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Resolution of the Cytochrome P-450-containing ω-Hydroxylation System of Liver Microsomes into Three Components*

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SUMMARY

The enzyme system in liver microsomes which catalyzes the ω-hydroxylation of fatty acids in the presence of molecular oxygen and a reduced pyridine nucleotide has been solubilized and resolved into fractions containing cytochrome P-450, a cytochrome P-450 reductase, and a heat-stable factor. The apparent K_m values of TPNH and DPNH are 2.0×10^{-5} M and 6.3×10^{-4} M, respectively. As a further indication of the role of cytochrome P-450, ω-hydroxylation is enhanced in microsomes prepared from animals previously treated with phenobarbital and is inhibited in the presence of carbon monoxide, with partial reversal of the inhibition by exposure to light.

The soluble preparation of cytochrome P-450 exhibits a carbon monoxide difference spectrum, an electron paramagnetic resonance spectrum, and substrate difference spectra similar to those previously attributed to the microsome-bound form. The difference spectrum obtained in the presence of laurate, with a peak at about 388 mμ and a trough at 419 mμ, is of the type given by hexobarbital rather than aniline. The K_s of laurate, determined from difference spectra, is 6.3×10^{-4} M, and the K_m, determined in the complete hydroxylation system, is 4.4×10^{-5} M. The partially purified reductase was shown to catalyze electron transfer from TPNH to cytochrome P-450 under anaerobic conditions. The TPNH-cytochrome *c* reductase activity of the enzyme preparations was found to be unrelated to their ability to reduce cytochrome P-450, and therefore to function in the hydroxylation system. The activity of the heat-stable fraction is attributed to a lipid component which enhances the rate of the hydroxylation reaction.

The liver microsomal enzyme system which catalyzes the ω-hydroxylation of fatty acids (3–5) has recently been obtained in a soluble form and resolved into three fractions: cytochrome P-450, TPNH-cytochrome *c* reductase, and a heat-stable component (6). P-450, the carbon monoxide-binding pigment of microsomes (7), was previously recognized as a component of the microsomal mixed function oxidase leading to the hydroxylation of steroids and the oxidative demethylation and hydroxylation of drugs (8–10), but earlier attempts to accomplish its solubilization had led to the extensive formation of P-420 (7), an altered form of the pigment possessing no hydroxylation activity.

The present paper is concerned with a comparison of the properties of our solubilized preparation of P-450 and those known to be characteristic of the microsomal bound form. Evidence is presented that the reductase fraction functions in the transfer of electrons from reduced pyridine nucleotide to P-450 and that the activity of the heat-stable fraction is due to a lipid component. The data in the present paper indicating that microsomal ω-hydroxylation is inhibited by carbon monoxide and stimulated by the prior administration of phenobarbital to the animals document our earlier report on the role of P-450 (6) and are in full accord with similar results recently presented by Das, Orrenius, and Ernster (11). Wada *et al.* (12) have also described the inhibition of microsomal ω-oxidation by CO but reported that phenobarbital treatment decreased stearate ω-hydroxylation.

EXPERIMENTAL PROCEDURE

Assay of Fatty Acid Hydroxylation—The activity of enzyme preparations in catalyzing fatty acid ω-hydroxylation was determined as follows. A reaction mixture containing 100 μmoles of potassium phosphate buffer, pH 7.7, 0.5 μmole of TPNH, 0.4 μmole of laurate-1-^{14}C (1.0×10^5 cpm), and the necessary microsomal fractions in a final volume of 1.0 ml was incubated for 20 min at 30°. When intact microsomes were used, the TPNH was replaced by a TPNH-generating system containing 1.0 μmole of TPN, 5 μmoles of glucose-6-P, and 0.2 unit (13) of glucose-6-P dehydrogenase. The radioactive ω-hydroxylaurate formed was isolated by silicic acid chromatography (14) and counted in a scintillation counter. The rate of the reaction was found to be proportional to the concentration of microsomal protein, as shown in Fig. 1, and constant for at least 30 min with either intact microsomes or the fractionated microsomal system, to be described, as shown in Fig. 2. The resolved enzyme system has a broad pH optimum in Tris buffer with maximal activity at about 7.4 and a pH optimum in phosphate buffer at about 7.6 (Fig. 3). Similar pH optima were observed with microsomal suspensions, but the specific activity in Tris buffer was only about half that in phosphate buffer. The addition of KCl, final concentration 0.05 M, to the Tris buffer increased the ac-

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tivity to that in phosphate, suggesting that the ionic strength of the microsomal suspension was too low for optimal activity. Presumably the reductase is the component affected, for Phillips and Langdon (15) have shown that the rate of reduction of cytochrome *c* or of dichlorophenolindophenol by this enzyme is enhanced at high ionic strength.

