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Sedimentation and Other Properties of the Reconstituted Liver Microsomal Mixed-Function Oxidase System Containing Cytochrome P-450, Reduced Triphosphopyridine Nucleotide—Cytochrome P-450 Reductase, and Phosphatidylcholine

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SUMMARY

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The effect of phosphatidylcholine, an essential component of the reconstituted liver microsomal enzyme system which hydroxylates drugs, on the sedimentation and other properties of cholate-solubilized cytochrome P-450 was studied. Upon centrifugation of the cytochrome P-450 fraction for 1 hr at 105,000 × *g* at 5°, 85–90% of the cytochrome P-450 remained in the supernatant fraction. The apparent sedimentation coefficient of the cytochrome P-450 was 13 S, whether this fraction was centrifuged alone or in the presence of the reductase fraction and phosphatidylcholine. When the concentration of bound cholate was reduced by dialysis to approximately 1 μg/mg of protein, corresponding to about 1 mole/mole of cytochrome P-450, almost full catalytic activity was retained and an apparent sedimentation coefficient of 18 S was observed. This value was not altered by the addition of the reductase fraction and the lipid. The molecular weight of the dialyzed cytochrome P-450 was judged to be about 350,000 by sedimentation velocity measurements in the ultracentrifuge, sucrose density gradient centrifugation, and gel exclusion chromatography, but this figure must be considered provisional in view of the heterogeneity of the preparation. The effect of sedimentation on the distribution of enzyme activity was also determined. After centrifugation of the complete reaction mixture containing the cytochrome P-450 fraction, the reductase fraction, phosphatidylcholine, TPNH, and benzphetamine for 1 hr at 105,000 × *g*, over 90% of the original hydroxylation activity remained in the supernatant layer. The resuspended pellet was inactive when supplemented with TPNH and substrate. When the reconstituted enzyme system was subjected to gel exclusion chromatography, the cytochrome P-450 and TPNH–cytochrome P-450 reductase were well separated from each other and from the phospholipid, thereby indicating that these components behave as separate entities rather than as a tightly associated complex. The solubilized cytochrome P-450 fraction was examined in the electron microscope and found to have the appearance of individual protein molecules rather than of highly complex structures. Similarly, a mixture of the cytochrome P-450 fraction, the reductase fraction, and phospholipid was devoid of structures which could be attributed to aggregation. These findings indicate that, although a dissociable complex containing cytochrome P-450, reductase, and phosphatidylcholine may function in catalysis, the phospholipid exerts its effect on drug hydroxylation without causing the formation of aggregates or membrane-like structures.

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INTRODUCTION

Cytochrome P-450 has been obtained in a solubilized form from liver microsomes by Lu and Coon (1) by treatment with deoxycholate in the presence of glycerol and other stabilizing agents. This pigment, in the presence of two additional, obligatory microsomal components, TPNH–cytochrome P-450 reductase and a heat-stable lipid fraction, catalyzes the hydroxylation of fatty acids and hydrocarbons (1, 2), drugs (3, 4), aniline (5, 6), and polycyclic aromatic carcinogens (7, 8). The functional lipid has been identified as phosphatidylcholine and shown to be essential for electron transfer from TPNH to cytochrome P-450, catalyzed by the reductase, as determined from the appearance of the reduced carbon monoxide difference spectrum under anaerobic conditions (9). Presumably, therefore, phospholipids are an integral component of this hydroxylating enzyme system in the endoplasmic reticulum. Of interest in this connection, Chaplin and Mannering (10) have reported that phospholipase C partially inhibits drug and aniline hydroxylation when added to liver microsomes, and Eling and DiAugustine (11) have found that prior treatment of microsomes with phospholipase C or D decreases the demethylation of benzphetamine.

This paper is concerned with some of the physical characteristics of the reconstituted liver microsomal drug-hydroxylating system. As judged by sedimentation measurements and electron microscopy, the resolved cytochrome P-450 has the properties of a soluble protein rather than of an aggregated or membrane-bound protein, even in the presence of the reductase and the phospholipid.

