from the absence of a shoulder at ~400 nm, but the existence of a minor peak at ~647 nm suggested that a small fraction of it was of a high-spin nature. The Soret peak of the ferrous form was shifted to 413 nm, and the absorbance was considerably lower. The carbon monoxide adduct of reduced P-450$_{C-M/F}$ had a peak at 449 nm with a virtual absence of cytochrome P-450.

Immunological Specificities. The chicken antibodies raised against the purified P-450$_{C-M/F}$ formed only one precipitin line in Ouchterlony double immunodiffusion analysis with purified

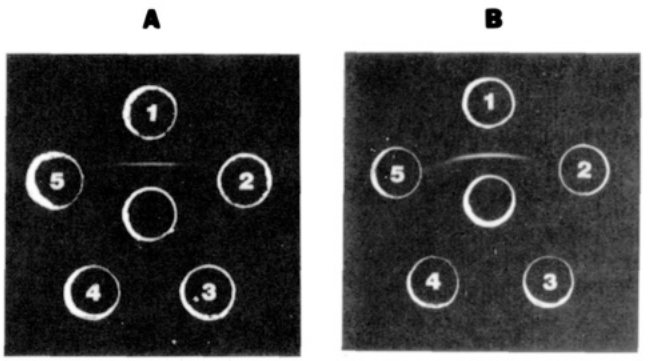

FIGURE 3: Ouchterlony double immunodiffusion analysis of cytochrome P-450$_{C-M/F}$. The Ouchterlony plates were incubated at room temperature overnight. On plate A, the center well contained anti-P-450$_{C-M/F}$; well 1, P-450$_{C-M/F}$; well 2, P-450$_c$; well 3, P-450$_b$; well 4, P-450$_i$; well 5, P-450$_h$. On plate B, the center well contained cytochrome P-450$_{C-M/F}$; well 1, anti-P-450$_{C-M/F}$; well 2, anti-P-450$_c$; well 3, anti-P-450$_b$; well 4, anti-P-450$_i$; well 5, anti-P-450$_h$. Two micrograms of purified P-450 isozymes was used in each well.

P-450$_{C-M/F}$ but did not form any precipitin line with either P-450$_b$, P-450$_h$, or P-450$_i$ (Figure 3A). Antibodies prepared against P-450b, P-450$_c$, P-450$_h$, and P-450$_i$ similarly did not cross-react with P-450$_{C-M/F}$ (Figure 3B). The antibody against P-450$_{C-M/F}$ also formed a single precipitin line with detergent-solubilized microsomes from untreated males and females, phenobarbital-treated males, and 3-methylcholanthrene-treated males and females as well as β-naphthoflavone-treated male rats (data not shown).

Western Blot Analysis. Liver microsomes from untreated male, female, phenobarbital-treated male, 3-methyl-

Table II: Oxidation Metabolism of Xenobiotics Catalyzed by Cytochrome P-450$_{C-M/F}$, P-450$_b$, and P-450$_c$

	P-450$_{C-M/F}$ [nmol of product formed min^{-1} (nmol of P-450)$^{-1}$]	P-450$_b$ [nmol of product formed min^{-1} (nmol of P-450)$^{-1}$]	P-450$_c$ [nmol of product formed min^{-1} (nmol of P-450)$^{-1}$]
ethylmorphine N-demethylation	19.0	19.0	12.7
benzphetamine N-demethylation	84.7	143.2	64.5
7-ethoxycoumarin deethylation	0.02	1.02	5.30
7-ethoxyresorufin deethylation	0.03	0.18	120.4
arylhydrocarbon hydroxylase	0.01	0.67	10.86

[a] Purification of P-450$_{C-M/F}$, P-450$_b$, and P-450$_c$ and reconstitution assays were carried out as described under Materials and Methods. Data are the mean of triplicate determinations.

