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Purification and Characterization of Three Forms of Microsomal Cytochrome P-450 in Liver from 3-Methylcholanthrene-Treated Guinea Pigs

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SUMMARY

One molecular form of cytochrome P-450$_{IIA}$ from liver microsomes of guinea pigs treated with 3-methylcholanthrene was purified to a specific content of 17.4 nmoles/mg of protein. The difference spectrum of reduced hemoprotein-carbon monoxide complex of this cytochrome exhibits an absorption maximum at 448 nm. The absolute absorption spectrum of the oxidized form of this hemoprotein suggests a high-spin state of heme iron. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the purified protein shows a single band of polypeptide stained with Coomassie brilliant blue at the position corresponding to M_r 54,000. On the other hand, the other two forms of cytochrome P-450, cytochrome P-450$_I$ and P-450$_{IIB}$, were also separated and purified to specific contents of 8.7 and 5.2 nmoles/mg of protein, respectively. Both cytochrome P-450$_I$ and P-450$_{IIB}$ exhibit absorption maxima at 450 nm in the difference spectrum of reduced hemoprotein-carbon monoxide complex, and a low-spin state of ferric iron in the heme. The spectrophotometrical property of cytochrome P-450$_I$ and P-450$_{IIB}$ was clearly different from that of cytochrome P-450$_{IIA}$. Molecular activities of a reconstituted aryl hydrocarbon hydroxylase (EC 1.14.14.1) containing, respectively, cytochrome P-450$_I$, P-450$_{IIA}$, and P-450$_{IIB}$ were 0.224, 0.250, and 0.395 (moles per minute per mole of cytochrome P-450), and were estimated to be one-tenth that of cytochrome P-448 induced in rat liver by 3-methylcholanthrene, indicating the presence of the low inducibility by 3-methylcholanthrene of aryl hydrocarbon hydroxylase in liver microsomes of guinea pigs.

INTRODUCTION

The microsomal monooxygenase system containing P-450[1] plays a critical role in the metabolic activation of many kinds of chemical carcinogens (1–3). The activity of this enzyme system sometimes appears to be a determinant factor among those which affect the sensitivity of animals to chemical carcinogens. For example, polycyclic hydrocarbon carcinogenesis and the inducibility of microsomal AHH by MC correlate with each other and are inherited from parents in the same genetic manner by their offspring in several strains of mice (4–6).

We have recently examined the strain difference in the inducibility of liver microsomal AHH by MC in four strains of guinea pigs (7), a species known to be resistant to some chemical carcinogens that require metabolic activation to exert their carcinogenicities (3, 8–10). Compared with the strains of mice and rats whose AHH activities in liver microsomes are enhanced up to 10-fold or more (4–6, 11), all four strains of guinea pigs examined were relatively refractory in the induction of AHH by treatment with MC (7).

The elevated AHH activities in liver microsomes of mice or rats treated with MC are attributed to the induction of molecular forms of P-450 which have high catalytic activities for BP hydroxylation (12–14). As the blue spectral shift of the Soret region in the difference spectra of the reduced hemoprotein-CO complex of liver microsomes from MC-treated guinea pigs suggests the induction of a P-450 different from those which exist in nontreated animals (7), we tried to separate and purify this molecular form of P-450 from liver microsomes of MC-treated guinea pigs, and to characterize its molecular properties and catalytic activity for BP hydroxylation.

MATERIALS AND METHODS

Animals. The animals used were the Hartley strain of male guinea pigs weighing 250–300 g, and the Sprague-Dawley strain of male rats weighing 140–200 g. Guinea pigs were obtained from Tokyo Experimental Animal

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[1] The abbreviations used are: P-450, cytochrome P-450; AAH, aryl hydrocarbon hydroxylase; MC, 3-methylcholanthrene; BP, benzo[*a*]pyrene; PEG, polyethylene glycol 6000; AAF, 2-acetylaminofluorene; DLPC, dilauroyl glyceryl-3-phosphorylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NADPH-P-450 reductase, NADPH-cytochrome P-450 (cytochrome *c*) reductase; PB, phenobarbital; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

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Company, Ltd. (Tokyo, Japan), and rats were obtained from Charles River Japan Inc. (Atsugi, Japan).