METHODS

Preparation of resolved microsomal components. The TPNH–cytochrome P-450 reductase fraction and lipid (fraction B) were prepared as previously described from liver microsomes of rats which had been treated with injections of phenobarbital and hydrocortisone (9). Cytochrome P-450 was solubilized according to a modification of earlier procedures (1, 2, 7). Cytochrome P-450 preparations made in this manner are obtained in better yield and are more consistently free of cytochrome P-420 than earlier preparations which were solubilized with deoxycholate and then subjected to column chromatography on DEAE-cellulose (2). The following operations were carried out at 5° unless stated otherwise. Microsomes prepared as described previously (2) were deaerated by stirring the suspension under reduced pressure for 1 hr, flushed with nitrogen, and frozen. When stored in this manner the microsomal drug-demethylating enzyme system was stable for several months; this was judged using benzphetamine as the substrate. Thawed microsomes (800 mg of protein) were suspended in a solution prepared by mixing 14 ml of glycerol, 3.5 ml of 2 M Tris-chloride buffer (pH 7.7, measured at 4°), 0.7 ml of 0.1 M dithiothreitol, and water added to a final volume of 65 ml. The suspension was subjected to sonic oscillation with a 500-W ultrasonic disintegrator (Measuring and Scientific Equipment Company) at maximum output for eight 20-sec intervals while the temperature of the suspension was kept below 10° by use of a salt-ice bath. A solution of sodium cholate was added to give a final concentration of 1 mg/mg of protein. The mixture (final volume, 70 ml) was stirred for 10–20 min, and solid ammonium sulfate was added to 40% saturation. Stirring was continued for 10 min, and the precipitate was removed by centrifugation and discarded. Solid ammonium sulfate was then added to 50% saturation and stirring was continued for 10 min. The resulting precipitate was removed by centrifugation, dissolved in 15 ml of 0.1 M Tris buffer, pH 7.7, containing 20% glycerol and 0.1 mM dithiothreitol, and dialyzed overnight at 5° against the same buffer which had been flushed with nitrogen. The cytochrome P-450 preparation was diluted to 30 ml with the buffer mixture and subjected to sonic oscillation in a salt-ice bath for three 10-sec intervals. Solid ammonium sulfate was added to 40% saturation and then, after removal of the precipitate, to 50% saturation. The fraction precipitating between 40% and 50% saturation was dissolved in the buffer mixture, dialyzed overnight against the same solution, and centrifuged at 105,000 × *g* for 1 hr. The resulting supernatant fraction generally con-

tained 1.5–3.0 nmoles of cytochrome P-450 per milligram of protein. No cytochrome P-420 was detectable in this fraction.

Analytical procedures. Unless stated otherwise, the "standard buffer mixture" was 0.1 M Tris buffer, pH 7.7, containing 10% glycerol and 0.1 mM dithiothreitol. The rate of hydroxylation of benzphetamine was determined at 30° either by substrate-dependent TPNH oxidation or by formaldehyde formation, as previously described (4), except that $MgCl_2$ and semicarbazide were omitted from the reaction mixtures. Formaldehyde was determined by the method of Nash (12) as modified by Cochin and Axelrod (13). Benzphetamine was kindly donated by Dr. J. W. Hinman of the Upjohn Company, and a 1:5 mixture of mono- and dilauroylglyceryl-3-phosphorylcholine was generously provided by Dr. W. E. M. Lands. Gel exclusion studies were carried out at 5° using Sepharose 6B (Pharmacia) in a column 2.5 × 75 cm equipped with a flow adaptor and equilibrated with 0.1 M Tris buffer, pH 7.7, containing 5% glycerol and 0.1 mM dithiothreitol.

For the sucrose density gradient centrifugation procedure, a protein mixture in a volume not greater than 1.5 ml was layered onto 38 ml of a previously prepared 5–15% gradient of sucrose in 0.1 M Tris buffer, pH 7.7. After centrifugation at 150,000 × *g* for 14.5 hr at 5° in an SW 27 rotor, the tubes were punctured and 1.0-ml fractions were collected. The fractions were assayed for their cytochrome P-450 content by measurement of the reduced CO difference spectrum and also for their activity in catalyzing drug demethylation. *Escherichia coli* β-galactosidase was obtained from Boehringer, rabbit muscle aldolase from Pharmacia, and horse spleen apoferritin from Mann Research Laboratories. Pig heart fumarase was kindly provided by Ms. Marcia S. Flashner, and rabbit muscle pyruvate kinase by Dr. Michael Flashner.

