MOLECULAR PHARMACOLOGY, 9, 372-382

Induction of Drug Metabolism

VII. Differences in P-420 Hemoproteins from Untreated and 3-Methylcholanthrene-Treated Rats

D. W. SHOEMAN,[1] FLOIE M. VANE,[2] AND G. J. MANNERING

Department of Pharmacology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

(Received August 30, 1972)

SUMMARY

SHOEMAN, D. W., VANE, FLOIE M., AND MANNERING, G. J.: Induction of drug metabolism. VII. Differences in P-420 hemoproteins from untreated and 3-methylcholanthrene-treated rats. *Mol. Pharmacol.* **9**, 372-382 (1973).

Cytochromes P-450 and P_1-450 (P-448), found predominantly in hepatic microsomes from untreated and from 3-methylcholanthrene-treated rats, respectively, are reported to be distinct molecular entities. To test this hypothesis, a comparison was made of soluble P-420 hemoproteins obtained from membrane-bound P-450 hemoproteins by digesting microsomes with steapsin. Partially purified, soluble cytochromes P-420 and P_1-420 from microsomes from untreated and 3-methylcholanthrene-treated rats, respectively, were found to differ in their electrophoretic mobilities and in the molar absorbances of their carbon monoxide complexes (P-420, 110 mM^{-1} cm^{-1}; P_1-420, 134 mM^{-1} cm^{-1}); when caused to aggregate, cytochrome P-420 exhibited both type I (hexobarbital) and type II (aniline difference spectra, but aggregated cytochrome P_1-420 exhibited a type II difference spectrum only. That cytochrome P_1-450 is not simply a complex of cytochrome P-450 with 3-methylcholanthrene or its metabolites was demonstrated by the failure of soluble, purified cytochrome P_1-420 from rats treated with tritiated 3-methylcholanthrene to exhibit radioactivity. These studies support the view that cytochromes P-450 and P_1-450 are distinct molecular entities.

INTRODUCTION

A wide variety of drugs and other foreign compounds are known to stimulate the biotransformation of an equally wide variety of drugs and other foreign compounds (1). Of these agents, the polycyclic hydrocarbons form a special group because they exhibit considerable selectivity in the kinds of foreign compounds they cause to be metabolized at increased rates. For example, phenobarbital, the most frequently employed representative of the more general

This research was supported by United States Public Health Service Grant GM 15477. Part of this material appeared in abstract form [*Fed. Proc.* **29**, 738 (1970)] and in a thesis submitted by D. W. Shoeman in partial fulfillment of the requirements for the degree of Doctor of Philosophy to the Department of Pharmacology, University of Minnesota, 1970.

[1] United States Public Health Service Predoctoral Trainee (GM 01117). Present address, Clinical Pharmacology-Toxicology Center, University of Kansas Medical Center, Kansas City, Kansas 66103.

[2] Participant in this study while on leave from the Research Division, Hoffmann-La Roche, Inc., Nutley, New Jersey 07110.

class of inducers, stimulates the microsomal *N*-demethylation of both ethylmorphine and 3-methyl-4-methylaminoazobenzene, whereas the polycyclic hydrocarbons 3-methylcholanthrene and 3,4-benzpyrene stimulate the *N*-demethylation of 3-methyl-4-methylaminoazobenzene only (2–4). This relatively greater specificity of the inducing effects of the polycyclic hydrocarbons suggested not only that more than one mechanism of induction exists, but that more than one hepatic mixed-function oxidase system can exist or can be caused to occur in hepatic microsomes. A clear understanding of the mechanisms whereby these agents produce their inductive effects has not been achieved despite considerable investigative effort, but recent studies from our laboratory (2–9) have shown that the P-450 hemoprotein[3] component of the mixed-function oxidase system seen in microsomes after the administration of 3-methylcholanthrene differs from that seen in microsomes from untreated animals or animals treated with phenobarbital. This species of P-450 hemoprotein was first named cytochrome P_1-450 (2), but has since been referred to by some investigators as cytochrome P-448 because the absorption maximum of its carbon monoxide difference spectrum is at 448 nm rather than at 450 nm (10). Evidence for the existence of cytochrome P_1-450 has appeared in several recent publications (2–17). It is not known whether cytochrome P_1-450 occurs normally in liver and other tissues or whether it comes into being only when the animal is exposed to polycyclic hydrocarbons. If it exists normally in small amounts in the livers of untreated animals as a natural constituent or in response to polycyclic hydrocarbons in the environment, quantities are not great enough to be detected by methods currently available (8).

Cytochrome P-450 from untreated animals and cytochrome P_1-450 from 3-methylcholanthrene-treated animals might differ in their biochemical and physical properties because their proteins differ, because polycyclic hydrocarbons may cause a change in membrane lipids or other membrane constituents, thus causing the hemoprotein to associate differently with the membrane, or because differences in the protein may cause the hemoproteins to associate differently with membrane constituents. Solubilized and partially purified cytochromes P-450 and P_1-450 prepared in our laboratory (9) showed slightly different spectral characteristics. This suggests that the hemoproteins of the two cytochromes differ, but the preparations were not pure enough to exclude the possibility that the solubilized hemoproteins may have remained attached to certain membrane constituents and that a single hemoprotein may have been attached to certain membrane constituents when the source of microsomes was untreated animals, and to other membrane constituents when the source of microsomes was animals treated with 3-methylcholanthrene.