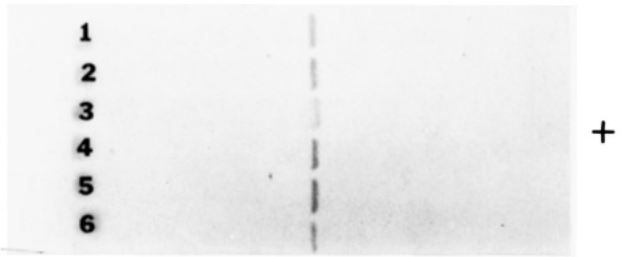

FIGURE 4: Immunostained Western blots of liver microsomes isolated from rats. Microsomes were isolated from rat livers according to the standard procedure. Each lane contained 5 μg of microsomal protein. (Lane 1) Untreated male; (lane 2) untreated female; (lane 3) phenobarbital-treated male; (lane 4) 3-methylcholanthrene-treated male; (lane 5) 3-methylcholanthrene-treated female; (lane 6) β-naphthoflavone-treated male.

cholanthrene-treated male and female, and β-naphthoflavone-treated male rats were resolved in SDS–polyacrylamide gels, electrophoretically transferred to nitrocellulose (Western blots), and probed for antibody reactivity with the specific antibody prepared against purified P-450$_{C-M/F}$ (Figure 4). The location of the antibody bound to the antigen was revealed with a secondary antibody conjugated to horseradish peroxidase, and the peroxidase activity was detected by the oxidation of 4-chloro-1-naphthol. The staining pattern of each blot in Figure 4 demonstrates the existence of only one molecular species corresponding to the molecular weight of P-450$_{C-M/F}$. The intensity of the peroxidase reaction was comparable for microsomes from untreated male and female and β-naphthoflavone-treated male rats, but it was slightly less for microsomes from phenobarbital-treated male rats. Microsomes from 3-methylcholanthrene-treated male and female rats appeared visually to contain an increased amount of P-450$_{C-M/F}$, but densitometry did not in fact confirm such an increase.

Rates of Metabolism of Prototype Substrates. The catalytic activity of P-450$_{C-M/F}$ toward five prototype substrates was compared to those of P-450$_b$ and P-450$_c$ (Table II). The specific activity of P-450$_{C-M/F}$ for ethylmorphine *N*-demethylase was higher than that of P-450$_c$ and comparable to that of P-450$_b$. Benzphetamine was metabolized at a rate intermediate between that of P-450$_b$ and P-450$_c$. Activities of 7-ethoxycoumarin deethylase, 7-ethoxyresorufin deethylase, and arylhydrocarbon hydroxylase were markedly lower in P-450$_{C-M/F}$ as compared with those of P-450$_b$ and P-450$_c$.

Steroid Hydroxylations. The regio- and stereospecificity of purified P-450$_{C-M/F}$ for hydroxylation of endogenous steroid substrates, e.g., P, T, AD, and E_2, as well as for the 7α-hydroxylation of cholesterol were examined by using radiometric analysis and/or autoradiographic methods. No activity was found for 7α-hydroxylation of cholesterol or 19-hydroxylation of AD by a radiometric analysis method that employed [7-^{3}H]cholesterol and [19-$^3H_3$]AD as substrates (data not shown). The autoradiographic analysis of products from the [^{14}C]steroid substrates P, AD, and T showed a few metabolites from each (Figure 5). 16α-HOP, 6β-HOP,

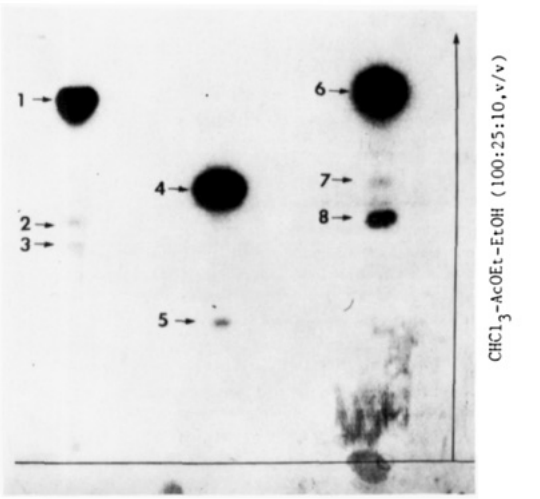

FIGURE 5: Autoradiograph of a thin-layer chromatogram of metabolites formed from androstenedione, testosterone, and progesterone by the purified cytochrome P-450$_{C-M/F}$. Reconstituted hydroxylation assays using the purified P-450$_{C-M/F}$ and autoradiography of the TLC plate were performed as described under Materials and Methods. TLC was performed with a mixture of chloroform–ethyl acetate–ethanol (100:25:10 v/v) in an ascending fashion. (Spot 1) AD; (spot 2) 6β-HOAD; (spot 3) unknown; (spot 4) T; (spot 5) 16α-HOT; (spot 6) P; (spot 7) 6β-HOP; (spot 8) 16α-HOP.