RESULTS

Effect of resolved components on sedimentation pattern of solubilized cytochrome P-450. The cytochrome P-450 fraction, judged to be soluble by the arbitrary criterion that it remains in the supernatant fraction after centrifugation at 105,000 × *g* for 1 or 2 hr, otherwise retains the characteristic properties of the membrane-bound pigment. Thus the soluble cytochrome P-450 preparation gives a CO difference spectrum when reduced enzymatically or chemically (2) and substrate difference spectra in the presence of substrates such as laurate, hexobarbital, benzphetamine, and aniline (2, 3, 14). Moreover, the ability to catalyze the hydroxylation of a variety of substrates is retained upon solubilization. The lipid fraction, although essential to the catalytic activity of the cytochrome P-450, does not alter the binding of substrates (2, 4). In an effort to elucidate the function of the lipid component, its effect on the sedimentation characteristics of the cytochrome P-450 was studied under a variety of conditions.

In preliminary experiments the cytochrome P-450 fraction (1–10 mg of protein per milliliter of the standard buffer mixture) was centrifuged at 105,000 × *g* at 5° for 1 hr in a Spinco preparative ultracentrifuge, and the supernatant fraction was found to contain 85–90% of the cytochrome P-450; the small pellet which had formed was resuspended and found to contain the remainder, including a variable amount of cytochrome P-420. Under similar conditions the reductase fraction alone yielded no pellet on centrifugation. Of particular interest, when a mixture of the cytochrome P-450 fraction, reductase fraction, and lauroyl-GPC[2] was centrifuged under similar conditions, the results were the same. In other experiments in which glycerol was omitted from the buffer mixture containing the cytochrome P-450 fraction, the supernatant layer contained only 40–45% of the cytochrome P-450, and the pellet contained the remainder, more than half of which had been converted to cytochrome P-420. Since glycerol is necessary to stabilize cytochrome P-450 in the solubilization and resolution procedure, these results were not unexpected.

Sedimentation velocity studies were carried out on the cytochrome P-450 preparation using a Spinco model E analytical

[2] The abbreviation used is: lauroyl-GPC, a 1:5 mixture of mono- and dilauroylglyceryl-3-phosphorylcholine.

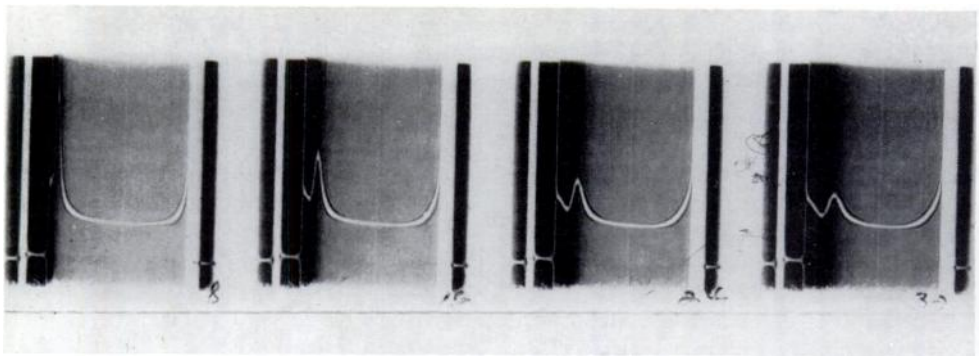

FIG. 1. *Sedimentation pattern of cytochrome P-450 fraction*

The cytochrome P-450 fraction (20 nmoles; 10 mg of protein per milliliter) was centrifuged under the conditions described in the text. The photographs, from left to right, were taken 8, 16, 24, and 32 min after full rotor speed had been attained.