Assay of TPNH-cytochrome C Reductase—The activity of the microsomal reductase fraction in reducing cytochrome *c* was estimated by a procedure based on that of Masters, Williams, and Kamin (16). The reaction mixture contained 0.1 μmole of TPNH, 0.04 μmole of cytochrome *c*, the reductase sample, and a sufficient amount (0.7 to 0.8 ml) of 0.05 M potassium phosphate buffer, pH 7.7, containing 1.0×10^{-4} M EDTA, to bring the final volume to 1.0 ml. The ionic strength in such mixtures was adequate for optimal activity. The reaction was initiated by the addition of TPNH and followed at 550 mμ at 25° with a spectrophotometer equipped with a Gilford multiple sample absorbance recorder. The rate of cytochrome *c* reduction was found to be

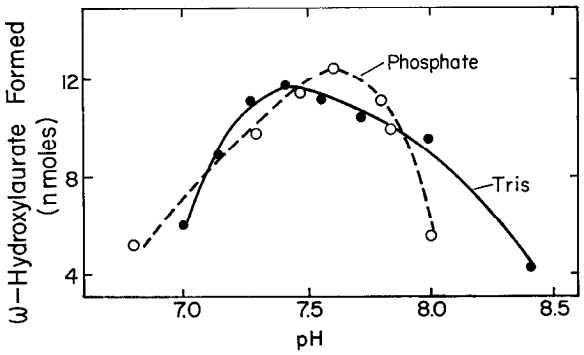

FIG. 3. ω-Hydroxylation as a function of pH. A resolved microsomal system containing P-450 and reductase fractions from DEAE-cellulose chromatography (0.40 and 0.18 mg of protein, respectively) and Fraction B (0.1 mg of lipid) was incubated with 0.1 M Tris or phosphate buffer. The pH values were measured in the complete reaction mixtures at 30°.

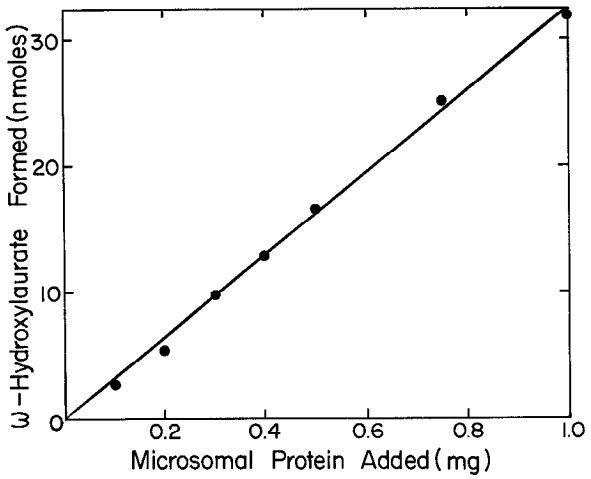

FIG. 1. Laurate ω-hydroxylation as a function of protein concentration in KCl-washed liver microsomes.

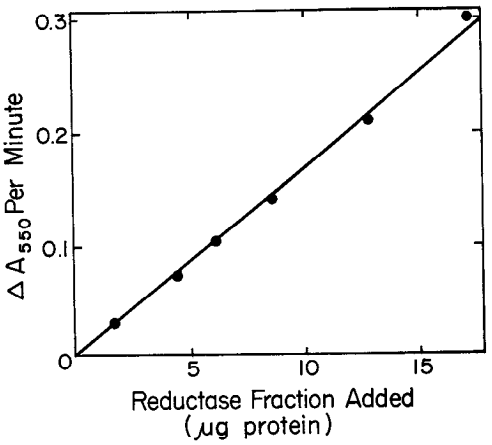

FIG. 4. Rate of cytochrome *c* reduction as a function of reductase concentration. The reductase preparation had a specific activity of 870.

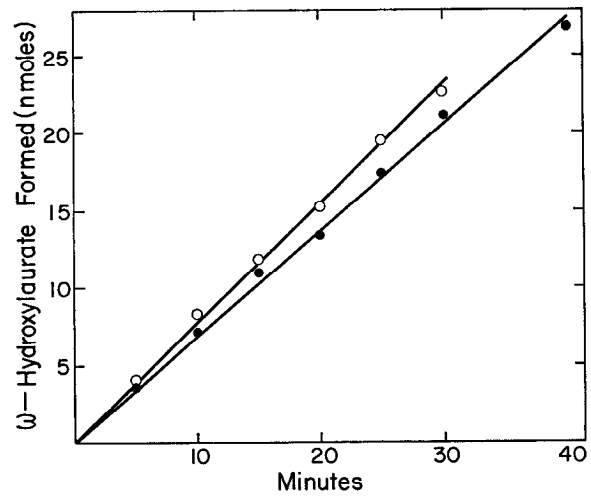

FIG. 2. Laurate ω-hydroxylation as a function of time with KCl-washed microsomes containing 0.5 mg of protein (○) or with a reconstituted enzyme system (●) containing fractions from chromatography on DEAE-cellulose: P-450 (0.22 nmole; 0.4 mg of protein), reductase (0.21 mg of protein), and Fraction B (containing 0.1 mg of lipid).

constant for at least 4 min and, as shown in Fig. 4, proportional to the amount of reductase added. One unit of reductase is defined as the amount catalyzing the reduction of 1.0 nmole of cytochrome *c* per min under these conditions.

Assay of P-450—Microsomal or solubilized preparations of P-450 in 0.1 M Tris or phosphate buffer, pH 7.7, were reduced by the addition of a few grains of solid dithionite. The sample cell was bubbled with CO for about 20 sec, and the CO difference spectrum was recorded in a Cary model 14 recording spectrophotometer with cuvettes of 1-cm light path. The P-450 concentration was calculated with the use of an extinction coefficient of 91 cm^{-1} mM^{-1} for $A_{450}-A_{490}$ (17).