Soluble P-420 hemoproteins, which can be prepared readily from membrane-bound P-450 hemoproteins by a variety of procedures (18, 19), can be obtained in a considerably higher state of purity than has thus far been attainable with P-450 hemoproteins. The current study was undertaken to determine whether inherent differences in cytochromes P-450 and P_1-450—that is, differences not due to associations with membrane constituents—would be retained in their more readily solubilized and purified P-420 hemoprotein derivatives, cytochrome P-420 and cytochrome P_1-420. Disc electrophoretic and spectral binding studies of solubilized, purified P-420 released by

[3] Throughout this communication a P-450 hemoprotein is considered to be any hemoprotein occurring in hepatic microsomes which in its reduced state and combined with carbon monoxide gives a difference spectrum with a λ_{max} in the *neighborhood* of 450 nm. Examples of P-450 hemoproteins are cytochrome P-450, found predominantly in hepatic microsomes from untreated rats, and cytochrome P_1-450 (or P-448), found predominantly in hepatic microsomes from rats treated with 3-methylcholanthrene. P-420 hemoproteins are derived from P-450 hemoproteins; in their reduced state and combined with carbon monoxide they give a difference spectrum with a λ_{max} at about 420 nm. Cytochrome P-420 is derived from cytochrome P-450; cytochrome P_1-420 is derived from cytochrome P_1-450.

steapsin digestion from microsomes from untreated and 3-methylcholanthrene-treated rats provided further evidence that cytochromes P-450 and P_1-450 are distinct chemical entities.

Schenkman and co-workers (20) suggested that polycyclic hydrocarbons or their metabolites may combine with the type I binding site of native cytochrome P-450 to produce a stable complex with the characteristics of cytochrome P_1-450. Although more recent studies show that this possibility is highly unlikely (8), the radioactivity of purified cytochrome P_1-420 from rats treated with tritiated 3-methylcholanthrene was measured in an attempt to resolve the question further.

METHODS

Treatment of animals. Male Holtzman rats (250–300 g) were treated intraperitoneally with 3-methylcholanthrene or with tritiated 3-methylcholanthrene (1 or 10 μCi/mg) dissolved in corn oil at a dose level of 20 mg/kg/day for 4 days. Control animals were given only corn oil. Animals were killed 20 hr after the last injection.

Preparation of purified P-420 hemoproteins. Microsomes were prepared as described previously (3) from pooled livers (200 g) which had been perfused *in situ* with cold 1.15% KCl solution to remove blood. Microsomes were suspended in phosphate buffer (0.1 M, pH 7.4) in a concentration equivalent to 1 g of fresh liver per milliliter of suspension. Cytochrome P-450 was partially converted to soluble cytochrome P-420 by adding steapsin (Sigma grade II) to the suspension to a final concentration of 0.07%, flushing with nitrogen, and incubating under nitrogen for 24 hr at 4°. The mixture was centrifuged at 105,000 $\times$ g_{max} for 1 hr. The supernatant fraction was desalted by passage through a Sephadex G-25 column (5 $\times$ 45 cm) and evaporated under reduced pressure to about one-fourth its volume in a rotary evaporator equipped with a condenser cooled with ice water (temperature of water bath, 40°). Aggregates of P-420 hemoprotein that formed as a result of the desalting and concentrating processes (21) were collected by centrifugation at 105,000 $\times$ g_{max} for 1 hr. This pellet was the source of P-420 hemoproteins in the electrophoresis studies. In several experiments the desalted, concentrated fraction was not centrifuged; instead, sucrose was added (final concentration, 0.5 M); the suspension was layered over a 1, 1.5, and 2 M discontinuous sucrose gradient and centrifuged under refrigeration for 16 hr at 26,000 rpm in a Spinco SW 27 rotor. P-420 hemoprotein was found in the material which formed a band above the 1.5 M layer.

Disc electrophoresis of P-420 hemoproteins. Disc electrophoresis of the purified, aggregated P-420 cytochromes was conducted as described by Ornstein (22) and by Davis (23), except that ammonium persulfate was used to catalyze polymerization rather than riboflavin, because P-420 hemoprotein was found to be unstable to light in the presence of riboflavin; also, urea was incorporated in the gels to prevent the formation of immobile particulate P-420 hemoprotein, which forms in solutions of low ionic strength (21). Currents of 4 mamp and 12–20 mamp were used in the analytical and preparative apparatus, respectively. Samples were dissolved in 8 M urea solution. Five-milliliter volumes of both upper and lower gels were employed in the preparative apparatus (Canalco), with a sample volume of 2.5 ml. The lowest 5 cm of the gel chamber were etched with hydrofluoric acid to aid in the retention of the urea-containing gels. The eluate was passed into a fraction collector through a Gilson ultraviolet absorbance monitor equipped with a 280-nm filter. Protein-containing bands in the analytical gels were revealed by staining with Amido black. Heme-containing bands were detected using the benzidine reaction.