ultracentrifuge equipped with schlieren optics. Centrifugations were performed at 250,000 × *g* with a rotor speed of 60,000 rpm and a bar angle of 60 degrees. The cytochrome P-450 fraction was centrifuged at 10° in the standard buffer mixture unless otherwise noted. The sedimentation coefficients obtained were corrected to standard conditions of 20° and water as solvent. Standard viscosity tables were used for the temperature correction (15), and the viscosity of the buffer mixture used was measured at 10° relative to the viscosity of water by use of an Ubbelohde viscometer. The density of the buffer mixture was calculated to be 1.02 g/ml. Since the cytochrome P-450 preparation is not homogeneous as judged by disc gel electrophoresis, it was anticipated that several distinct protein peaks would be observed upon ultracentrifugation. However, the cytochrome P-450 fraction gave a single broad peak, as shown in Fig. 1. Since the color moved with the single peak and these preparations contained only very small amounts of cytochrome b_5 (0.05 nmole/mg of protein, or less than 4% of the total heme present), it is concluded that the cytochrome P-450 had the same sedimentation characteristics as the other proteins present. The reductase fraction also gave a single broad peak but sedimented much more slowly than the cytochrome P-450 fraction. The apparent sedimentation coefficient of the cytochrome P-450 fraction was 12–13 S and that of the reductase fraction was 3–4 S, as indicated in Table 1. Since the cytochrome P-450 fraction contains phospholipids (0.04 μmole of lipid phosphorus per milligram of protein, determined on a 2:1 chloroform–methanol extract), the possibility must be considered that it is present as a low-density lipoprotein with a deceptively low *s* value. The sedimentation of the cytochrome P-450 was examined in the standard buffer mixture made either 5% or 10% in KBr in order to accentuate such a

TABLE 1

Sedimentation coefficients of microsomal components

The components were centrifuged at 60,000 rpm as described in the text. The concentrations were as follows: cytochrome P-450 fraction, 20–24 nmoles in 10–12 mg of protein; reductase fraction, 7–10 mg of protein; lauroyl-GPC, 0.08 mg; TPNH, 0.2 μmole; and benzphetamine, 0.5 μmole, in a final volume of 1.0 ml.

Microsomal components present	KBr added	$s_{20,w}$
	%	*S*
Cytochrome P-450 fraction		12
Reductase fraction		3[a]
Cytochrome P-450 fraction	5	13
Cytochrome P-450 fraction	10	13
Cytochrome P-450 fraction + reductase fraction + lipid		4, 13

[a] A minor peak ($s_{20,w}$ = 12 S) was also observed; it was due to the presence of a small amount of cytochrome P-450 in some of the reductase preparations.

possible buoyancy effect (see ref. 16). The small increase in $s_{20,w}$ which was observed is probably not significant, and in any case is in the opposite direction from that predicted for a low-density lipoprotein. As also shown in the table, when the cytochrome P-450 fraction was combined with the reductase fraction and phosphatidylcholine, only the same two peaks were observed; the areas under the peaks corresponded roughly to those obtained when the protein fractions were sedimented separately. In other experiments, not presented here, cytochrome P-450 preparations made by an earlier procedure involving sonication, solubilization by deoxycholate, and DEAE-cellulose column chromatography (6) were also examined in the ultracentrifuge and found to give similar results but to contain significant amounts of cytochrome P-420, and were therefore not studied in detail.

Cholate content and sedimentation of cytochrome P-450 fraction. In view of the possibility that the cholate content might alter the sedimentation properties of cytochrome P-450, this fraction was prepared in the presence of radioactive sodium cholate and subjected to several procedures designed to remove the detergent. As may be seen in Table 2, dialysis for 40 hr lowered the cholate content to 1.0 μg/mg of protein (corresponding to a 1:1 molar ratio of cholate to cytochrome P-450), but longer dialysis did not further decrease this ratio. The cytochrome P-450 was not denatured by dialysis, for almost full catalytic activity was retained at 40 hr, and the magnitude of the CO difference spectrum was reduced by only 14%, even after 60 hr of dialysis. Upon ultracentrifugation and gel chromatography the cholate content was decreased almost to the same extent, but the cytochrome P-450 was partially denatured and the recovery was only 50% and 70%, respectively. The finding of a cholate to cytochrome P-450 ratio close to 1.0 does not necessarily imply that the detergent was bound to this pigment, since other proteins in the preparation might also bind cholate.