Resolution of Microsomal Enzyme System—Frozen rabbit liver from male animals fasted 24 hours was minced with scissors and homogenized with 4 volumes of 0.25 M sucrose in a Waring Blendor for 2 min at 4°. The following operations were carried out at the same temperature. The homogenate was centrifuged for 25 min at 10,000 × *g*, and the supernatant fluid was filtered through two layers of cheesecloth. The centrifugation at low speed was repeated, and the supernatant layer was then centrifuged at 105,000 × *g* for 2 hours. The supernatant fluid was discarded, and the lipid adhering to the sides of the tube was removed. The translucent red microsomal pellet was suspended

in 0.25 M sucrose in a Potter-Elvchjem homogenizer and stored in the frozen state. In some experiments, particularly when it was desired to obtain a P-450 preparation with minimal contamination by hemoglobin, the liver was homogenized in 1.15% KCl and the microsomal pellet was resuspended in isotonic KCl and again precipitated by centrifugation. The resulting "KCl-washed" microsomes were then suspended in 0.25 M sucrose at a final protein concentration of 30 mg of protein per ml; such preparations were used where stated specifically in the text.

A microsomal suspension containing 1.95 g of protein in 65 ml of 0.25 M sucrose was treated with deoxycholate in the presence of glycerol, KCl, dithiothreitol, and potassium citrate buffer in the manner described previously (6), and the precipitate formed upon centrifugation for 2 hours at 105,000 × *g* was discarded. In typical experiments the supernatant layer contained at least 90% of the protein and 30 to 50% of the ω-hydroxylation activity originally present in the microsomes. The pH of this preparation was 7.4 to 7.5. Deoxycholate, glycerol, and citrate appear to be necessary for optimal solubilization of the enzyme system, whereas dithiothreitol is added as a precautionary measure to avoid the oxidation of enzyme sulfhydryl groups and KCl to raise the ionic strength, thereby enhancing the activity of the reductase. Other buffers, such as pyrophosphate, glycine, Tris, and phosphate proved to be much less effective than citrate. The microsomal extract was filtered through glass wool to give a clear, faintly reddish yellow solution having a protein concentration of 12 mg per ml, diluted with 3 volumes of deionized water, and put onto a DEAE-cellulose column (5 × 45 cm) previously equilibrated with 0.1 M Tris buffer, pH 7.7, containing 10^{-4} M dithiothreitol and 0.05% deoxycholate. The column was washed with 880 ml of the same buffer mixture containing 0.10 M KCl, thereby removing hemoglobin. A reddish yellow fraction containing P-450 was then eluted with 1 liter of buffer mixture containing 0.20 M KCl and the reductase with 1 liter of a similar solution containing 0.30 M KCl. A fraction containing the heat-stable factor was eluted from the column with 1.5 liters of a similar solution containing 0.50 M KCl. This stepwise elution was found to give better results than the salt gradient used previously. It may be noted that "Fraction A" obtained with a salt gradient (6) contained both P-450 and the reductase and "Fraction B" the heat-stable component. Until the identity of the latter is established, we will refer to it as Fraction B or the lipid fraction. The protein concentration of microsomal suspensions and crude extracts was determined by the method of Lowry *et al.* (18) and that of other preparations according to Warburg and Christian (19).

Attempts at purification of the P-450 have so far proved unsuccessful, but it could be concentrated and further freed of the reductase by adsorption on alumina C_γ gel at a gel to protein ratio (dry weight) of 2.0, following by washing with 0.1 M phosphate buffer, pH 7.7, and elution of the P-450 by 0.5 M buffer; such preparations were stable for about 2 weeks when sorted in the frozen state. The heat-stable factor was routinely stored in the cold to avoid bacterial contamination. An aliquot was extracted with ether, and the dry weight remaining after evaporation of the ether was taken to represent the lipid content.

Purification of TPNH-cytochrome c Reductase—To the reductase fraction (180 ml containing 240 mg of protein) were added 9.6 ml of alumina C_γ gel (50 mg per ml). The mixture was stirred for 30 min and centrifuged, and the supernatant solution was discarded. The gel was then stirred for 30 min with 15 ml of 0.02 M phosphate buffer, pH 7.7, containing 10^{-4} M dithiothreitol and EDTA, and the mixture was centrifuged and the supernatant solution was discarded. The reductase was eluted by stirring the gel for 30 min with 6 ml of a similar solution containing 0.25 M phosphate buffer, the operation was repeated, and the combined eluates were dialyzed overnight against 2 liters of a similar solution containing 0.02 M phosphate buffer. The dialyzed preparation was put onto a column of hydroxylapatite (1.7 × 3.5 cm) which had been equilibrated with 0.02 M phosphate buffer containing dithiothreitol and EDTA. A similar 0.1 M buffer solution was passed through the column, and the bulk of the reductase was then eluted by a similar 0.2 M buffer solution. The most active fraction, which was light yellow in color, had a specific activity of about 800 in the TPNH-dependent reduction of cytochrome *c*, representing about 100-fold purification from the microsomal extract. The purified enzyme, which was more stable than the crude reductase, was stable for at least 2 weeks in the frozen state.

Materials—Glucose-6-P dehydrogenase was obtained from Calbiochem; frozen rabbit, rat, and guinea pig livers from Pel-Freez Biologicals, Rogers, Arkansas; horse heart cytochrome *c*, TPNH, TPN, DPNH, alumina C_γ gel, and DEAE-cellulose from Sigma; lauric acid-1-^{14}C from Nuclear-Chicago; sodium deoxycholate from Difco; and BioGel HTP (hydroxylapatite) from Bio-Rad Laboratories (Richmond, California). The DEAE-cellulose (capacity, 0.83 meq per g) was thoroughly washed before further use with the following solutions in the order given: 0.5 N NaOH, 0.1 N HCl, 0.5 N NaOH, and water.