Determination of 3-methylcholanthrene. The pellets remaining after digestion with 0.07% steapsin or the purified, particulate P-420 hemoprotein obtained from microsomes from rats treated with 3-methylcholanthrene or tritiated 3-methylcholanthrene were examined for their contents of 3-methylcholanthrene, using a combination of thin-layer and gas-liquid chromatography by procedures similar to those used by Gammal *et al.* (24) for the determination of 7,12-

dimethylbenz[a]anthracene. Aqueous suspensions (1–5 ml) of these preparations were acidified to pH 1 with concentrated HCl and extracted three times with 2 volumes of ether. The ether extracts were dried over anhydrous sodium sulfate contained in a 50-ml Erlenmeyer flask. The decanted ether was evaporated in a conical tube under nitrogen on a water bath (50°). The residue was dissolved in about 50 μl of ethanol and transferred to thin-layer chromatographic plates coated with silica gel (Brinkmann precoated or Kontes preparative) which had been activated for 30 min at 105°. The chromatograms were developed at room temperature with benzene. 3-Methylcholanthrene was located under ultraviolet light. The areas of gel containing 3-methylcholanthrene were scraped from the plate and extracted with 3 ml of ethanol which was evaporated to dryness in a conical tube. The residue was dissolved in 25 μl of ethanol, 5 μl of which were then injected into a Barber-Coleman gas chromatograph equipped with an argon ionization detector. The 6 foot × 5 mm column was packed with 2% OV-210 on Gas-Chrom Q and operated at 240°. The flash heater and detector were maintained at 260°. The column was loaded with 50 μg of 3-methylcholanthrene prior to each analysis. A standard curve derived from known amounts of 3-methylcholanthrene spotted on the thin-layer plates was determined daily. Recovery of 3-methylcholanthrene added to tissue was determined to be about 65%.

Determination of radioactivity. Exactly 0.25 ml of suspensions of microsomes or fractions of microsomes from rats treated with tritiated 3-methylcholanthrene, which contained 1–2 mg of protein per milliliter, was dissolved in 1 ml of Nuclear-Chicago Solubilizer and allowed to stand for 1 hr with occasional shaking. In order to prevent quenching, heme was bleached by adding 30 μl of glacial acetic acid (analytical reagent) and 20 μl of 30% hydrogen peroxide (analytical reagent) and allowing the mixture to stand for an additional hour. Fifteen milliliters of toluene containing 0.4% 2,5-diphenyloxazole and 0.05% *p*-bis[2-(5-phenyloxazolyl)]benzene (Packard Instrument Company) were added, and the activity was determined in a Packard Tri-Carb scintillation counter. External standard ratio-efficiency correlations were determined using microsomes from untreated rats and tritiated toluene or tritiated 3-methylcholanthrene as standards, with nitromethane as a quenching agent. Absolute activities were calculated from the correlation and the automatic external standard ratio with the aid of a computer, using a modification of the program written by Grower and Bransome (25). Samples were counted for a length of time which ensured a statistical error of no more than 5%.

Determination of heme. The heme content of microsomes and fractions of microsomes was determined by the method of Omura and Sato (26). Recovery of hemin chloride added to the preparations showed that the preparations did not interfere with the assay of hemin.

Determination of cytochrome b_5. The cytochrome b_5 content of purified P-420 hemoprotein preparations was determined using cytochrome b_5 reductase prepared by the method of Strittmatter and Velick (27). NADH reduction of cytochrome b_5 was coupled to the reduction of added cytochrome *c* in the presence of excess reductase. As little as 0.006 nmoles of cytochrome b_5 can be determined by this procedure. Crude cytochrome b_5 used in the assay was prepared by the method of Sargent and Vadlamudi (28) and assayed by the method of Omura and Sato (26).

Determination of carbon monoxide and drug binding spectra. The carbon monoxide difference spectra of reduced P-450 and P-420 hemoproteins were determined by the method of Omura and Sato (26) as described previously (3). The difference spectrum produced by the binding of aniline or hexobarbital to particulate P-420 hemoprotein was determined by the method of Remmer *et al.* (29) as described previously (6).

RESULTS

Properties of P-420 hemoproteins from untreated and 3-methylcholanthrene-treated rats. The results of a typical fractionation

TABLE 1

Purification of P-420 hemoproteins from untreated and 3-methylcholanthrene-treated rats
Data were obtained from a typical experiment.

Fraction[a]	Source of microsomes			
	Untreated rats		3-Methylcholanthrene-treated rats	
	Recovery of P-420	P-420 content	Recovery of P-420	P-420 content
	%	*nmoles/mg protein*	%	*nmoles/mg protein*
Supernatant after steapsin treatment	43		35	
Eluate from Sephadex G-25	43	1.9	35	0.41
Aggregated P-420 hemoprotein	6.7	10.3	2.2	7.6
P-420 hemoprotein from sucrose gradient fractionation	3.1	12.4	1.4	9.8

[a] The fractions can be identified from the description of the purification procedure given in METHODS.