Chemicals. The sources of chemicals, reagents, and resins used were as follows: corn oil, ammonium persulfate, and Coomassie brilliant blue R-250, from Nakarai Chemicals, Ltd. (Kyoto, Japan); sodium cholate, PEG, cytochrome *c* (from horse heart, Type III), and DLPC, from Sigma Chemical Company (St. Louis, Mo.); tetrasodium salt of NADPH and disodium salts of NADH and $NADP^+$, from Oriental Yeast Company, Ltd. (Tokyo, Japan); AAF, acrylamide, *N*,*N*′-methylenebisacrylamide, and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, from Eastman Kodak Company (Rochester, N. Y.); phenobarbital sodium, from E. Merk A.G. (Darmstadt, West Germany); Emulgen 913, from Kao-Atlas Company, Ltd. (Tokyo, Japan); phenylmethylsulfonyl fluoride, from Calbiochem-Behring Corporation (La Jolla, Calif.); (+)-Benzphetamine hydrochloride, from Upjohn Company (Kalamazoo, Mich.); bromophenol blue, from Junsei Chemical Company, Ltd. (Tokyo, Japan); DEAE-cellulose (DE-52), from Whatman Ltd. (Maidstone, Kent, England); hydroxyapatite (Bio-Gel HT), from Bio-Rad Laboratories (Richmond, Calif.); CM-Sephadex C-50, 2′5′-ADP-Sepharose 4B, and low molecular weight calibration kit (for electrophoresis), from Pharmacia Fine Chemicals AB (Uppsala, Sweden); 3-hydroxybenzo[*a*]pyrene, from Dr. N. Kinoshita, Kyushu University (Fukuoka, Japan); *N*-hydroxy-AAF, from Dr. T. Matsushima, Tokyo University (Tokyo, Japan); and the remainder, from Wako Pure Chemical Company, Ltd. (Osaka, Japan).

Assay procedures. The concentrations of P-450 and cytochrome b_5 were measured by the method of Omura and Sato (15), using extinction coefficients of 91 and 185 cm^{-1} mM^{-1}, respectively. The activities of NADPH-P-450 reductase and NADH-cytochrome b_5 reductase were assayed by the method of Vermilion and Coon (16) and of Takesue and Omura (17), respectively; 1 unit of enzyme activity was defined to be the amount of enzyme that reduced 1 μmole of substrate in 1 min. Protein concentration was determined by the method of Lowry *et al.* (18).

Treatment of animals. All animals were housed in equipment which was conditioned for air, light (from 7:00 a.m. to 7:00 p.m.), and temperature (23–25°), and fed animal chow (Oriental RC-4 for guinea pigs and Oriental MF for rats) ad libitum for at least 1 week before an experiment began. The animal chow for guinea pigs contained ascorbic acid at a concentration of 50 mg/kg. MC was dissolved in corn oil and injected i.p. at a dose of 25 mg/kg of body weight every 24 hr. The final dose of MC was 50 mg/kg of body weight for guinea pigs and 75 mg/kg of body weight for rats. PB was dissolved in 0.9% NaCl solution and injected i.p. at a dose of 75 mg/kg of body weight every 24 hr to a final dose of 300 mg/kg of body weight.

Preparation of liver microsomes. Twenty-four hours after the final injection of MC or PB, the animals were killed by decapitation and the livers were excised immediately. Liver microsomes were prepared by the method of West *et al.* (19), and stored at −80° under N_2 until use.

Separation and purification of P-450 of guinea pigs. All of the procedures for separation and purification except those indicated otherwise were performed at 0–4°. Liver microsomes from MC-treated guinea pigs were treated with sodium cholate according to the method of West *et al.* (19) and centrifuged at 125,000 × *g* for 60 min. The supernatant was fractionated with PEG at a concentration range of 9–20%. The material pelleted with PEG was dissolved in 50 ml of 10 mM potassium phosphate (pH 7.4) which contained 20% glycerol, 0.5% sodium cholate, 0.2% Emulgen 913, and 0.1 mM EDTA (Buffer A), stirred at room temperature (20–25°) for 30 min, and applied to a DEAE-cellulose column (2.6 × 30 cm) equilibrated with Buffer A. After washing the column with 200 ml of Buffer A, elution was carried out by the linear gradient of KCl concentration from 0 to 250 mM in 500 ml of Buffer A. This column chromatography was performed at room temperature. Two main fractions containing P-450 were obtained, each of which was further purified as follows.