TABLE I

Fatty acid hydroxylation in hepatic microsomal preparations

Phenobarbital-treated rats were injected intraperitoneally with 10 mg of the drug per 100 g of body weight daily for 5 days. Rabbits were injected with 6 mg/100 g of body weight for 4 days and killed 2 days later. The liver microsomes of control animals injected with 0.9% NaCl had a specific activity of 0.99 for the rat and 2.10 for the rabbit.

Condition of animal	ω-Hydroxylation activity		
	Guinea pig	Rat	Rabbit
	nmoles/min/mg protein		
Fed		1.21	1.38
Fasted 24 hrs	0.75	1.13	2.38
Fasted 72 hrs	0.80	1.25	2.18
Phenobarbital-treated		2.36	5.25

RESULTS

Factors Influencing ω-Hydroxylation Activity in Hepatic Microsomes—Various hepatic microsomal preparations were examined for ω-hydroxylation activity with the results shown in Table I. The rabbit appeared to be the best of the species tested, and the activity of the rabbit liver microsomes was found to be enhanced by fasting. However, whereas Wada *et al.* (12) recently noted an increase in P-450 when rats were fasted, our data showed no such increase in ω-hydroxylation activity in this species. The animals to which phenobarbital was administered showed increased laurate hydroxylation activity. Furthermore, the reaction was effectively inhibited by CO, and this inhibition was partially reversed by light, both in microsomal suspensions and in the resolved system, as indicated in Table II. At the

1.5:1 CO/O_2 ratio employed, the inhibition in the resolved system was 85%, and this was reduced to 66% when the system was exposed to light. Since drug induction and CO inhibition with reversal by exposure to light are characteristic of hemoprotein P-450, as described by other investigators (10, 20), our data indicate that this pigment is involved in ω-oxidation.

Requirements for Fatty Acid Hydroxylation—The effect on the reaction rate of varying the concentration of the individual microsomal components is shown in Fig. 5. Since the various components have not yet been obtained entirely free of one another, it was necessary to determine the amount of product formed in the absence of each and to correct the data accordingly. The results indicate that the rate of the reaction is proportional to the concentration of the P-450, reductase, and heat-stable fractions under conditions where each of the other two components is present in excess. It is evident that each of the three microsomal components may be assayed within a limited range by its ability to support ω-hydroxylation and that high levels of the lipid component are inhibitory.

DPNH is inferior to TPNH in the hydroxylation reaction, being only 13% as active when both were tested at a concentration of 2×10^{-4} M and 58% as active as saturating levels of each; the minimal saturating concentrations are about 3×10^{-3} M for DPNH and 2×10^{-4} M for TPNH. From the plots in Fig. 6 the K_m values of TPNH and DPNH were found to be 2.0×10^{-5} and 6.3×10^{-4} M, respectively.

In view of the ability of a similar enzyme system obtained from *Pseudomonas oleovorans* to hydroxylate both alkanes and fatty acids (21), the activity of the resolved microsomal system toward octane was determined. Octane hydroxylation was found to occur at about one-fifth the rate of laurate hydroxylation and to require all three of the microsomal components for maximum activity. Das, Orrenius, and Ernster (11) have recently reported the hydroxylation of heptane by rat liver microsomal suspensions.

Properties of Heat-stable Fraction—The activity of Fraction B, as determined by its stimulating effect on the ω-hydroxylation of laurate, is unaffected by heating for 2 hours at 100° at neutral pH or for 10 min at 50° in 0.1 N HCl or H_2SO_4 or by ashing. The activity is completely extracted into a few volumes of ether or chloroform at neutral pH, and after evaporation of the organic solvent the residue may be taken up in Tris buffer to give a colorless but faintly turbid solution which is devoid of protein as judged by the Lowry method. This solution exhibits all of the stimulating effect in the hydroxylation assay originally present in Fraction B. The solubility properties indicate that the activity is due to one or more lipid components. Attempts to

TABLE II

Inhibition by carbon monoxide

Reaction mixtures containing 100 μmoles of Tris buffer (pH 7.4), 5 μmoles of glucose-6-P, 1.0 μmole of TPN, 0.2 unit of glucose-6-P dehydrogenase, 0.4 μmole of radioactive laurate, and either a microsomal suspension (0.8 mg of protein) or fractions from DEAE-cellulose containing P-450 and the reductase (0.24 mg of protein) and the heat-stable component (0.12 mg of lipid) were incubated at 37° for 12 min. Where indicated, the tubes were flushed with a gas mixture containing O_2, CO, and N_2 in the ratio 2:3:5 during the incubation, and the reaction mixture was exposed to light from an 1100-watt bulb at a distance of about 12 inches in the water bath used to maintain a constant temperature.

Gas phase	Exposed to light	Hydroxylation activity	
		Microsomal suspension	Resolved system
		nmoles product formed/min/mg total protein	
Air	−	3.18	3.47
O_2-CO-N_2	−	1.09	0.52
O_2-CO-N_2	+	1.81	1.18