[b] Percentage recovery = [(nmoles of P-420 hemoprotein in the fraction)/(nmoles of P-450 hemoprotein in unfractionated microsomes)] × 100. The amounts of P-450 hemoprotein from microsomes from 200 g of wet liver from untreated and 3-methylcholanthrene-treated rats were 2520 and 5560 μmoles, respectively. Calculations of the P-450 hemoprotein content of microsomes from both untreated and 3-methylcholanthrene-treated rats were based on a millimolar extinction coefficient of 91 mM^{-1} cm^{-1} (30); calculations of the P-420 hemoprotein content of the fractions were based on a millimolar extinction coefficient of 110 mM^{-1} cm^{-1} (30). Values for P-420 from microsomes from rats treated with 3-methylcholanthrene can be recalculated to accommodate the assumption that P-420 hemoprotein from these microsomes is P_1-420 by substituting an extinction coefficient of 134 for 110 mM^{-1} cm^{-1} (see the text).

study are summarized in Table 1. The aggregated material contained a high concentration of P-420 hemoprotein. The large loss of P-420 hemoprotein could be almost accounted for in the supernatant fractions obtained from centrifugation of the aggregated material. Although there was abundant cytochrome b_5 in the supernatant fraction recovered from the aggregates, none was detectable in the aggregates from either source of microsomes using the sensitive enzymatic method described in METHODS. The absence of cytochrome b_5 enabled determinations of the extinction coefficients of the P-420 hemoproteins. The extinction of P-420 hemoprotein from untreated rats (cytochrome P-420) was calculated on the basis of its heme content and $\Delta A_{420} - A_{500}$ to be 110 ± 4 mM^{-1} cm^{-1}, which agrees with the value determined by Omura and Sato (30). The extinction of P-420 hemoprotein from 3-methylcholanthrene-treated rats (cytochrome P_1-420) was calculated to be 134 ± 9 mM^{-1} cm^{-1}. The two extinction coefficients differ significantly ($t = 2.7$, $df = 12$, $p < 0.05$). It has been reported previously that the absolute oxidized, reduced, and reduced carbon monoxide spectra of cytochromes P-420 and P_1-420 do not show qualitative differences (31).

A mean concentration of about 15 nmoles of cytochrome P-420 per milligram of protein was observed in the particulate material obtained by sucrose gradient fractionation. This is about 2.5 times more concentrated than the purified cytochrome P-420 obtained by Omura and Sato (31). In a preparation of cytochrome P-420 containing 15 nmoles of heme per milligram of protein, the molecular weight of cytochrome P-420 cannot exceed 70,000.

Aggregated cytochrome P-420 is in the form of microtubules (21). It exhibited a typical type I (hexobarbital) binding spectrum with λ_{max} = 385 nm, λ_{min} = 429 nm, and an isosbestic point at 405 nm (Fig. 1). It also gave a type II (aniline) binding spectrum with typical λ_{max} and λ_{min} values of 385 nm and 429 nm, respectively, but it was more symmetrical than that usually seen with microsomes and thus its isosbestic point was at 400 nm rather than at 420 nm. Electron micrographs showed the aggregates of cytochrome P_1-420 to be amorphous, with only rare incidence of microtubules,

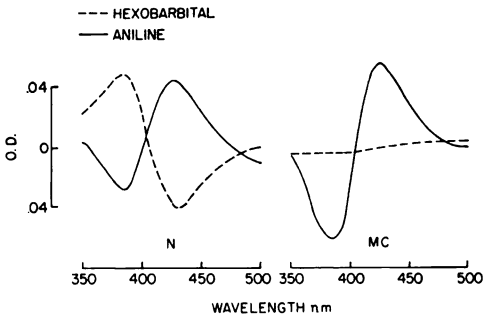

FIG. 1. *Drug binding to aggregated P-420 hemoproteins from hepatic microsomes from untreated (N) and 3-methylcholanthrene (MC)-treated rats*

Microsomes from a given source were placed in reference and sample cuvettes (1 mg of protein per milliliter), and a baseline of equal light absorbance at all wavelengths was established using a dual-beam spectrophotometer. Spectra were recorded after addition of saturating amounts of the type I compound (hexobarbital; final concentration, 1.6 mM) or the type II compound (aniline; final concentration, 10 mM) to the sample cuvette. The aggregated cytochrome P-420 preparation contained 7.7, and the aggregated cytochrome P_1-420 preparation, 8.1 nmoles of P-420 hemoprotein per milligram of protein, based on an extinction coefficient of 110 mM^{-1} cm^{-1}.

which conceivably could have been due to the presence of small amounts of cytochrome P-420. These aggregates showed a type II difference spectrum but no type I difference spectrum (Fig. 1). The magnitudes (ΔA/[P-420]) of the binding spectra produced with P-420 hemoproteins are about one-fourth those seen with membrane-bound P-450 hemoproteins.