The fraction eluted at the flow-through region (Fraction I) was treated with saturated ammonium sulfate solution to recover P-450 without using PEG. The precipitated material at 50% saturation of ammonium sulfate was dissolved in 20 ml of 10 mM potassium phosphate (pH 7.4) which contained 20% glycerol and 0.2% Emulgen 913, and dialyzed against 2000 ml of the same buffer. The dialysate was applied to a hydroxyapatite column (2.5 × 8.0 cm) equilibrated with the buffer used for the dialysis. After washing the column with the equilibration buffer, the concentration of potassium phosphate was increased linearly from 10 to 300 mM in a total volume of 250 ml. The fraction of P-450 eluted from the hydroxyapatite column was dialyzed against 50 times the volume of 10 mM potassium phosphate solution (pH 7.25) which contained 20% glycerol and 0.2% Emulgen 913, and applied to a CM-Sephadex C-50 column (2.5 × 10 cm) equilibrated with the buffer used for the dialysis. After washing the column with the equilibration buffer, P-450 was eluted by the linear gradient of the concentration of potassium phosphate from 10 to 300 mM in a total volume of 250 ml. The fraction of P-450 eluted from the CM-Sephadex C-50 column was dialyzed against 100 times the volume of 10 mM potassium phosphate solution containing 20% glycerol, and applied again to a hydroxyapatite column (1.5 × 8.0 cm) equilibrated with the buffer used for the dialysis. After washing the column with the equilibration buffer until the absorbance at 280 nm of the eluate reached below 0.01, the concentration of potassium phosphate was increased to 500 mM to elute P-450. The P-450 purified by these procedures was designated as $P\text{-}450_I$.

The fraction that contained P-450 eluted from the DEAE-cellulose column at the beginning of the gradient of the KCl concentration (Fraction II) was further purified as follows. This fraction was diluted 3-fold with 20% glycerol and applied to a hydroxyapatite column (2.5 × 8.0 cm) equilibrated with 10 mM potassium phosphate (pH 7.4) containing 20% glycerol. After washing the column with 50 ml each of 10 mM and 40 mM potassium phosphate (pH 7.4), each of which contained 20% glycerol and 0.2% Emulgen 913, the concentration of potassium

phosphate was increased stepwise to 80 mM and 150 mM. The fractions containing P-450 eluted from the hydroxyapatite column with 80 mM potassium phosphate (Fraction IIA) and 150 mM potassium phosphate (Fraction IIB) were further purified individually by the same procedures as follows. P-450 eluted from the hydroxyapatite column was dialyzed against 50 times the volume of Buffer A and chromatographed again on a DEAE-cellulose column under the same conditions as those for the first DEAE-cellulose column chromatography except that the column size was reduced to 2.0 × 30 cm and that the linear gradient elution was carried out from 0 to 100 mM KCl in 250 ml of Buffer A. P-450 eluted from this column as a single peak was dialyzed against 100 times the volume of 20 mM potassium phosphate solution (pH 7.25) containing 20% glycerol and applied to a CM-Sephadex C-50 column (1.5 × 8.0 cm) equilibrated with the buffer used for the dialysis. After washing the column with the equilibration buffer until the absorbance at 280 nm of the eluate decreased below 0.01, the concentration of potassium phosphate was increased to 300 mM to elute P-450. The preparations of P-450 purified from Fraction IIA and Fraction IIB by these procedures were designated as P-450$_{IIA}$ and P-450$_{IIB}$, respectively.

Separation and purification of P-450 of rats. P-448 and P-450, which are used as an arbitrary terms in this report to indicate the molecular form of P-450 predominantly induced in rat liver by MC and PB, respectively (13, 14), were purified from liver microsomes of MC- and PB-treated rats by the same procedures as those used for the purification of P-450$_{IIB}$ except that the concentration of PEG used to fractionate the solubilized supernatant was 8–14%.

Purification of NADPH-P-450 reductase. NADPH-P-450 reductases of guinea pigs and rats were purified from liver microsomes of PB-treated animals by the procedure of Taniguchi *et al.* (20). We used a DEAE-cellulose column instead of a DEAE-Sephadex A-25 column. Purified preparations of P-450s and NADPH-P-450 reductase were stored at −80° until use.

SDS/PAGE. SDS/PAGE of the purified hemoproteins was performed by the method of Laemmli (21) with a slab gel. The concentrations of acrylamide of the spacer and lower gel were 3.0% and 7.5%, respectively. Samples were electrophoresed by a constant current of 20 mamp for 6 hr. The electrophoresed polypeptides were stained with Coomassie brilliant blue.

Reconstitution of monooxygenation. Components of a reconstituted AHH with purified enzymes were as follows: 50 μmoles of Hepes (pH 7.4), 0–40 pmoles of P-450, 0.3 unit of NADPH-P-450 reductase, 30 μg of DLPC, 80 nmoles of BP, and 0.4 μmole of NADPH. The reaction volume was 1 ml. P-450, NADPH-P-450 reductase, and DLPC were first mixed in this order, and the mixture was kept at room temperature (20–25°) for 1 min. The other components (except BP) were then added. The reaction was started by the addition of BP, which was dissolved in methanol at a concentration of 2 mM, and stopped after 5 min by the addition of 1 ml of ice-cold acetone and 3.25 ml of *n*-hexane. Hydroxylated derivatives of BP formed were quantitated by the method of Nebert (22).