16α-HOT, and 6β-HOAD were identified by comparison with the migration of authentic steroid standards, and the amounts of products after extraction constituted 1.2%, 0.2%, 0.5%, and 0.4% of the original steroid substrate, respectively. A metabolite of AD, representing 0.3% of the recovered substrate, migrating just after the 6β-hydroxylated standard on the TLC plate, was not identified. 2-Hydroxylated metabolites from these steroids were undetectable or insignificant (<0.2%).

In contrast, significant amounts of E_2 were metabolized by P-450$_{C-M/F}$ as determined by the release of ^{3}H from specific positions in E_2 that were quantitated as [^{3}H]H_2O. By use of autoradiographic analysis, metabolites of [^{14}C]E_1 and [^{14}C]E_2 produced by P-450$_{C-M/F}$ were identified. P-450$_{C-M/F}$ metabolized E_2 to 2-HOE$_2$ (3.3% of the recovered substrate), E_3 (4.5%), and E_1 (0.2%) and 2-HOE$_1$ (0.1%), 6-oxoE$_2$ (0.4%), 6α-HOE$_2$ (0.4%), 6β-HOE$_2$ (0.2%), 15α-HOE$_2$ (0.4%), and 2-HOE$_3$ (0.2%) (Figure 6A) and E_1 to 2-HOE$_1$ (2.1%), 16α-HOE$_1$ (2.8%), 6α-HOE$_1$ (0.2%), and 15α-HOE$_1$ (1.0%) (Figure 6B). There was no significant radioactivity in the areas corresponding to authentic 4-HOE$_2$ or 4-HOE$_1$. These results correspond to those of authentic 4-HOE$_2$ or 4-HOE$_1$. These results were consistent with the radiometric analyses of the E_2 2- and 16α-hydroxylations.

Kinetic Analysis of E_2 Hydroxylation. By use of radiometric analysis that employed E_2 labeled with tritium at the specific position, kinetic analysis of the two major E_2 hydroxylations, i.e., 2- and 16α-, were examined. The Lineweaver–Burk plot of the E_2 hydroxylations revealed an apparent K_m of 8 μM and a V_{max} of 0.88 nmol (nmol of P-450)$^{-1}$