Electrophoresis of P-420 hemoproteins. Figure 2 illustrates the analytical scale disc electrophoresis of aggregated P-420 hemoproteins from microsomes from untreated and 3-methylcholanthrene-treated rats dissolved in 8 M urea solution, and of a mixture of the two preparations. Cytochrome P-420 and cytochrome P_1-420 migrated at different rates. That the bands represented P-420 hemoproteins was verified by preparative disc electrophoresis, in which large enough quantities of the cytochromes were collected in the eluates to permit their characterization spectrally. Typical results obtained with a preparation of cytochrome P-420 are shown in Fig. 3. Similar results were observed with the cytochrome P_1-420 preparation. Preparative disc electrophoresis did not improve the purity of the P-420 cytochromes, and recoveries of the cytochromes after electrophoresis were poor (Table 2).

Binding of 3-methylcholanthrene to microsomes and to purified cytochrome P_1-420. Microsomes from 3-methylcholanthrene-treated rats were analyzed for their content of 3-methylcholanthrene using a method which combined thin-layer and gas-liquid chromatography as described in METHODS. The molar ratio of 3-methylcholanthrene to P-450 hemoprotein was found to be 0.085 ± 0.005. This is about twice that estimated by Schenkman *et al.* (20) from the absorbance of methylcholanthrene-like material extracted from microsomes. Microsomes and aggregated cytochrome P_1-420 from animals which had received tritium-labeled 3-methylcholanthrene were analyzed for radioactivity. The microsomes contained radioactivity equivalent to 0.094 ± 0.007 mole[4] of 3-methylcholanthrene per mole of cytochrome P_1-450. The amount of 3-methylcholanthrene in microsomes measured directly did not differ significantly from that estimated from measurement of radioactivity. No radioactivity was detected in the aggregated cytochrome P_1-420 prepared from these microsomes. Consideration of the confidence limits of the 3-methylcholanthrene analyses places an upper limit of 0.02 for the molar ratio of metabolites of 3-methylcholanthrene to P-450 hemoprotein in microsomes from 3-methylcholanthrene-treated rats.

Microsomes were incubated with tritiated 3-methylcholanthrene to determine what effect the compound might have on the binding spectra of cytochrome P-450. Microsomes from 50 g of liver were incubated at 35° for 30 min in 0.05 M phosphate buffer (pH 7.4) containing tritiated 3-

[4] This value was obtained by using the extinction coefficient for cytochrome P-420 (110 mM^{-1} cm^{-1}); if the extinction coefficient for cytochrome P_1-420 (134 mM^{-1} cm^{-1}) derived in the text is used, the value becomes 0.112 mole.

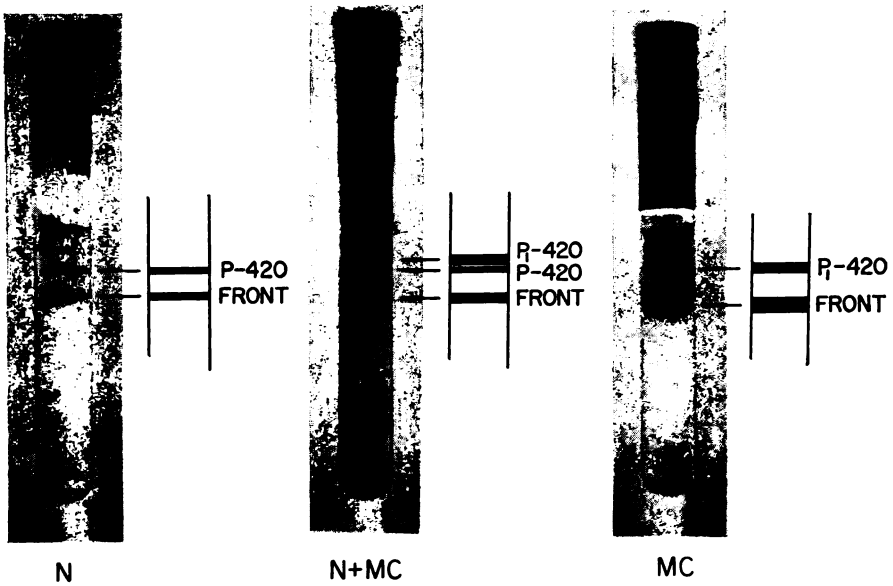

FIG. 2. *Analytical scale disc electrophoresis of P-420 hemoproteins*

Aggregated P-420 hemoprotein preparations from the livers of untreated (N) and 3-methylcholanthrene (MC)-treated rats were dissolved in 8 M urea. Duplicate samples of each preparation, as well as mixtures of the two (N + MC), were subjected to disc electrophoresis as described in the text. One of the gels was stained to reveal proteins; the other, to reveal heme. The photographs are of the protein-stained gels; the diagrams are composite representations of bands revealed by both staining procedures. The lowest band (shown in both the photograph and the diagrams) did not combine with carbon monoxide; the highest band (shown in the photograph but not in the diagram) contained no heme. The dark masses at the tops of the gels seen in the photographs are stacking gels.

methylcholanthrene (0.05 M) which had been emulsified by sonication (total volume of incubation mixture, 25 ml). After incubation, the microsomes were recovered by centrifugation at 100,000 × *g* for 60 min. The microsomes contained 3.3 moles of 3-methylcholanthrene per mole of P-450 hemoprotein. This is about 30 times that found in microsomes from animals treated with tritiated 3-methylcholanthrene; yet the carbon monoxide, ethyl isocyanide, and type I binding (hexobarbital) spectra shown by these microsomes were no different qualitatively or quantitatively from those produced by control microsomes, thus demonstrating that it is not possible to convert cytochrome P-450 to a P-450 hemoprotein with spectral characteristics similar to cytochrome P_1-450 by incubating microsomes with 3-methylcholanthrene. Similar results were obtained by Gnosspelius and co-workers (32) using 3,4-benzpyrene.