N-Hydroxylation of AAF was assayed according to the method of Hinson *et al.* (23). The 3.0-ml incubation mixture contained 186 μmoles of potassium phosphate (pH 7.4), 0.12 pmole of P-450, 0.44 unit of NADPH-P-450 reductase, 50 μg of DLPC, 1.2 μmoles of AAF, and 6 μmoles of NADPH. After a 20-min incubation at 37°, the reaction was stopped by the addition of 3.0 ml of cold acetone, followed by extraction twice with 5 ml of ethyl ether. The ether extracts were evaporated under nitrogen and the residues were dissolved in 50 μl of methanol. The sample was injected onto the column of Zorbax BP using a Trirotar high-pressure liquid chromatograph (Japan Spectroscopic Company, Tokyo, Japan). Absorbance was monitored at 546 nm using the dual beam Model UA-5 photometer (Instrumentation Specialties Company, Lincoln, Nebr.).

Benzphetamine *N*-demethylase was assayed by the formation of formaldehyde. The standard assay mixture (1.5 ml) contained 250 μmoles of Hepes (pH 7.4), 0.18 pmole of P-450, 0.3 unit of NADPH-P-450 reductase, 5 μg of DLPC, 7.5 μmoles of benzphetamine, 5 μmoles of NADPH, 0.2 μmole of KCl, and 0.015 μmole of $MgCl_2$. Reaction was started by the addition of NADPH and stopped after 10 min by the addition of 2.25 ml of 12.5% trichloroacetic acid (24). The amount of formaldehyde formed was measured according to the method of Werringloer (25).

RESULTS

Separation and purification of P-450. P-450 in liver microsomes prepared from MC-treated guinea pigs was almost completely solubilized with sodium cholate at the concentration of 3 mg/mg of protein (Table 1). The solubilized supernatant was then fractionated with PEG. Since this step was aimed at reducing the sample volume before application to the first DEAE-cellulose column chromatography, the concentration of PEG (9–20%) was determined so that most of P-450 could be recovered from the solubilized supernatant. The material precipi-

TABLE 1

Purification of a cytochrome P-450$_{IIA}$ of guinea pigs from liver microsomes

Microsomes were prepared from the Hartley strain of male guinea pigs treated with MC, and P-450$_{IIA}$ was purified as described under Materials and Methods.

Procedure	Protein	Cytochrome P-450		
		Total content	Specific content	Recovery[a]
	mg	*nmoles*	*nmoles/mg protein*	%
Microsomes	2920	4910	1.68	100
Solubilization with cholate	2610	4820	1.85	98.2
PEG fractionation	1280	3660	2.86	74.5
DEAE-cellulose column (room temperature)	267	1250	4.67	25.5
Hydroxyapatite column	46.0	320	6.95	6.53
DEAE-cellulose column (room temperature)	26.0	236	9.07	4.81
CM-Sephadex C-50 column	4.94	85.9	17.4	1.75

[a] Ratio of the amount of cytochrome P-450 recovered to the total content of cytochrome P-450 in microsomes.

tated with PEG was chromatographed on a DEAE-cellulose column in the presence of sodium cholate and Emulgen 913 at room temperature according to the method of West *et al.* (19), and three main fractions of hemoprotein were obtained (Fig. 1). Both the fraction eluted in the flow-through region (Fraction I) and that eluted at the beginning of the gradient of KCl concentration (Fraction II) contained P-450, and the fraction eluted finally from the column contained cytochrome b_5, but not P-450. The difference spectra of reduced hemoprotein-CO complex of Fraction I and Fraction II had their Soret maxima at 450 nm and 448 nm, respectively, indicating that the molecular species of P-450 induced by MC in guinea pig liver was eluted into Fraction II. Thus this fraction was further purified by hydroxyapatite column chromatography (Fig. 2). By increasing the concentration of potassium phosphate stepwise, Fraction II was resolved into two fractions of P-450, one of which was eluted by 80 mM (Fraction IIA) and the other by 150 mM (Fraction IIB). The difference spectra of reduced hemoprotein-CO complex of Fraction IIA exhibited its Soret maximum at 448 nm, and of Fraction IIB at 450 nm, suggesting that the molecular species of P-450 induced by MC in guinea pig liver was collected into Fraction IIA. This P-450 (Fraction IIA) was purified again by DEAE-cellulose column chromatography, which consisted of almost the same method as performed the first time, followed by CM-Sephadex C-50 column chromatography. In these latter two steps a single major peak of P-450 was observed on the chromatographic profile, respectively. The molecular species of P-450 purified from Fraction IIA was designated as P-450$_{IIA}$. The specific contents of the purified P-450$_{IIA}$ were 16–18 nmoles/mg of protein with about 2% recoveries from microsomes. A result of a typical purification of P-450$_{IIA}$ is shown in Table 1.