Sedimentation studies were then carried out on a cytochrome P-450 preparation from which the cholate had been largely removed by dialysis, with the results shown in Table 3. Extensive dialysis increased the sedimentation coefficient of the cytochrome P-450 fraction to 18 S. Longer dialysis or passage through a gel filtration column prior to the usual period of dialysis had no additional effect on the sedimentation coefficient, nor did the omission of glycerol from the buffer during dialysis. As also shown in Table 3,

TABLE 2

Effect of various treatments on cholate content of cytochrome P-450 fraction

Sodium[*carboxyl*-^{14}C]cholate (New England Nuclear, 46 μCi/μmole) was diluted with carrier to a specific activity of 1.8×10^4 cpm/mg and used in the solubilization and fractionation procedure. The cytochrome P-450 fraction, which was prepared by the usual steps, including centrifugation at 105,000 × *g* for 1 hr, contained 13.5 nmoles of cytochrome P-450 and 6.5 mg of protein per milliliter. In the procedures indicated, the standard buffer mixture was used. The solution was changed at the end of each 20-hr period of dialysis. Ultrafiltration was carried out using a 10-ml Amicon cell with a PM-30 membrane. In each step the protein solution was concentrated to one-half the original volume. The Sephadex G-50 column (1.4 × 30 cm) was equilibrated with the buffer solution before the protein was applied. Aliquots (1.5 ml) were removed at various stages of the fractionation procedure, mixed with 15 ml of Aquasol (New England Nuclear), and counted at ambient temperature in a Unilux scintillation counter.

Sample analyzed	Molar ratio, cholate to cytochrome P-450
Experiment 1	
Starting material	18.3
Preparation dialyzed against buffer solution containing 10% glycerol for 20 hr	2.7
Same, for 40 hr	1.0
Same, for 60 hr	1.0
Experiment 2	
Starting material	24.6
Preparation subjected to ultrafiltration in buffer solution containing 20% glycerol, 7 times	1.6
Same, 11 times	1.5
Experiment 3	
Starting material	25.8
Preparation subjected to column chromatography on Sephadex G-50	2.2
Same, repeated	1.4

TABLE 3

Sedimentation of cytochrome P-450 after dialysis

The conditions were as described in Table 1, except that the cytochrome P-450 and reductase fractions were individually dialyzed for two 20-hr periods at 5° against the standard buffer mixture with a change of the solution between these periods.

Components present	Conditions	$s_{20,w}$
		S
Cytochrome P-450 fraction		18
Cytochrome P-450 fraction	No glycerol present	18[a]
Cytochrome P-450 fraction	Passed through Sepharose 6B column before dialysis	18
Cytochrome P-450 fraction + reductase fraction + lipid + benzphetamine + TPNH		4, 18

[a] Although the omission of glycerol had no apparent effect on the sedimentation coefficient, more of the cytochrome P-450 was found in the pellet, as stated in the text.

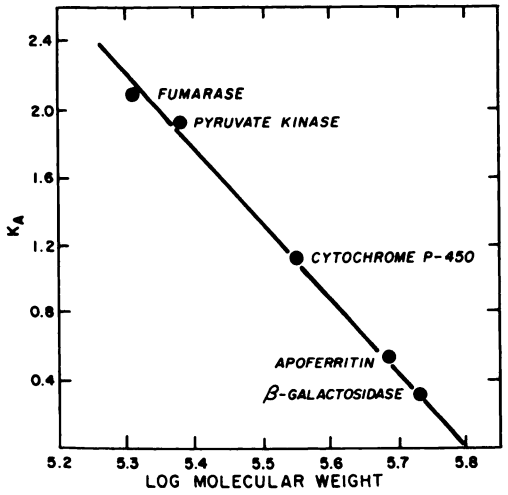

FIG. 2. *Estimation of molecular weight of cytochrome P-450 by gel filtration*

The logarithm of the molecular weight of the various proteins is given as a function of the partition coefficient (K_A). The various proteins were applied to a column of Sepharose 6B and eluted in 0.1 M Tris buffer, pH 7.7, containing 5% glycerol and 0.1 mM dithiothreitol. The molecular weights of the standard proteins were as follows: fumarase, 204,000; pyruvate kinase, 240,000; apoferritin, 480,000; and β-galactosidase, 540,000.