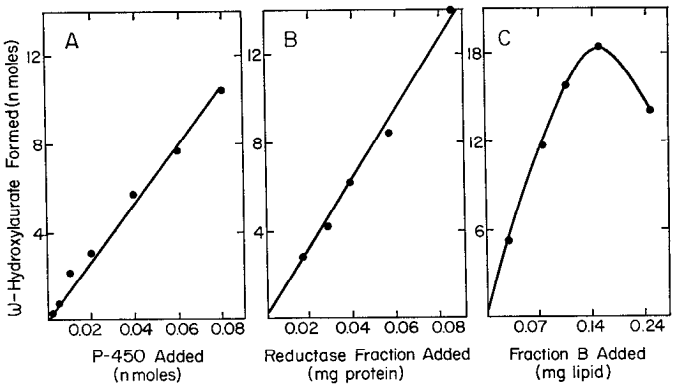

FIG. 5. Effect of concentration of microsomal components on rate of laurate ω-oxidation. In Experiment A, the reductase (specific activity, 800; 0.084 mg of protein) and Fraction B (0.1 mg of lipid) were in excess and P-450 (alumina C_γ gel eluate) was varied. In Experiment B, P-450 (alumina C_γ gel eluate; 0.1 nmole) and Fraction B (0.1 mg of lipid) were in excess and the reductase (specific activity, 800) was varied. In Experiment C, Fraction B was varied and P-450 (DEAE-cellulose eluate; 0.4 mg of protein) and the reductase (DEAE-cellulose eluate; 0.34 mg of protein) were in excess; Tris buffer, pH 7.4, was used in place of the usual phosphate buffer, but the KCl content of the reductase and P-450 fractions provided adequate ionic strength. The data are corrected for product formation in the absence of the component being varied: 4.7, 2.5, and 9.3 nmoles in Experiments A, B, and C, respectively.

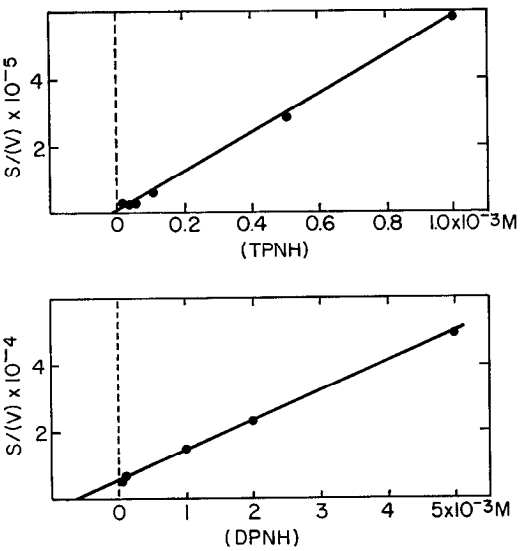

FIG. 6. Hofstee plots indicating K_m values of DPNH and TPNH. P-450 (alumina C_γ gel eluate; 0.1 nmole), the reductase (specific activity, 800; 0.087 mg of protein), and Fraction B (0.1 mg of lipid) were used in the standard radioactive assay. The concentrations are expressed as molarities and the velocities as nanomoles of product formed in 20 min.

replace this fraction by the following phospholipid preparations over a wide range of concentrations have proved unsuccessful: rat liver lecithin or phosphatidyl ethanolamine, bovine phosphatidyl serine or sphingomyelin, egg yolk lecithin, pig heart phosphatidyl ethanolamine or plasmalogen, or cardiolipin. Although the possibility might be considered that the active component in Fraction B is derived from the deoxycholate preparation used rather than from the microsomal lipids, deoxycholate failed to replace the unknown factor when tested at concentrations from 0.01 to 0.3% in the hydroxylation assay system.

Properties of Reductase—The reductase was purified about 100-fold from the microsomal extract, as judged by its ability to catalyze cytochrome *c* reduction in the presence of TPNH. Cytochrome *c* reduction is not a reliable index of the activity of the reductase in the hydroxylation system, however, since the latter activity is much more labile than the former during purification and storage. As a result, typical 100-fold purified preparations of the cytochrome *c* reductase appeared to have undergone only a 10-fold increase in activity as judged by the hydroxylation assay, and occasional preparations lost all of the latter activity. Furthermore, a homogeneous preparation of calf liver TPNH-cytochrome *c* reductase generously furnished by Dr. H. Kamin exhibited no significant activity in the hydroxylation system when tested at the same concentration or as high as 12 times the concentration of our cytochrome *c* reductase preparation. On the other hand, it was not inhibitory, even at the higher concentration, when added in the presence of the usual reductase fraction. Since Kamin's preparation had been solubilized by treatment with a lipase (22), it may have different physical properties from that obtained by our procedure. The ability to transfer electrons in the hydroxylation system is therefore considered to be a better indication than cytochrome *c* reduction of the native state of the reductase. Evidence for the role of this enzyme as an electron carrier in ω-oxidation was also provided by Wada *et al.* (12), who observed the inhibitory effect of adding the antibody against TPNH-cytochrome *c* reductase to microsomal suspensions.

Properties of Solubilized Cytochrome P-450—The P-450 preparation obtained by fractionation of the microsomal extract exhibits many of the properties already known to be characteristic of this pigment in microsomes. The activity of the P-450 fraction in catalyzing ω-hydroxylation is stable to dialysis against deionized water or buffers or to lyophilization, but it is largely lost after storage for several weeks at 0° or in the frozen state. The dialyzed and lyophilized preparation remains soluble as judged by the observation that it is not sedimented upon centrifugation for 2 hours at 105,000 × *g*.