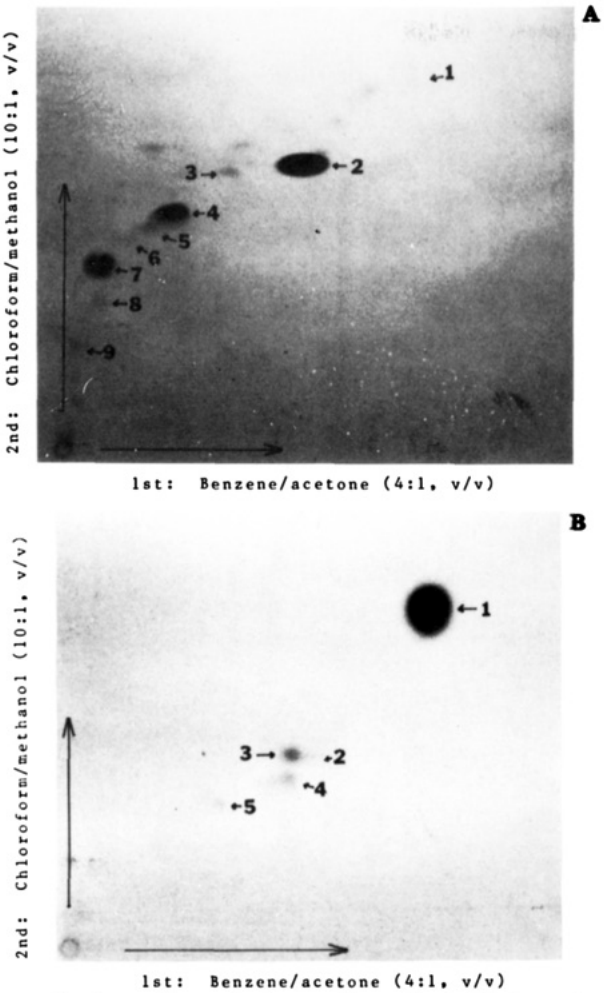

FIGURE 6: Autoradiograph of a two-dimensional thin-layer chromatogram of estrogen metabolites formed by the purified P-450$_{C\text{-}M/F}$. A reconstituted hydroxylation assay and autoradiography of the TLC plate were performed as described under Materials and Methods. Chromatography was developed with benzene–acetone (4:1 v/v) in the primary dimension and with chloroform–methanol (10:1 v/v) in the secondary dimension. (A) E_2 as substrate. (Spot 1) E_1; (spot 2) E_2; (spot 3) 6-oxoE_2; (spot 4) 2-HOE_2; (spot 5) 6α-HOE_2; (spot 6) 6β-HOE_2; (spot 7) E_3; (spot 8) 15α-HOE_2; (spot 9) 2-HOE_3. (B) E_1 as substrate. (Spot 1) E_1; (spot 2) E_2; (spot 3) 16α-HOE_1; (spot 4) 2-HOE_1; (spot 5) 15α-HOE_1.

min^{-1} for the 2-hydroxylation and an apparent K_m of 48 μM and a V_{max} of 0.34 nmol (nmol of P-450)$^{-1}$ min^{-1} for the 16α-hydroxylation.

NH_2-Terminal Amino Acid Sequence Analysis. The further distinction of P-450$_{C\text{-}M/F}$ as a new species of P-450 was provided by amino acid sequencing. Comparison of the first 20 residues of P-450$_{C\text{-}M/F}$ with those of the 14 P-450's for which NH_2-terminal amino acid sequence data have been published revealed that there was no significant homology between P-450$_{C\text{-}M/F}$ and the other P-450's (Table III). For example, no other P-450's had a Met-Glu-Leu sequence at the N-terminus. P-450$_{C\text{-}M/F}$ purified from 3-methylcholanthrene-treated male rats showed an amino acid sequence identical with that of P-450$_{C\text{-}M/F}$ purified from untreated male animals.

Discussion

The present paper describes the purification of a new form of cytochrome P-450 that we have termed P-450$_{C\text{-}M/F}$ because it is constitutively present in the liver of both untreated male and female rats. The purification of this cytochrome was accomplished by a combination of PEG cuts, DE52 chromatography, and hydroxylapatite chromatography. Purified P-450$_{C\text{-}M/F}$ had a M_r of 48 500, and its ferric form showed essentially a typical low-spin type spectrum having a major Soret peak at 417 nm with two minor peaks occurring at 530 and 570 nm; however, the presence of a minor peak at ~647 nm suggested a small amount of a high-spin form. P-450$_{C\text{-}M/F}$ was unusual in that it bound relatively weakly to hydroxylapatite even at a low ionic strength, while most other P-450's are known to bind to hydroxylapatite well.

A specific antibody was generated in chicken eggs by injecting a homogeneously purified P-450$_{C\text{-}M/F}$ preparation into two hens. The response to antigenic stimuli is highly developed in the fowl on both the cellular and humoral levels, and similar to the colostra of mammals, yolks of birds' eggs provide a supply of antibodies to the newborn (Polson et al., 1980). The antibody generated in eggs can be isolated from the egg yolk by PEG cuts with a greater purity than IgG isolated from sera of mammals (Polson et al., 1980). The IgY prepared from hens that had been immunized with P-450$_{C\text{-}M/F}$ was shown to be monospecific on the basis of the lack of its cross-reactivity with P-450$_b$, P-450$_c$, P-450$_h$, and P-450$_i$. Similarly, antibodies prepared against P-450$_b$, P-450$_c$, P-450$_h$, and P-450$_i$ did not cross-react with P-450$_{C\text{-}M/F}$. By use of an immunoblotting technique, it was found that P-450$_{C\text{-}M/F}$ was present in the liver of both male and female rats in significant quantities (10–20% of total microsomal P-450 on the basis of total P-450 quantitated by the CO difference spectrum and for P-450$_{C\text{-}M/F}$ by immunoblots), and it was not inducible by phenobarbital, 3-methylcholanthrene, or β-naphthoflavone treatment of animals. Thus, the term "P-450$_{C\text{-}M/F}$" has tentatively been proposed for this molecule, since it is a P-450 isozyme constitutively present in the liver of both untreated male and female animals. Cytochrome P-450$_{C\text{-}M/F}$ was also demonstrated in the male kidney using an immunohistochemical technique (data not shown).