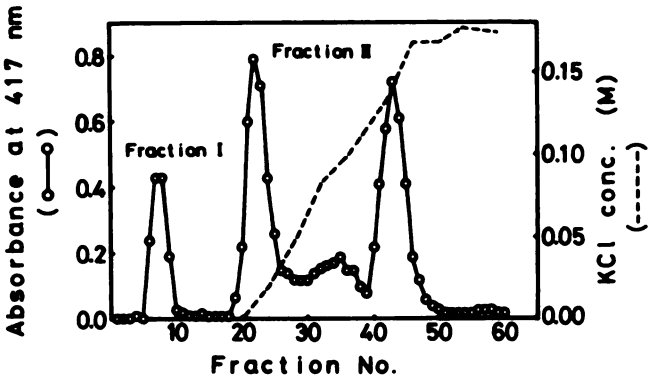

FIG. 1. *Elution profile of DEAE-cellulose column chromatography after PEG fractionation*

Material precipitated from the solubilized supernatant with PEG was dissolved in 50 ml of 10 mM potassium phosphate (pH 7.4) which contained 20% glycerol, 0.5% sodium cholate, 0.2% Emulgen 913, and 0.1 mM EDTA (Buffer A), stirred at room temperature for 30 min, and applied to a DEAE-cellulose column (2.6 × 30 cm) equilibrated with Buffer A. After washing the column with 20 ml of Buffer A, the KCl concentration was increased linearly from 0 to 250 mM in 500 ml of Buffer A. Elution of hemoproteins was monitored by absorbance at 417 nm (○——○), and the concentration of KCl (- - -) was measured by the conductivity of the eluate. Each fraction contained 20 g of the eluate. All of the procedures accompanying this chromatography were performed at room temperature (20–25°).

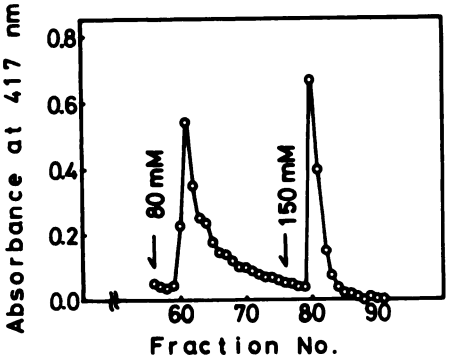

FIG. 2. *Elution profile of hydroxyapatite column chromatography after DEAE-cellulose column chromatography*

Fraction II of the eluate from the DEAE-cellulose column (Fig. 1) was diluted 3-fold with 20% glycerol and applied to a hydroxyapatite column (2.5 × 8.0 cm) equilibrated with 10 mM potassium phosphate (pH 7.4) containing 20% glycerol. After washing the column with 50 ml each of 10 and 40 mM potassium phosphate (pH 7.4) that contained 20% glycerol and 0.2% Emulgen 913, the concentration of potassium phosphate was increased stepwise to 80 and 150 mM as indicated by the *arrows*. Elution of hemoproteins was monitored by absorbance at 417 nm (○——○). Each fraction contained 10 g of the eluate.

The Fraction IIB eluted by 150 mM potassium phosphate from the hydroxyapatite column (Fig. 2) was further purified by the same steps as those for the purification of P-450$_{IIA}$ performed after the hydroxyapatite column chromatography, and a partially purified preparation, designated as P-450$_{IIB}$, with a specific content of 5.2 nmoles/mg of protein, was obtained.

Fraction I, eluted at the flow-through region of the first DEAE-cellulose column chromatography (Fig. 1), was further purified by the first hydroxyapatite, CM-Sephadex C-50, and the second hydroxyapatite column chromatography as described under Materials and Methods. In these procedures, P-450 obtained from Fraction I always eluted as a single peak, and a partially purified preparation, designated as P-450$_I$, with a specific content of 8.7 nmoles/mg of protein, was obtained.

The specific contents of P-448 and P-450 purified from rat livers were 20.2 and 14.7 nmoles/mg of protein, respectively.

Spectral properties of P-450 of guinea pigs. Figure 3 shows the difference spectra of reduced hemoprotein-CO complex of the three purified preparations of guinea pig P-450. The absorption maxima in the Soret region of P-450$_I$ and P-450$_{IIB}$ were both at 450 nm, and of P-450$_{IIA}$ at 448 nm.