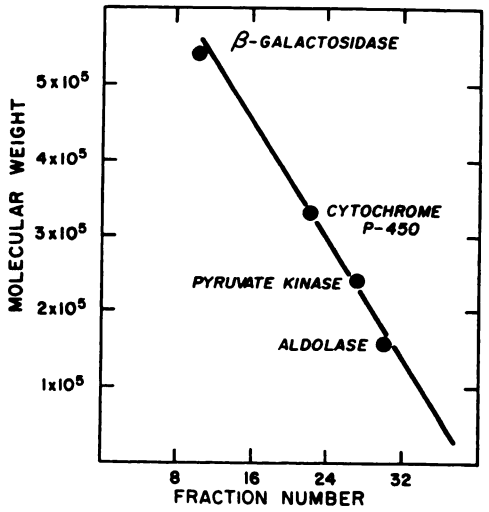

FIG. 3. *Sucrose density gradient study of cytochrome P-450*

The marker proteins used were β-galactosidase, pyruvate kinase, and aldolase (mol wt 158,000), and the procedure was carried out as described in the text.

centrifugation of a mixture of dialyzed P-450 and similarly dialyzed reductase, along with phosphatidylcholine, drug (benzphetamine), and TPNH, gave the same two major protein peaks. The manner in which dialysis increases the rate of sedimentation of the cytochrome P-450 fraction is not understood.

Apparent molecular weight of solubilized cytochrome P-450. Attempts were made to estimate the molecular weight of the dialyzed cytochrome P-450 sedimenting at $s_{20,w}$ = 18 S. The diffusion coefficient was determined using a synthetic boundary cell, and the $D_{20,w}$ value was calculated to be 4.6 × 10^{-7} cm² sec^{-1}. Using the sedimentation and diffusion coefficients and assuming a partial specific volume of 0.72 ml/g, an apparent

TABLE 4

Effect of centrifugation on hydroxylation activity of reconstituted microsomal enzyme system

The complete reaction mixture, containing, per milliliter, cytochrome P-450 fraction (0.4 nmole and 0.3 mg of protein in experiment A; 0.9 nmole and 0.4 mg of protein in experiment B), reductase fraction (0.2 mg of protein in experiment A and 0.1 mg in experiment B; specific activity, 1190 nmoles of cytochrome *c* reduced per minute per milligram of protein), lauroyl-GPC (0.1 mg), benzphetamine (1.0 μmole), TPNH (0.15 μmole in experiment A and none in experiment B), and phosphate buffer, pH 7.7 (100 μmoles), was centrifuged at 105,000 × *g* for 1 hr, at 5° in experiment A and at 20° in experiment B, in a Spinco preparative centrifuge. The supernatant fraction was decanted, and the small pellet was resuspended in 1.0 ml of the standard buffer mixture. The production of formaldehyde from benzphetamine was determined by assaying the fractions in the usual manner at 30°. TPNH (0.1 μmole) was added in all cases to initiate the reaction, and benzphetamine (1.0 μmole) was added when the resuspended pellet was assayed to ensure that the substrate concentration was saturating. Additional benzphetamine had no effect on the activity of the supernatant fraction, nor did variations in the TPNH concentration from 0.1 to 0.3 mM. Where indicated, the supernatant fraction and resuspended pellet were supplemented (before assay) with the cytochrome P-450 and reductase fractions in the same amounts as in the starting reaction mixture. Lauroyl-GPC was not added to the supernatant fraction because the phosphatidylcholine concentration was already optimal, and additional amounts inhibited benzphetamine hydroxylation.

Components added to assay mixture	Hydroxylation activity[a]	
	A. Centrifuged at 5°	B. Centrifuged at 20°
	% maximal rate	
Supernatant fraction		
None	93	80
Cytochrome P-450 fraction	101	
Reductase fraction	119	
Pellet		
None	0	11
Cytochrome P-450 fraction	0	
Reductase fraction	15	
Pellet + supernatant fraction	104	99

[a] The maximal activity (100%) corresponds to 4.0 nmoles of formaldehyde produced per minute at 30° in experiment A and 3.6 in experiment B in reaction mixtures which were kept at the temperature indicated for 1 hr but were not centrifuged. No significant amount of formaldehyde was produced during the centrifugations.

molecular weight of 350,000 was calculated for the cytochrome P-450. The molecular weight range was confirmed by two additional methods. As shown in Fig. 2, the cytochrome P-450 fraction was subjected to gel filtration in the presence of other proteins of known molecular weight. By this method it showed an apparent molecular weight of 360,000. In addition, sucrose density gradient centrifugation was carried out with proteins of known molecular weight as markers, and, as indicated by the data in Fig. 3, the cytochrome P-450 was estimated to have a molecular weight of 330,000. Despite the good agreement in the values determined by these methods, it must be emphasized that the apparent molecular weight was determined on preparations containing both lipid and contaminating proteins and that, with all three techniques used, the cytochrome P-450 fraction gave a broad peak. Although the molecular weight must therefore be considered provisional, the evidence clearly indicates that the resolved cytochrome P-450 behaves as a soluble protein rather than as membrane-bound or particulate material.