DISCUSSION

The view that cytochrome P_1-450 is a species of P-450 hemoprotein distinguishable from cytochrome P-450 is based on several kinds of evidence.

1. The relative sizes of the 430 and 455 nm maxima seen in the spectrum produced by the ethyl isocyanide complex with reduced membrane-bound cytochrome P-450 differ from those seen with membrane-bound cytochrome P_1-450 (2, 4, 15). The locations of the second spectral peaks of the two cytochromes differ slightly; with cytochrome P-450 the maximum is at 455 nm, and with cytochrome P_1-450 it is at 453 nm (14). This difference was also observed in soluble preparations of the two cytochromes (9, 31).

2. The maximum of the spectrum produced by the carbon monoxide complex with reduced membrane-bound cytochrome P-450 is seen at 450 nm; with membrane-bound

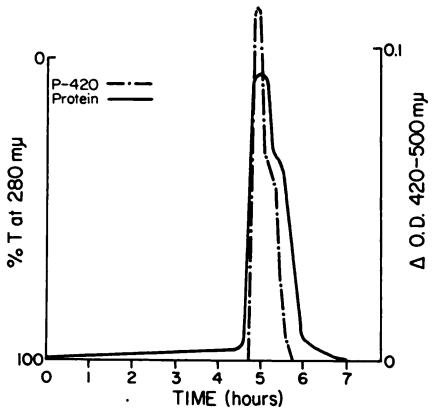

FIG. 3. *Preparative scale disc electrophoresis of cytochrome P-420*

Aggregated cytochrome P-420 from livers of untreated rats was dissolved in 8 M urea and subjected to disc electrophoresis as described in the text. The apparatus was flushed at a rate of 1 ml/min, and the eluate was passed through an ultraviolet flow monitor into a fraction collector. The output of the flow monitor (——) and the magnitude of the carbon monoxide difference spectrum (-·-·-) of the corresponding fraction are plotted as functions of time.

TABLE 2

Preparative disc electrophoresis of P-420 hemoproteins from untreated (cytochrome P-420) and 3-methylcholanthrene-treated (cytochrome P_1-420) rats

Details of the procedure are given in METHODS. Results were derived from a typical experiment.

Cytochrome	P-420 hemoprotein applied to column		P-420 hemoprotein recovered in eluate	
	mg protein	*nmoles cytochrome*	*mg protein*	*nmoles cytochrome*
P-420	20	105	13	21
P_1-420	39	140	26	20

cytochrome P_1-450 it is seen at 448 nm (10). This difference was also observed in soluble preparations of the two cytochromes (9, 31).

3. Membrane-bound cytochrome P-450 produces a type I binding spectrum with hexobarbital; membrane-bound cytochrome P_1-450 does not (6). This difference was also observed in soluble preparations of the two cytochromes (9, 31).

4. The spectral extinction coefficient of soluble cytochome P-450 is about 25% lower than that of soluble cytochrome P_1-450 (9, 31).

The current study adds to this evidence by showing that the soluble, partially purified P-420 hemoprotein derivatives of cytochromes P-450 and P_1-450 differ in their electrophoretic mobilities and in their spectral extinction coefficients. Aggregated P-420 hemoproteins from the two sources also differed in drug binding characteristics; aggregated cytochrome P-420 showed both type I (hexobarbital) and type II (aniline) difference spectra whereas cytochrome P_1-420 showed the type II difference spectrum only. This is in keeping with what is known about the binding characteristics of membrane-bound cytochrome P_1-450, namely, that it is lacking or highly deficient in a type I binding site, at least for certain type I compounds such as hexobarbital (6, 8). It is of some interest that cytochrome P-420 shows a type I difference spectrum only when in the aggregated state. Whether more or fewer specific structural conformities produced by aggregation are required for spectral binding, or whether aggregation simply causes a very high local concentration of cytochrome that may be required before binding spectra become detectable, is a matter for conjecture. In a previous study (21) it was observed that cytochrome P-420 aggregated in the form of uniform microtubules, whereas aggregates of P_1-420 were amorphous. This might suggest that aggregates of P-420 hemoproteins must be in the microtubular form in order to show a type I difference spectrum. This is not the case, however, because aggregates of cytochrome P-420 from another species, the cat, did not assume tubular forms, and these amorphous aggregates produced the type I spectrum with hexobarbital.[5]