Figure 4 shows the absolute absorption spectra of the three preparations of guinea pig P-450 in the absence of Emulgen 913. The absorption maxima of oxidized P-450$_I$ and P-450$_{IIB}$ were at 415, 530, and 565 nm, suggesting the presence of a low-spin state of ferric iron in the heme of these molecular forms of P-450. On the other hand, the absorption maxima of oxidized P-450$_{IIA}$ were at 394 and 645 nm, suggesting the presence of a high-spin state of ferric iron in the heme.

SDS/PAGE of the purified preparations of P-450. Figure 5 is a result of SDS/PAGE of purified P-450s of guinea pigs and rats. Of the three preparations of P-450 from guinea pigs, only P-450$_{IIA}$ showed a single band of polypeptide stained with Coomassie brilliant blue, as

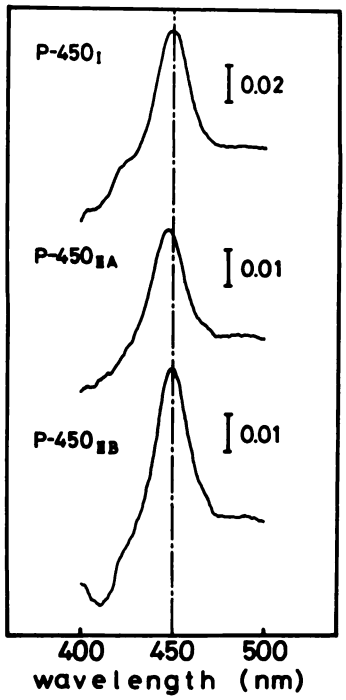

FIG. 3. *Difference spectra of reduced hemoprotein-CO complex of P-450s purified from liver microsomes of MC-treated guinea pigs*

A purified preparation of each cytochrome was diluted with 100 mM potassium phosphate (pH 7.4) containing 20% glycerol, and a difference spectrum of dithionite-reduced hemoprotein complexed with carbon monoxide to dithionite-reduced hemoprotein was recorded at room temperature according to the method of Omura and Sato (15). Concentrations of the hemoproteins applied were as follows: P-450$_{I}$, 0.68 μM; P-450$_{IIA}$, 0.32 μM; P-450$_{IIB}$, 0.43 μM.

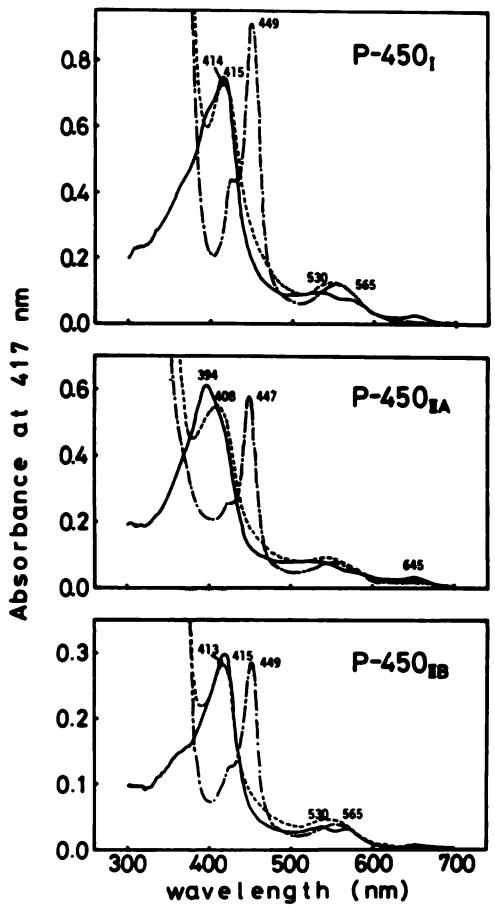

FIG. 4. *Absolute absorption spectra of P-450s purified from liver microsomes of MC-treated guinea pigs*

A purified preparation of each cytochrome was diluted with 300 mM potassium phosphate (pH 7.25) containing 20% glycerol, and the absolute absorption spectra of the oxidized form (——), the dithionite-reduced form (- - -), and the reduced form complexed with carbon monoxide (—·—) were recorded at room temperature. In the reference cuvette was placed 300 mM potassium phosphate (pH 7.25) containing 20% glycerol. Concentrations of the hemoproteins applied were as follows: P-450$_{I}$, 7.67 μM; P-450$_{IIA}$, 5.27 μM; P-450$_{IIB}$, 3.11 μM.

expected from the results showing a higher value of the specific contents. The M_r of P-450$_{IIA}$ was estimated to be 54,000. Although P-450$_{I}$ and P-450$_{IIB}$ showed several bands of polypeptides in each lane when stained with Coomassie brilliant blue, the most deeply stained bands in the lanes of P-450$_{I}$ and P-450$_{IIB}$ migrated at the positions corresponding to M_r values of 54,000 and 52,000, respectively. The rat P-448, the specific content of which was 20.2 nmoles/mg of protein, somehow showed a minor band at the position corresponding to 49,000 M_r in addition to a main band calculated as 56,000 M_r.