Distribution of drug-hydroxylating activity upon centrifugation. Although examination of the reconstituted microsomal system by ultracentrifugation indicated that no gross change in the structural properties of the solubilized cytochrome P-450 occurred upon the addition of the lipid fraction, it was important to ascertain whether the hydroxylating activity also remained in the supernatant fraction. The results obtained when a complete reaction mixture containing the cytochrome P-450 fraction, reductase fraction, lipid, TPNH, and substrate (benzphetamine) was prepared at room temperature and centrifuged for 1 hr at 105,000 × *g* at 5° and the resulting supernatant fraction and pellet were then examined for catalytic activity are shown in Table 4, experiment A. As judged by formaldehyde production, the supernatant fraction re-

tained almost all the drug-demethylating activity. Supplementation of the supernatant fraction with cytochrome P-450 gave only slightly greater activity, whereas supplementation with reductase gave significant stimulation. In experiments not presented here it was shown that the reductase, unlike the cytochrome P-450, is somewhat unstable under the conditions of centrifugation. Accordingly, it may become the rate-limiting component in such experiments. The resuspended pellet was devoid of activity in this experiment, and in other similar experiments no more than 15% of the original activity has ever been recovered in this fraction. The data also show that supplementation of the resuspended pellet with cytochrome P-450 was without effect, whereas supplementation with reductase gave slight but significant activity. Finally, the combination of pellet and supernatant fraction was not significantly better than the supernatant fraction alone. These results are believed to rule out the possibility that the function of the lipid is to generate a sedimentable aggregate or membrane-like structure which might be postulated as essential for hydroxylation activity.

In experiment B a mixture of the cytochrome P-450 fraction, reductase fraction, and lipid was centrifuged for 60 min at 20°. The supernatant fraction exhibited 80% of the original benzphetamine demethylation activity, and the suspended pellet only 11%. Apparently, therefore, a lipid-initiated aggregation of cytochrome P-450 occurs neither at this higher temperature, at which significant catalytic activity occurs, nor in the cold. At 20° this reconstituted system exhibits 65% of the activity observed at 30°. Sedimentation studies were not carried out at 30° because of the extensive loss of activity when the complete enzyme system is kept at this temperature for 1 hr or longer.

Distribution of drug-hydroxylating activity upon gel exclusion chromatography. The possibility of the occurrence of lipid-induced aggregation was also examined by the technique of gel filtration. The cytochrome P-450 fraction, reductase fraction, and microsomal lipid, in proportions closely approximating those used in catalytic assay mixtures, were combined and incubated at 30° for 30 min. The mixture was then applied to a Sepharose 6B column at 4°, and the fractions eluted were analyzed for protein, cytochrome P-450, and TPNH–cytochrome *c* reductase, with the results shown in Fig. 4. The void volume