Utilizing P-450$_{C\text{-}M/F}$ in a reconstituted system, it was possible to demonstrate a high substrate specificity and turnover rates for ethylmorphine and benzphetamine N-demethylation. The specific activity of P-450$_{C\text{-}M/F}$ for ethylmorphine N-demethylation was as high as that of P-450$_b$, which in turn was 50% higher than that of P-450$_c$. Benzphetamine N-demethylation by P-450$_{C\text{-}M/F}$ was ~40% less than that of that of P-450$_b$ but was ~30% higher than that of P-450$_c$. The substrate specificity of P-450$_{C\text{-}M/F}$ for 7-ethoxycoumarin, 7-ethoxyresorufin, and benzo[*a*]pyrene was in sharp contrast to that of P-450$_c$ in that P-450$_{C\text{-}M/F}$ exhibited only 0.4, 0.02, and 0.1%, respectively, of the activity of P-450$_c$. P-450$_b$ had also substantially weaker activity than P-450$_c$ toward these substrates, which is consistent with earlier findings of others (Ryan et al., 1982b), but they were much higher than the activity associated with P-450$_{C\text{-}M/F}$.

P-450$_{male\text{-}specific}$ (Kamataki et al., 1983; Haniu et al., 1984; Waxman, 1984), P-450$_{female\text{-}specific}$ (Kamataki et al., 1983; Waxman et al., 1985), and RLM5 (Cheng & Schenkman, 1982) are known to exist in untreated rat liver. They also perform several steroid hydroxylation reactions including 2- and 16α-hydroxylations of T, AD, and P (Kamataki et al., 1983; Cheng & Schenkman, 1983; Jansson et al., 1985). Not only the oxidative metabolism of androgens but also the 2- and 4-hydroxylations of E_2 have been described (Dannan et al., 1986). Thus, we examined the ability of P-450$_{C\text{-}M/F}$ to oxidatively metabolize steroid substrates. Our data show that P-450$_{C\text{-}M/F}$ exhibits little activity toward T, AD, P, and cholesterol, while it exhibited significant activity with respect to the 2- and 16α-hydroxylations of E_1 and E_2. The significant

Table III: Amino-Terminal Sequence Analysis of Cytochrome P-450$_{C\text{-}M/F}$[a]

cytochrome	position 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
P-450$_{C\text{-}M/F}$	Met	Glu	Leu	—	Ile	Asn	Asp	Thr	Leu	Trp	Ala	Val	Met	Ile	Phe	Thr	Ala	Ile	Phe	Leu
P-450$_a$	Met	Leu	Asp	Thr	Gly	Leu	Leu	Leu	Val	Val	Ile	Leu	Ala	Ser	Leu	Ser	Val	Met	Leu	Leu
P-450$_b$	Met	Glu	Pro	Ser	Ile	Leu	Leu	Leu	Leu	Ala	Leu	Leu	Val	Gly	Phe	Leu	Leu	Leu	Leu	Val
P-450$_c$	Met	Pro	Ser	Val	Tyr	Gly	Phe	Pro	Ala	Phe	Thr	Ser	Ala	Thr	Glu	Leu	Leu	Leu	Ala	Val
P-450$_d$	Met	Ala	Phe	Ser	Gln	Tyr	Ile	Ser	Leu	Ala	Pro	Glu	Leu	Leu	Leu	Ala	Thr	Ala	Ile	Phe
P-450$_e$	Met	Glu	Pro	Ser	Ile	Leu	Leu	Leu	Leu	Ala	Leu	Leu	Val	Gly	Phe	Leu	Leu	Leu	Leu	Val
P-450$_f$	Met	Asp	Leu	Val	Thr	Phe	Leu	Val	Leu	Thr	Leu	Ser	Ser	Leu	Ile	Leu	Leu	Ser	Leu	Trp
P-450$_g$	Met	Asp	Pro	Val	Val	Val	Leu	Leu	Leu	Ser	Leu	Phe	Phe	Leu	Leu					
P-450$_h$	Met	Asp	Pro	Val	Leu	Val	Leu	Val	Leu	Thr	Leu	Ser	Ser	Leu	Leu	Leu	Leu	Ser	Leu	Trp
P-450$_i$	Met	Asp	Pro	Phe	Val	Val	Leu	Val	Leu	Ser	Leu	Ser	Phe	Leu	Leu	Leu	Leu	Tyr	?	Trp
P-450$_j$	Ala	Val	Leu	Gly	Ile	Thr	Ile	Ala	Leu	Leu	Val	Trp	Val	Ala	Thr	Leu	Leu	Val	Ile	
PB-1/PB-C	Met	Asp	Leu	Val	Met	Leu	Leu	Val	Leu	Thr	Leu	Thr	?	Leu	Ile	Leu	Leu	?	Ile	Trp
2a/PCN-E	Met	Asp	Leu	Ile	Phe	Met	Leu	Glu	Thr	Ser	Ser	Leu	Leu	Leu	Ala					
P-450$_{PCN}$	Met	Asp	Leu	Leu	Ser	Ala	Leu	Thr	Leu	Glu	Thr	Trp	Val	Leu	Leu	Ala	Val	Val	Leu	Val
RLM2	Met	Leu	Asp	Thr	Gly	Leu	Leu	Leu	Val	Val	Ile	Leu	Ala	Ser	Leu	Ser	Val	Met	Phe	Leu