Reconstitution of monooxygenation by the purified enzyme preparations. With the purified preparations of P-450 and NADPH-P-450 reductase, the AHH system was reconstituted and the molecular activities for BP hydroxylation were compared among guinea pig P-450$_{I}$, P-450$_{IIA}$, and P-450$_{IIB}$, and rat P-448. In all of the AHH systems reconstituted by guinea pig enzymes and by rat enzymes, hydroxylated derivatives of BP were formed proportionally to the amount of P-450, at least in the range of 0–40 pmoles in the reaction mixture. As shown in Table 2, the molecular activity of each form of guinea pig P-450 is clearly lower than that of rat P-448.

The formation of *N*-hydroxy metabolites from AAF in the reconstituted enzyme system composed of guinea pig P-450$_{IIA}$ was also very low, compared with that of rat P-448, as shown in Table 2. Furthermore, the activity of benzphetamine *N*-demethylation in the reconstituted enzyme was measured, because benzphetamine is one of the favorite substrates for the monooxygenation system using rat liver P-450 (13). The *N*-demethylation activity in the enzyme system using either guinea pig P-450$_{I}$ or P-450$_{IIA}$ was apparently lower than that using rat P-450. From the experiments herein reported it was observed that there was relatively low catalytic activity in the reconstituted enzyme system composed of the purified guinea pig P-450s and NADPH-P-450 reductase. As one of a critical factor in the low catalytic activity, P-450 may be considered in the guinea pig liver, because no change in the AHH activity was detected, whenever mutual change of the purified NADPH-P-450 reductase in the reconstituted enzyme system was applied between guinea pig and rat (data not shown).

DISCUSSION

As we have recently reported (7), the difference spectrum of the reduced hemoprotein-CO complex of liver microsomes from MC-treated guinea pigs has its Soret maximum at about 448 nm. This suggests that treatment of guinea pigs with MC may induce at least one molecular form of P-450 which is different from P-450(s) present in

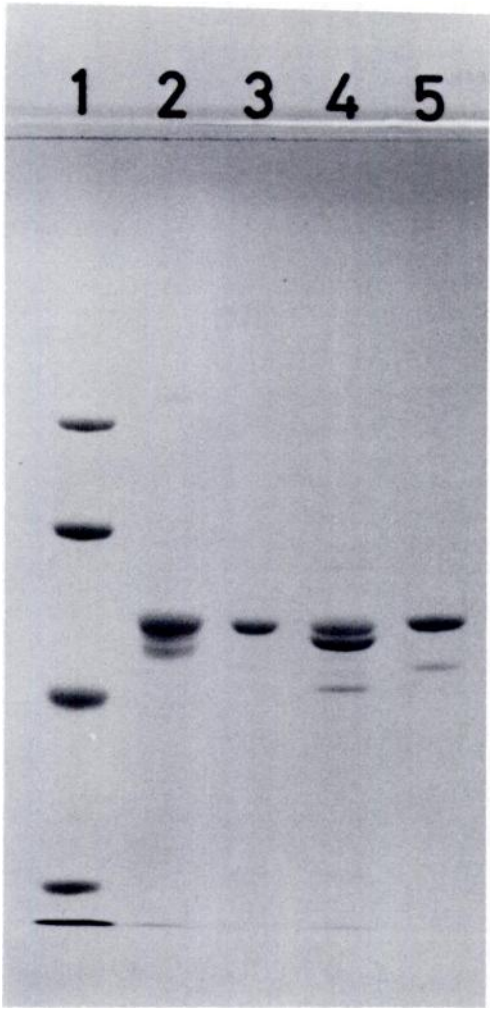

FIG. 5. *SDS/PAGE of P-450s purified from liver microsomes of MC-treated animals*