Carbon Monoxide Difference Spectrum—The CO difference spectrum of a typical P-450 preparation, as given in Fig. 7, shows the expected peak at 450 mμ and only a slight absorbance at 420 to 422 mμ attributable to the presence of a trace of P-420. In another P-450 preparation containing a significant amount of P-420 it was found that the amount of the latter which is detected is dependent upon the order in which dithionite and CO are added (Fig. 8*A*). Presumably P-420 is labile to oxygen in the presence of dithionite but is stabilized as the CO complex, as proposed by Omura and Sato (23). Of particular interest, as shown in Fig. 8*B*, a similar CO difference spectrum is obtained when TPNH, as the final addition, serves as the reductant in the presence of the purified reductase. The enzymatic reduction was carried out anaerobically because reduced P-450 is readily

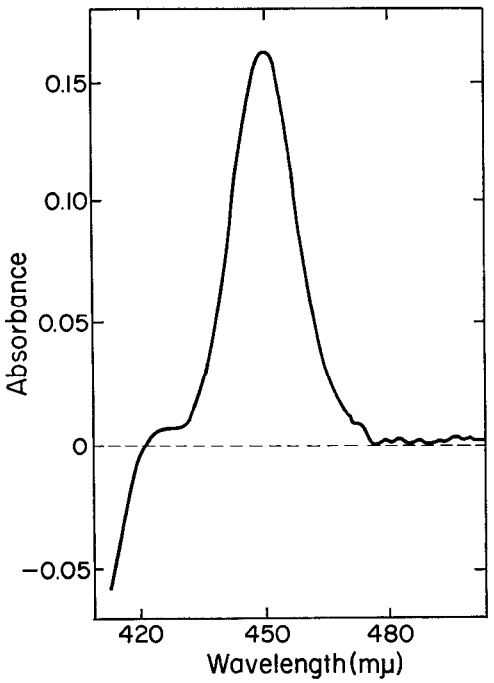

FIG. 7. Carbon monoxide difference spectrum of P-450 preparation, determined as described in the text. The protein concentration was 2.9 mg per ml.

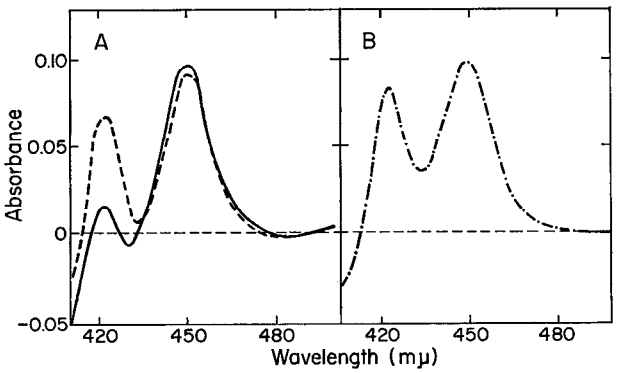

FIG. 8. Chemical and enzymatic reduction of P-450 as determined by CO difference spectra. In Experiment A, P-450 (alumina C_γ eluate; 1.0 nmole; 1.9 mg of protein) in 0.25 M phosphate buffer, pH 7.7, was reduced by dithionite prior to the addition of CO (——) or exposed to CO prior to dithionite reduction (– – –), and the difference spectrum was recorded 10 min after the last addition. In Experiment B, a mixture of the P-450 preparation, the purified reductase (0.85 mg of protein), and Fraction B (0.1 mg of lipid) in phosphate buffer in the main compartment of a cell was bubbled with CO, and 0.2 ml of 0.01 M TPNH (previously saturated with CO) was placed in a side arm. The cell was evacuated and flushed with CO, and the system was closed before the TPNH was tipped in; the final volume was 1.1 ml. At 10 min the reaction was about 54% complete. The spectrum shown was recorded at 60 min, when the reaction was complete.

autoxidized. No change in the extent or rate of P-450 reduction was noted upon omission of the lipid fraction, but the initial phase of the reaction was too rapid to permit determination of true initial rates. In other experiments it was found that the highly purified cytochrome *c* reductase furnished by Dr. Kamin had no significant activity in reducing P-450.

Electron Paramagnetic Resonance Spectrum—A concentrated

preparation of the solubilized P-450 was submitted to electron paramagnetic resonance spectrometry at a temperature near that of liquid helium. The spectrum shown in Fig. 9 is very similar to that reported for microsomal bound P-450 or "Fe_x" by Mason and his associates (24–26) and is indicative of a low spin ferric hemoprotein. The measured g values were $g_x = 1.92$, $g_y = 2.25$, and $g_z = 2.42$. The signal at $g = 2$ has been observed with submicrosomal particles containing P-450 and has been attributed to some component other than P-450 on the basis of power saturation characteristics (27). The absence of cytochrome b_5 in our preparation is shown by the lack of a signal at $g = 3.1$, indicated by an *arrow* in the figure. We are indebted to Dr. Graham Palmer for these measurements.

Laurate Difference Spectrum—The addition of certain hydroxylatable substrates to microsomes results in spectral shifts, as reported by others (28, 29), but the effect of laurate has not been studied. As shown in Fig. 10, the addition of laurate to the P-450 fraction gave a difference spectrum with a peak at about 388 mμ and a trough at 419 mμ. Fraction B was included in such experiments because it appeared to stabilize the P-450 in the presence of laurate, but the lipid fraction by itself gave no spectral change. A similar Type I spectrum was obtained with hexobarbital in the resolved system, whereas with aniline the typical Type II spectrum was seen (30). The magnitude of the difference spectrum was shown to vary with the laurate concentration, and the data from such experiments were used to determine the K_s of laurate (Fig. 11). The value obtained, 6.3×10^{-4} M, appeared to be the same in the presence or absence of Fraction B. These results apparently indicate that the lipid reaction does not enhance substrate binding to P-450. In other experiments the K_m of laurate in the complete hydroxylation system was determined to be 4.4×10^{-5} M.