[a] Heme-extracted P-450$_{C\text{-}M/F}$ (8 nmol) was prepared, subjected to Edman degradation, and analyzed by a gas-phase sequencer as described under Materials and Methods. Assays were repeated 5 times with three different batches of P-450$_{C\text{-}M/F}$ preparations, and results were comparable. An amino acid residue at position 4 has not been unequivocally assigned. The P-450$_{C\text{-}M/F}$ sequence obtained is compared to those of the rat liver P-450's for which amino acid sequences have been reported.

activity of P-450$_{C\text{-}M/F}$ with respect to estrogen hydroxylation is also noteworthy in that the cytochrome did not hydroxylate another aromatic polycyclic compound, benzo[*a*]pyrene.

A TLC method for the separation of estrogen metabolites was developed that is capable of separating E_1, 2-, 4-, 6α-, 15α- and 16α-HOE$_1$, 6-oxoE$_1$, E_2, 2-, 4-, 6α-, 6β-, and 15α-HOE$_2$, 6-oxoE$_2$, E_3, E_4, and 2-HOE$_3$. By use of this system together with the above-listed authentic standards, the major oxidation products of P-450$_{C\text{-}M/F}$ were identified to be 2- and 16α-hydroxylated estrogens but not 4-hydroxylated estrogens. It should be noted that, in this pattern of metabolism, P-450$_{C\text{-}M/F}$ can carry out the hydroxylation of aliphatic and aromatic carbons on the same steroid molecule. In view of the fact that there is a significant correlation between the level of 16α-HOE$_1$ and the incidence of mammary tumor (Fishman et al., 1980; Bradlow et al., 1985), production of 16α-HOE$_1$ (2.8%) from E_1 by P-450$_{C\text{-}M/F}$ is noteworthy. Neither P-450$_b$ nor P-450$_c$ produced E_3, although both produced some 2-HOE$_2$.

2-Hydroxylation and 4-hydroxylation of E_2 have been described with P-450$_{UT\text{-}A}$ and P-450$_{PCN\text{-}E}$ (Dannan et al., 1986); however, 16α-hydroxylation was not demonstrated for these cytochromes. P-450$_{UT\text{-}A}$ and P-450$_{PCN\text{-}E}$ are known to be the major forms that are responsible for E_2 2- and 4-hydroxylations in rat hepatic microsomes (Cheng & Schenkman, 1984). It has been reported that P-450 RLM5 as well as microsomal preparations is capable of forming E_1, E_3, and 2-HOE$_2$ (Cheng & Schenkman, 1984). RLM5 is considered to be identical with UT-A or P-450$_h$, and it is distinct from P-450$_{C\text{-}M/F}$ as judged from amino acid sequence data (Table III). Rates of 16α-hydroxylation of P are substantially greater by RLM5 than by P-450$_{C\text{-}M/F}$, while P-450$_{C\text{-}M/F}$ preferentially hydroxylates E_2. Perhaps in vivo in animals where other steroids are present in competing concentrations the major activity of RLM5 may be directed toward P hydroxylation and the activity of P-450$_{C\text{-}M/F}$ may largely be directed toward E_2 hydroxylation.