A purified preparation of each cytochrome was treated and electrophoresed on a polyacrylamide slab gel containing SDS, according to the method of Laemmli (21). Concentrations of acrylamide in the spacer and lower gel were 3.0% and 7.5%, respectively. Treated polypeptides were electrophoresed by a constant current of 20 mamp for 6 hr at room temperature and stained with Coomassie brilliant blue. *Lane 1*, standard proteins for relative molecular mass: phosphorylase *b* (94,000 M_r), albumin (67,000 M_r), ovalbumin (43,000 M_r), carbonic anhydrase (30,000 M_r), and soybean trypsin inhibitor (20,100 M_r [dye front]); *Lane 2*, P-450$_I$ of guinea pig (7.0 μg); *Lane 3*, P-450$_{IIA}$ of guinea pigs (4.0 μg); *Lane 4*, P-450$_{IIB}$ of guinea pigs (6.0 μg); *Lane 5*, P-448 of rats (5.0 μg). Electrophoretic migration is from *top* to *bottom*.

livers of nontreated animals. Therefore, we examined the difference spectrum of each fraction in each step for purification of P-450 from liver microsomes of MC-treated guinea pigs, in order to distinguish the molecular form which is inducible by MC and which has the Soret maximum at a shorter wave length than 450 nm from the others which have the Soret maxima at 450 nm or more longer wavelength. In the course of separation and purification, the fraction which contains P-450 with the Soret maxima at about 448 nm was acquired as only one fraction. It is therefore concluded that the final P-450 preparation eluted from the CM-Sephadex C-50 column, designated as P-450$_{IIA}$, is one of the molecular forms of P-450 induced by MC in guinea pig liver.

Except for P-450$_{IIA}$, we also obtained two other molecular forms of P-450 from liver microsomes of MC-treated guinea pigs, namely P-450$_I$ and P-450$_{IIB}$. These two species of P-450 of guinea pigs are different from each other in their molecular properties such as relative molecular mass on SDS/PAGE (Fig. 5), and different from P-450$_{IIA}$ in their spectral characteristics (Figs. 3 and 4). These results demonstrate that there are at least three molecular forms of P-450 which are different from one another in their molecular properties in liver microsomes prepared from MC-treated guinea pigs. P-450$_{IIA}$ is clearly the protein induced by MC, judging from its absorption maximum of the difference spectrum of the reduced hemoprotein-CO complex (Fig. 3), but it is not clear only from the data presented in this report whether P-450$_I$ and P-450$_{IIB}$ are induced or constitutive molecular forms of P-450.

TABLE 2

Metabolism in reconstituted guinea pig and rat liver cytochrome P-450 systems utilizing NADPH and NADPH-cytochrome P-450 reductase

Assay methods are described under Materials and Methods for the metabolism of the various substrates by the indicated fractions.

Substrate	Guinea pigs			Rats	
	P-450$_I$	P-450$_{IIA}$	P-450$_{IIB}$	P-448	P-450
	moles formed/min/mole P-450				
BP	0.224	0.250	0.395	2.92	ND
AAF	ND[a]	9.4	ND	43.6	ND
Benzphetamine	5.17	6.53	ND	ND	15.2

[a] Not determined.

The molecular activities of P-450$_I$, P-450$_{IIA}$, and P-450$_{IIB}$ for BP hydroxylation in a reconstituted system were not different from each other, and were all about one-tenth that of P-448 of rat liver. Thus it is concluded that the low inducibility of microsomal AHH of guinea pig liver results from the low catalytic activity of P-450 induced in liver in this species.

It seems likely that P-450$_{IIA}$ of guinea pigs has properties similar to a P-450 in rabbit liver, such as P-448, that is induced by MC [Kawalek *et al.* (26)], or Form 4 of P-450, by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [Johnson *et al.* (27)], or P-450$_{LM4}$, by β-naphthoflavone [Haugen *et al.* (28)]. The reactions that this molecular form of P-450 in rabbit liver catalyzes are *N*-hydroxylation of AAF (29), hydroxylation of acetanilide (27), and *O*-deethylation of 7-ethoxycoumarin (27). It has been known that the *N*-hydroxylation of AAF is the first step in the chain of reactions to activate AAF metabolically (2, 3) and that the resistance of guinea pigs to the hepatocarcinogenic action of AAF resides in the lack of, or very low, activity of microsomal monooxygenase of this species to *N*-hydroxylate the compound (3, 8). As shown in Table 2, the catalytic activity of guinea pig P-450$_{IIA}$ for *N*-hydroxylation of AAF in a reconstituted monooxygenase system was very low as compared with that of rat P-448.

In neonatal rabbits, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induces microsomal AHH in the liver, and a P-450 which is different from so-called rabbit P-448, and which has a high catalytic activity for BP hydroxylation, has been separated and purified as Form 6 of P-450 (30). Therefore, there is a possibility that a specific molecular form of P-450 that hydroxylates BP efficiently will appear at a certain developmental stage of guinea pig liver.

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