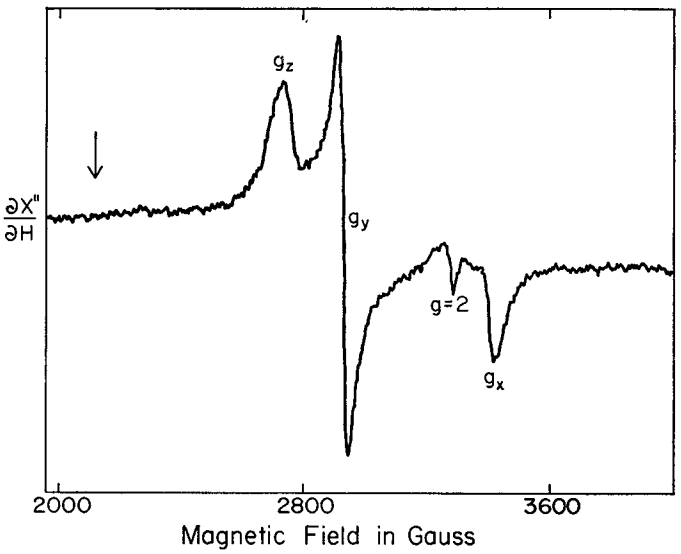

FIG. 9. Electron paramagnetic resonance spectrum of oxidized P-450. The P-450 preparation, which had been eluted from a column of hydroxylapatite with 0.5 M phosphate buffer, pH 7.7, was dialyzed extensively against deionized water and concentrated by lyophilization. The resulting dry powder was suspended in a small quantity of 0.1 M Tris buffer, pH 7.7, to give a thick paste containing about 29 mg of protein and 18 nmoles of P-450 per 0.1 ml, and the spectrum was recorded with a Varian electron paramagnetic resonance spectrometer with a variable temperature attachment, under the following conditions: microwave frequency, 9.195 GHz; modulation frequency, 100 kHz; modulation amplitude, 12 gauss; time constant, 1 sec; scan speed, 500 gauss per min; and temperature, 37° K.

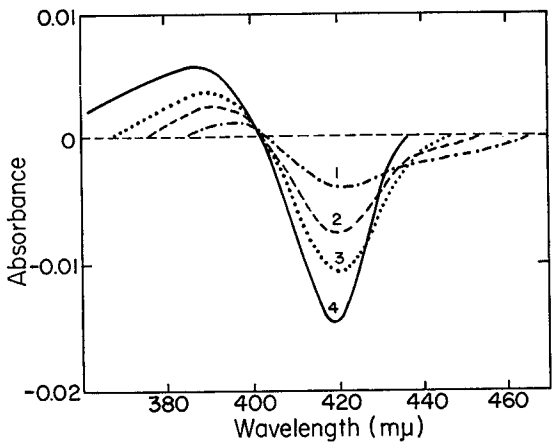

FIG. 10. Laurate-induced change in P-450 spectrum. Difference spectra due to the addition of laurate (at a final concentration of 0.1, 0.2, 0.3, and 0.5 mM in *Curves 1* to *4*, respectively) to a solution containing P-450 from an animal induced with phenobarbital (alumina C_γ gel eluate; 1.7 nmoles; 0.92 mg of protein), Fraction B (0.12 mg of lipid), and phosphate buffer, pH 7.7, in a final volume of 1.0 ml. The spectra were recorded immediately after the final addition of laurate, since with time further changes occurred which may be attributed to the detergent properties of the substrate.

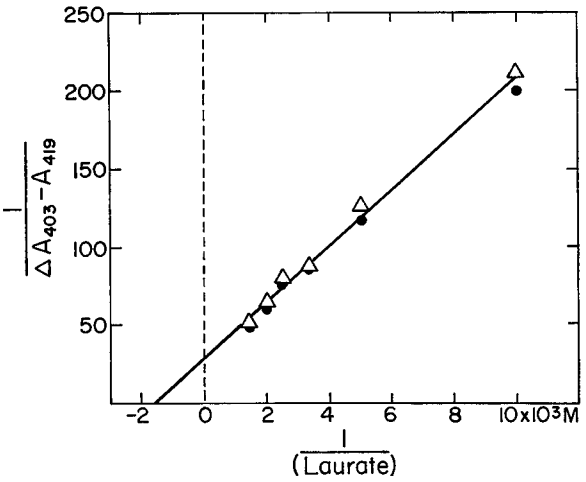

FIG. 11. Inverse plot for determination of K_s of laurate. The magnitude of the spectral change induced by varying concentrations of laurate was determined as $A_{403} - A_{419}$ under conditions similar to those described for Fig. 10, either in the presence (△) or in the absence (○) of Fraction B.

DISCUSSION

Resolution of the hepatic microsomal hydroxylation system has established the requirement for three components: cytochrome P-450, which binds the substrate and apparently catalyzes the hydroxylation reaction; TPNH-cytochrome *c* reductase, which was previously isolated from liver acetone powder (31) and from liver microsomes by lipase treatment (22) or tryptic digestion (15) and whose biological role has been uncertain; and the heat-stable lipid fraction. Since the reduction of cytochrome *c* is unrelated to the ability of various reductase preparations to function in the over-all hydroxylation reaction, and therefore to reduce P-450, TPNH-cytochrome P-450 reductase is the most

suitable name for this component. Our detergent-solubilized TPNH-cytochrome P-450 reductase preparation obviously differs in some important respect from the lipase-solubilized TPNH-cytochrome *c* reductase of Williams and Kamin (22). Possibly the latter preparation is partially denatured, retaining activity only toward cytochrome *c*, which is considered to be an artificial electron acceptor since it occurs in mitochondria rather than microsomes (32).