In contrast to P-450$_{UT\text{-}A}$ and P-450$_{PCN\text{-}E}$, which display both 2- and 4-hydroxylation activity toward E_2, P-450$_{C\text{-}M/F}$ displayed 2-hydroxylation activity but not significant 4-hydroxylation activity. It should be noted that both P-450$_{UT\text{-}A}$ (Guengerich et al., 1982; Dannan et al., 1983; Waxman, 1984) and microsomal E_2 2-hydroxylation are markedly suppressed by β-naphthoflavone treatment (Dannan et al., 1986). P-450$_{C\text{-}M/F}$ activity for E_2 2-hydroxylation was found to be only slightly suppressed by β-naphthoflavone treatment of animals.

Larrey et al. (1984) have reported the existence of a noninducible form of cytochrome P-450 isozyme, termed P-450$_{UT\text{-}H}$, in the liver of male and female Sprague-Dawley rats. P-450$_{UT\text{-}H}$ had a M_r of 52000, while the M_r of P-450$_{C\text{-}M/F}$ was 48 500. These and other findings on their mixed-function oxidase profiles indicate that P-450$_{C\text{-}M/F}$ is distinct from P-450$_{UT\text{-}H}$.

It is known that the liver of male rats oxidizes E_2 to E_1, 6-HOE$_2$, E_3 (Breuer et al., 1960), and the catechol estrogen 2-HOE$_2$ (Numazawa et al., 1979). 2-Hydroxylation is also quantitatively much greater in male rats than in humans (Yasukochi & Masters, 1976; Barbieri et al., 1978; Ball et al., 1978; Fishman, 1983). By use of homogeneously purified preparations of P-450's, RLM5 has been shown to catalyze the 16α-hydroxylation of E_2 (Cheng & Schenkman, 1984). Since RLM5 has been shown to be male-specific, P-450$_{C\text{-}M/F}$ represents the first P-450 molecule in female animals that expresses 16α-hydroxylation activity. The 16α-hydroxylase pathway is significant in that it produces E_3 and 16α-HOE$_1$, biologically active uterotropic hormones. Thus, the oxidative transformation of E_2 by P-450$_{C\text{-}M/F}$ not only produces a catecholestrogen such as 2-HOE$_2$ but also leads to the formation of the unterotropic hormone E_3. In this respect, the metabolism of E_2 by P-450$_{C\text{-}M/F}$ can be considered as extending the biological activity of the parent hormone, since it catalyzes the metabolic transformation of E_2 to an active uterotropic metabolite. This would thus be another example of the phenomenon of parent steroid hormones being converted by transformation to biologically active derivatives (Kappas & Palmer, 1963; Schneider et al., 1983).

In view of the fact that P-450$_{UT\text{-}A}$ and P-450$_{PCN\text{-}E}$ exhibit 2-hydroxylase activity toward E_2, the 2-hydroxylation activity observed with P-450$_{C\text{-}M/F}$ may be considered to represent a general property of these cytochrome P-450 species for aromatic carbon hydroxylation at the 2-position of E_2. In contrast, the lack of 4-hydroxylase activity and the significant 16α-hydroxylase activity appear to be a distinctive characteristic feature of P-450$_{C\text{-}M/F}$.

A number of P-450 isozymes have been purified from liver microsomes of rats treated with various drugs and chemicals. Several P-450's have been structurally characterized, and some of them have been proposed as being identical on the basis of their inducibility by certain drugs and chemicals, inhibition by specific inhibitors, substrate specificity, immunological cross-reactivity, NH_2-terminal amino acid sequences, and

nucleotide sequence data (Kawajiri et al., 1984). All of these criteria for identity should be considered indirect, however, except for immunological reactivity, the amino acid sequence, and nucleotide sequence data. Even immunological cross-reactivity may not recognize the difference between closely related P-450 isozymes (Waxman & Walsh, 1982; Kawajiri et al., 1984; Ryan et al., 1982a). Thus, until nucleotide sequence data become available for all purified P-450 molecules, the NH_2-amino acid sequence data must be considered to be the most critical criterion for structural comparisons among different P-450's (Nebert et al., 1987). As shown in Table III, NH_2-terminal amino acid sequence data have been reported for 14 different P-450's to date. Most P-450's have Met at the NH_2-terminus and are enriched in Asp, Glu, and Leu. Comparison of the data for the previously reported 14 P-450 species with those for P-$450_{C\text{-}M/F}$ in Table III shows that P-$450_{C\text{-}M/F}$ has unique amino acid residues which do not exist at the comparable positions in the other P-450's. In particular, no other P-450's have a Met-Glu-Leu sequence at the N-terminus.