Much evidence has accumulated to show that cytochrome P_1-450 is not simply a relatively stable complex of cytochrome P-450 with 3-methylcholanthrene or one or more of its metabolic derivatives (8). One of the more convincing arguments supporting this view was the observation that purified,

[5] D. W. Shoeman and G. J. Mannering, unpublished observations.

soluble cytochrome P_1-450 from rats treated with tritiated 3-methylcholanthrene contained radioactivity equivalent to only 0.04 mole of 3-methylcholanthrene per mole of P-450 hemoprotein (9, 31). However, the possibility remained that even this small amount of the polycyclic hydrocarbon in combination with cytochrome P-450 could have accounted for the small differences seen in spectral characteristics of the impure cytochromes P-450 and P_1-450 preparations. The current finding that cytochrome P_1-450 from animals treated with tritiated 3-methylcholanthrene is devoid of radioactivity within the limits of measurement, while still differing from cytochrome P-420 in its electrophoretic migration, binding to drugs, and spectral extinction coefficient, strengthens the previous conclusion that cytochrome P_1-450 is a distinct chemical entity not to be equated with a complex of polycyclic hydrocarbon and cytochrome P-450.

Hildebrandt and Estabrook (12) concluded that treatment of animals with 3-methylcholanthrene caused an increase in microsomal cytochrome P-446 and that this hemoprotein had an extinction coefficient of 218 mM^{-1} cm^{-1}. Microsomes were also thought to contain cytochrome P-454 with an extinction coefficient of 56 mM^{-1} cm^{-1}. The absorption maximum of the spectrum observed at 450 nm was considered to result from the sum of the spectra produced by P-446 and P-454. If P-446 is P_1-450, as implied, and we accept 91 mM^{-1} cm^{-1} as the extinction coefficient for cytochrome P-450 (30), then one would have expected the extinction coefficient of P_1-420 to be about twice that of P-420 rather than the 120% observed in the current study. While it can be argued that changes in the extinction coefficient of P_1-450 may not be reflected in P_1-420, or that this value may be attenuated, it should be pointed out that the technique of Kinoshita and Horie (33), as used by Hildebrandt and Estabrook, does not enable the determination of the extinction coefficient of specific P-450 hemoproteins when they occur together in microsomes. By this technique, spectra are recorded in a double-beam spectrophotometer with microsomes from 3-methylcholanthrene-treated animals in the sample cuvette and microsomes from untreated animals in the reference cuvette. By diluting the preparations to contain the same concentration of cytochrome b_5, the only cytochrome other than P-450 hemoproteins assumed to be present in microsomes in meaningful concentration, it was believed that the resulting difference spectrum represented the absolute spectrum of the induced cytochrome (P-446 or P_1-450). The extinction was then calculated on the basis of heme content. The following considerations demonstrate why this kind of experiment cannot yield extinction coefficients for two hypothetical species of cytochromes contained in the same preparation of microsomes, assuming that they do exist. The only data provided in these studies were the change in the difference optical density on the addition of carbon monoxide to reduced microsomes in both cuvettes, cytochrome b_5 concentrations in the preparations, and total heme content. If we let P^u_{446}, P^m_{446} and P^p_{446} equal the concentrations of cytochrome P-446 in microsomes from untreated, 3-methylcholanthrene-treated, and phenobarbital-treated animals, respectively; P^u_{454}, P^m_{454}, and P^p_{454}, the concentrations of cytochrome P-454 in microsomes from untreated, 3-methylcholanthrene-treated, and phenobarbital-treated animals, respectively; X, the extinction coefficient of P-446; and Y, the extinction coefficient of P-454, then the following relationships are obtained with the technique of Kinoshita and Horie, using the three sources of microsomes.

From difference spectra determinations:

$$\mathrm{OD} = P^m_{446}(X) + P^m_{454}(Y) - [P^u_{446}(X) + P^u_{454}(Y)] \quad (1)$$

$$\mathrm{OD} = P^p_{446}(X) + P^p_{454}(Y) - [P^u_{546}(X) + P^u_{454}(Y)] \quad (2)$$

From heme and cytochrome b_5 analyses: For microsomes from untreated animals,

$$P^u_{446} + P^u_{454} = [\text{heme}] - [b_5] \quad (3)$$

For microsomes from 3-methylcholanthrene-treated animals,

$$P^m_{446} + P^m_{454} = [\text{heme}] - [b_5] \quad (4)$$

For microsomes from phenobarbital-treated animals,

$$P^{p}_{446} + P^{p}_{454} = [\text{heme}] - [b_5] \qquad (5)$$

Because there are five equations involving eight unknowns a unique solution is not possible and no basis is apparent to us for the result that x equals 218.

Jefcoate and Gaylor (34) obtained an extinction coefficient of 143 mM^{-1} cm^{-1} for microsomal particles from 3-methylcholanthrene-treated rabbits which had been freed of cytochrome b_5. If we assume that microsomes from 3-methylcholanthrene-treated animals contain only cytochrome P_1-450, then the number of unknowns in Eqs. 1–5 is reduced and a unique solution for the extinction coefficient of cytochrome P_1-450 exists. Using the data of Hildebrandt and Estabrook, the extinction coefficient is calculated to be 138 mM^{-1} cm^{-1}. Thus the extinction coefficient of 143 observed by Jefcoate and Gaylor is consistent with the hypothesis that almost all the P-450 hemoprotein in their microsomal particles was cytochrome P_1-450.