That the solubilized form of P-450 retains the properties of the native microsomal bound form is indicated by the carbon monoxide difference spectrum, the substrate difference spectra obtained with hexobarbital, aniline, and laurate, and the electron paramagnetic resonance spectrum, as well as by its ability to undergo enzymatic reduction and, most importantly, to catalyze hydroxylation reactions. The role of the lipid fraction and the identity of the active component or components are under study. Attempts to implicate this fraction in electron transfer from TPNH to P-450 or in substrate binding to P-450 have so far proved negative. Lipid requirements have previously been shown for microsomal enzymes such as DPNH-cytochrome *c* reductase (33), glucose 6-phosphatase (34), ATPase (35), and stearyl-CoA desaturase (36), as well as for several enzymes from other sources (37–40).

To our knowledge, cytochrome P-450 has not previously been separated from liver microsomes in either a soluble or a particulate form capable of catalyzing hydroxylation reactions. However, Maclennan, Tzagoloff, and McConnell (41) have used *tert*-amyl alcohol to obtain a soluble complex containing P-450 which was purified about 5 fold from beef liver microsomes. Of particular interest, Miyake, Gaylor, and Mason (27) have isolated submicrosomal particles containing P-450 but largely devoid of P-420 and cytochrome b_5 from rabbit liver microsomes by the use of Lubrol and glycerol. Their preparation was reduced anaerobically in the presence of CO by the adrenodoxin system in bovine adrenal mitochondria, but not by TPNH or DPNH, even on the addition of TPNH-cytochrome *c* reductase. In similar studies, Nishibayashi and Sato (42) obtained hepatic microsomal particles containing P-450 as the sole heme constituent by treating rabbit liver microsomes with Nagarse. These preparations were used to determine the absolute spectrum of P-450 (27, 42), and Mason's group used their cytochrome b_5-free preparation to confirm that P-450 (Fe_x) is a low spin protoheme in its oxidized state.

The hepatic microsomal hydroxylation system differs in important respects from other, somewhat similar enzyme systems. Fatty acid and alkane ω-hydroxylation in *Pseudomonas oleovorans* requires rubredoxin as an electron carrier (21, 43, 44), and hemoprotein P-450 is not involved, as judged by attempts to inhibit the reaction with carbon monoxide or to detect the carbon monoxide complex in preparations of the ω-hydroxylase purified over 100-fold from bacterial extracts (45). In contrast, a soluble form of cytochrome P-450 is functional in camphor hydroxylation in enzyme preparations from *Pseudomonas putida*, as shown by Katagiri, Ganguli, and Gunsalus (46), and in octane oxidation in extracts of a *Corynebacterium*, as shown by Cardini and Jurtshuk (47), and has also been obtained from nitrogen-fixing *Rhizobium* bacteroids by Appleby (48). As shown by Omura *et al.* (49), steroid 11-β-hydroxylase activity in an enzyme system obtained from adrenal cortex mitochondria requires a nonheme iron protein (adrenodoxin) as well as a reductase and a particulate form of cytochrome P-450.

The remarkably broad specificity of the cytochrome P-450-containing hydroxylation system of liver microsomes is indicated by its ability to attack fatty acids and alkanes, as well as steroids (50), including testosterone (51) and bile acids (52), carcinogenic polycyclic hydrocarbons (53), and a variety of drugs and other foreign substances (53, 54). Although multiple forms of hepatic microsomal P-450 have not been distinguished with certainty by spectral methods (55, 56), catalytically different forms may nonetheless exist. The possibility may also be considered, in view of the lipid requirement for laurate hydroxylation, that the lipid environment in the endoplasmic reticulum is the primary determinant of substrate specificity.

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CORRECTIONS

In the paper by Achilles Dugaiczyk and John J. Eiler (Vol. 244, No. 10, Issue of May 25, 1969, page 2750), in Table I on page 2753, the calculation of the ϵ_{max} values in Line 2 for 5-carboxyuracil, one H_2O crystallization was not taken into account. The correct values for anhydrous 5-carboxyuracil are: pH 0 to 2, "11,950; 11,100"; pH 6.6, "11,200; 8,950"; pH 11.5 to 13, "10,350; 13,700." These values are correctly represented in Fig. 3.

In the paper by Irwin M. Arias, Darrell Doyle, and Robert T. Schimke (Vol. 244, No. 12, Issue of June 25, 1969, page 3303), in Table IV on page 3311, the unit of measure given as "cpm" should be "cpm $\times$ 10^{-5}." In addition, under the column headed "Experiment 1 (1-day phenobarbital treatment) (170 g body weight)," the value for the ^3H counts for "Cytochrome b_5" given as "0.067" should be "0.0067."

In the paper by Anthony Y. H. Lu, Karen W. Junk, and Minor J. Coon (Vol. 244, No. 13, Issue of July 10, 1969, page 3714), on page 3717, left-hand column, the first sentence of the paragraph entitled "Properties of Heat-stable Fraction" should be replaced by the following sentence:

> "The activity of Fraction B, as determined by its stimulating effect on the ω-hydroxylation of laurate, is unaffected by heating for 2 hours at 100° at neutral pH or for 10 min at 50° in 0.1 N HCl or H_2SO_4 but is destroyed by ashing."

Resolution of the Cytochrome P-450-containing ω-Hydroxylation System of Liver Microsomes into Three Components

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