These data establish that P-$450_{C\text{-}M/F}$ is a cytochrome isozyme which has not been previously described. P-$450_{C\text{-}M/F}$ could potentially be important physiologically in that it is normally present in the liver of both male and female rats and is capable of carrying out the oxidative metabolism of E_2, particularly along the pathway leading to the formation of E_3, an active uterotropic metabolite of the parent hormone.

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Nonrandom Assembly of Chromatin during Hydroxyurea Inhibition of DNA Synthesis†

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ABSTRACT: Incubation of MSB-1 chicken lymphoblastoid cells with hydroxyurea leads to a rapid 25-fold decrease in the incorporation of [^{3}H]thymidine into DNA and a 5-fold decrease of [^{3}H]lysine into the nucleosome core histones. I have investigated whether the distortion in the normal proportion of histone–DNA synthesis results in alterations in the nucleosome assembly process and find that neither the stoichiometry of new histone synthesis nor the deposition is appreciably changed during hydroxyurea incubation. Protein cross-linking and micrococcal nuclease digestion show that the histones synthesized during hydroxyurea treatment form octamer structures and are assembled into typical nucleosome particles. Minor nucleosome subpopulations are found which exhibit altered sensitivity to nuclease digestion and which are depleted in new histones H3 and H4. When MSB-1 cells incubated in hydroxyurea are pulsed briefly with density-labeled amino acids and [^{3}H]lysine, the radiolabeled core histone octamers formed are as dense as individual monomer histones. These results suggest that the newly synthesized histone octamers are uniformly dense and do not contain mixtures of new and old histones. Thus, histones synthesized during hydroxyurea incubation are deposited nonrandomly and do not exchange with preexisting histones.

The assembly of chromatin in vivo reflects the interplay of several regulated processes, including the coordinated synthesis of histones and DNA during S phase and the synthesis of S-phase histone subtypes (and basal histone variants) in nucleosomal stoichiometry (DePamphilis & Wassarman, 1980; Wu & Bonner, 1981; Delegeane & Lee, 1982; Wu et al., 1982, 1983; Sariban et al., 1985). The ordered nature of chromatin replication is evident also in the sequential deposition of histones H3/H4 prior to H2A/H2B during nucleosome formation (Worcel et al., 1978; Senshu et al., 1978) and the nonrandom assembly and segregation of nucleosomes (Seale, 1976; Weintraub, 1976; Leffak et al., 1977; Weintraub et al., 1978; Prior et al., 1980; Roufa & Marchionni, 1982; Leffak 1983b, 1984; Trempe & Leffak, 1985; Kumar & Leffak, 1986).

The synthesis of histones can be partially uncoupled from that of DNA by using chemicals which specifically inhibit either protein or DNA synthesis [reviewed in DePamphilis and Wassarman (1980) and in Sariban et al. (1985)]. In HeLa and Ehrlich ascites tumor cells, the inhibition of histone synthesis by cycloheximide is correlated with a 60–80% decrease in DNA synthesis. Under these conditions, the segregation of preexisting histones occurs normally at the level of mono- and oligonucleosomes (Pospelov et al., 1982; Seale & Simpson, 1975), and new histones are deposited nonrandomly immediately after removal of the cycloheximide block (Leffak, 1983b).

Both cytosine arabinoside (ara-C)[1] and hydroxyurea (HU) are able to cause a rapid decrease of greater than 95% in [^{3}H]thymidine incorporation into DNA with a concomitant, selective decrease of [^{3}H]lysine incorporation into the core histones by 60–80% (Leffak, 1983a; Louters & Chalkely, 1985). Here again, several features of normal chromatin replication persist. During ara-C incubation, the molar stoichiometry of new core histones is maintained, and bulk

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[1] Abbreviations: ara-C, cytosine arabinoside; Na_2EDTA, disodium ethylenediaminetetraacetate; HU, hydroxyurea; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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