REFERENCES

1. G. J. Mannering, *in* "Selected Pharmacological Testing Methods" (A. Burger, ed.), pp. 51–119. Dekker, New York, 1968.
2. N. E. Sladek and G. J. Mannering, *Biochem. Biophys. Res. Commun.* **24**, 668–674 (1966).
3. N. E. Sladek and G. J. Mannering, *Mol. Pharmacol.* **5**, 174–185 (1969).
4. N. E. Sladek and G. J. Mannering, *Mol. Pharmacol.* **5**, 186–199 (1969).
5. G. J. Mannering, N. E. Sladek, C. J. Parli, and D. W. Shoeman, *in* "Microsomes and Drug Oxidations" (J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts, and G. J. Mannering, eds.), pp. 303–330. Academic Press, New York, 1969.
6. D. W. Shoeman, M. D. Chaplin, and G. J. Mannering, *Mol. Pharmacol.* **5**, 412–419 (1969).
7. C. J. Parli and G. J. Mannering, *Mol. Pharmacol.* **6**, 178–183 (1970).
8. G. J. Mannering, *Metabolism* **20**, 228–245 (1971).
9. T. Fujita and G. J. Mannering, *Chem.-Biol. Interactions* **3**, 264–265 (1971).
10. A. P. Alvares, G. Schilling, W. Levin, and R. Kuntzman, *Biochem. Biophys. Res. Commun.* **29**, 521–526 (1967).
11. R. Kuntzman, W. Levin, M. Jacobson, and A. H. Conney, *Life Sci.* **7**, 215–224 (1968).
12. A. G. Hildebrandt and R. W. Estabrook *in* "Microsomes and Drug Oxidations" (J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts, and G. J. Mannering, eds.), pp. 331–347. Academic Press, New York, 1969.
13. A. Hildebrandt, H. Remmer, and R. W. Estabrook, *Biochem. Biophys. Res. Commun.* **30**, 607–612 (1968).
14. A. P. Alvares, G. Schilling, W. Levin, and R. Kuntzman, *J. Pharmacol. Exp. Ther.* **163**, 417–424 (1968).
15. K. Bidleman and G. J. Mannering, *Mol. Pharmacol.* **6**, 697–701 (1970).
16. Y. Imai and P. Siekevitz, *Arch. Biochem. Biophys.* **144**, 143–159 (1971).
17. A. Y. H. Lu, R. Kuntzman, S. West, M. Jacobson, and A. H. Conney, *J. Biol. Chem.* **247**, 1727–1734 (1972).
18. Y. Imai and R. Sato, *Eur. J. Biochem.* **1**, 419–426 (1967).
19. G. J. Mannering, *in* "Fundamentals of Drug Metabolism and Drug Disposition" (B. N. La Du, H. G. Mandel, and E. L. Way, eds.), pp. 206–252. Williams & Wilkins, Baltimore, 1971.
20. J. B. Schenkman, H. Greim, M. Zange, and H. Remmer, *Biochim. Biophys. Acta* **171**, 23–31 (1969).
21. D. W. Shoeman, J. G. White, and G. J. Mannering, *Science* **165** 1371–1372 (1969).
22. L. Ornstein, *Ann. N. Y. Acad. Sci.* **121**, 321–349 (1964).
23. B. J. Davis, *Ann. N. Y. Acad. Sci.* **121**, 404–427 (1964).
24. E. B. Gammal, K. K. Carroll, B. H. Muhlstock, and E. R. Plunkett, *Proc. Soc. Exp. Biol. Med.* **119**, 1087–1089 (1965).
25. M. F. Grower and E. D. Bransome, *Anal. Biochem.* **31**, 159–173 (1969).
26. T. Omura and R. Sato, *J. Biol. Chem.* **239**, 2370–2378 (1964).
27. P. Strittmatter and S. F. Velick, *J. Biol. Chem.* **221**, 277–286 (1956).
28. J. R. Sargent and B. P. Vadlamudi, *Biochem. J.* **107**, 839–849 (1968).
29. H. Remmer, J. Schenkman, R. W. Estabrook, H. Sasame, J. Gillette, S. Narasimhulu, D. Y. Cooper, and O. Rosenthal, *Mol. Pharmacol.* **2**, 187–190 (1966).

30. T. Omura and R. Sato, *J. Biol. Chem.* **239**, 2379–2385 (1964).
31. T. Fujita, D. W. Shoeman, and G. J. Mannering, *J. Biol. Chem.* **248**, 2192–2201 (1973).
32. Y. Gnosspelius, H. Thor, and S. Orrenius, *Chem.-Biol. Interactions* **1**, 125–137 (1969–1970).
33. T. Kinoshita and S. Horie, *J. Biochem.* **61**, 26–34 (1967).
34. C. R. E. Jefcoate and J. L. Gaylor, *Biochemistry* **8**, 3464–3472 (1969).