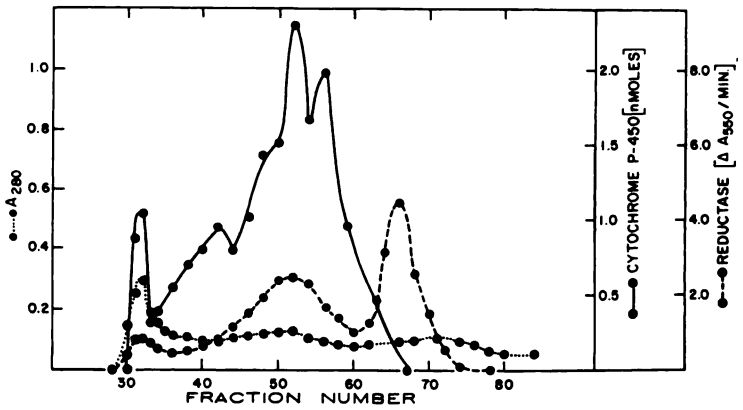

FIG. 4. *Gel chromatography of reconstituted microsomal enzyme system*

A mixture of the cytochrome P-450 fraction (60 nmoles; 31 mg of protein), reductase fraction (2.6 mg of protein), and microsomal lipid from which deoxycholate had largely been removed by cholestyramine treatment (9) (6.2 mg) in a final volume of 5.0 ml was incubated at 30° for 30 min. The mixture was then applied to a Sepharose 6B column at 4° and eluted with 0.1 M Tris buffer, pH 7.7, containing 5% glycerol and 0.1 mM dithiothreitol. Fractions of 4.8 ml each were collected and analyzed for cytochrome P-450, TPNH–cytochrome *c* reductase activity (measured as ΔA_{550}), and protein (measured as A_{280}). The high A_{280} readings in tubes 28–34 are not significant because of the turbidity present (see the text).

(tubes 28–34) contained only small amounts of cytochrome P-450 and the reductase and was somewhat turbid, whereas all subsequent samples were clear. Most of the cytochrome P-450 was eluted later in a broad peak along with some reductase. Reductase of lower molecular weight was subsequently eluted in a peak which was free of cytocrome P-450.

The ability of the components separated on the column to support hydroxylation activity was determined as follows. Fractions A (tubes 30–33), B (tubes 46–60), and C (tubes 62–72) were concentrated in an Amicon ultrafiltration apparatus, supplemented with benzphetamine and TPNH, and assayed for formaldehyde production. Fraction A required supplementation by phosphatidylcholine and TPNH–cytochrome P-450 reductase for activity, and then exhibited a specific activity of 1.9 nmoles of formaldehyde produced per minute per nanomole of cytochrome P-450. Fraction B was similarly dependent upon these two added components and then had a specific activity of 6.7. Clearly, therefore, the higher molecular weight cytochrome P-450 in fraction A was much less active than the lower molecular weight material in fraction B. Fractions B and C, when combined in equal amounts of protein, had a specific activity of 1.2 and were entirely dependent upon added lipid. It is apparent that, of the two major cytochrome *c* reductase peaks, only the one in fraction C possessed cytochrome P-450-reducing ability as well. In confirmation of the sedimentation data already presented, these results obtained by gel exclusion chromatography support the concept that the resolved microsomal components behave as separate entities rather than as a tightly associated complex.

Electron microscopy. Enzyme preparations were subjected to negative staining as described by Valentine *et al.* (17), except that 2% phosphotungstate was used at pH 7.4 and 0°. We are grateful to Dr. Robert H. Gray, who examined the preparations in an AEI EM-6B electron microscope. As shown in Fig. 5a, the microsomal suspension had the typical appearance of negatively stained membranes, but the resolved cytochrome P-450 fraction exhibited only much smaller structural features, primarily particles with a diameter of about 10 nm, which would correspond roughly to the apparent molecular weight as determined by sedimentation and gel filtration. The electron micrographs of the cytochrome P-450 fraction (Fig. 5b) resemble those of other detergent-solubilized membrane proteins, e.g., the Na^+- and K^+-activated ATPase of brain microsomes (18). Of particular interest, as shown in Fig. 5c, a mixture of the cytochrome P-450 fraction, reductase fraction, and phospholipid at concentrations equal to those employed in the usual catalytic assay for hydroxylation activity showed about the same particle size as the cytochrome P-450 fraction alone. Similar results were obtained when a cytochrome P-450 preparation solubilized with deoxycholate and subjected to column chromatography on DEAE-cellulose (1) was substituted for the cholate-solubilized preparation described in the present paper. Furthermore, electron microscopy gave no indication of membrane formation in a reconstituted enzyme system to which benzphetamine and TPNH had been added or which had been incubated for 30 min at 25° prior to negative staining. These results are in accord with the other evidence already presented on the physical properties of the preparations.

DISCUSSION

The results presented provide no indication that phosphatidylcholine causes the resolved cytochrome P-450 to form aggregates or membrane-like structures. Upon ultracentrifugation of the reconstituted enzyme system, almost all this pigment remained in the supernatant fraction, as did the drug hydroxylation activity. Furthermore, examination of the cytochrome P-450 fraction or of the reconstituted system by electron microscopy revealed no structural features other than those expected of soluble proteins. A mixture of cytochrome P-450, reductase, and lipid was readily separated into its components by ion-exchange chromatography as described previously (1), or on the basis of molecular weight differences by gel exclusion chromatography, as shown in the present studies. On the other

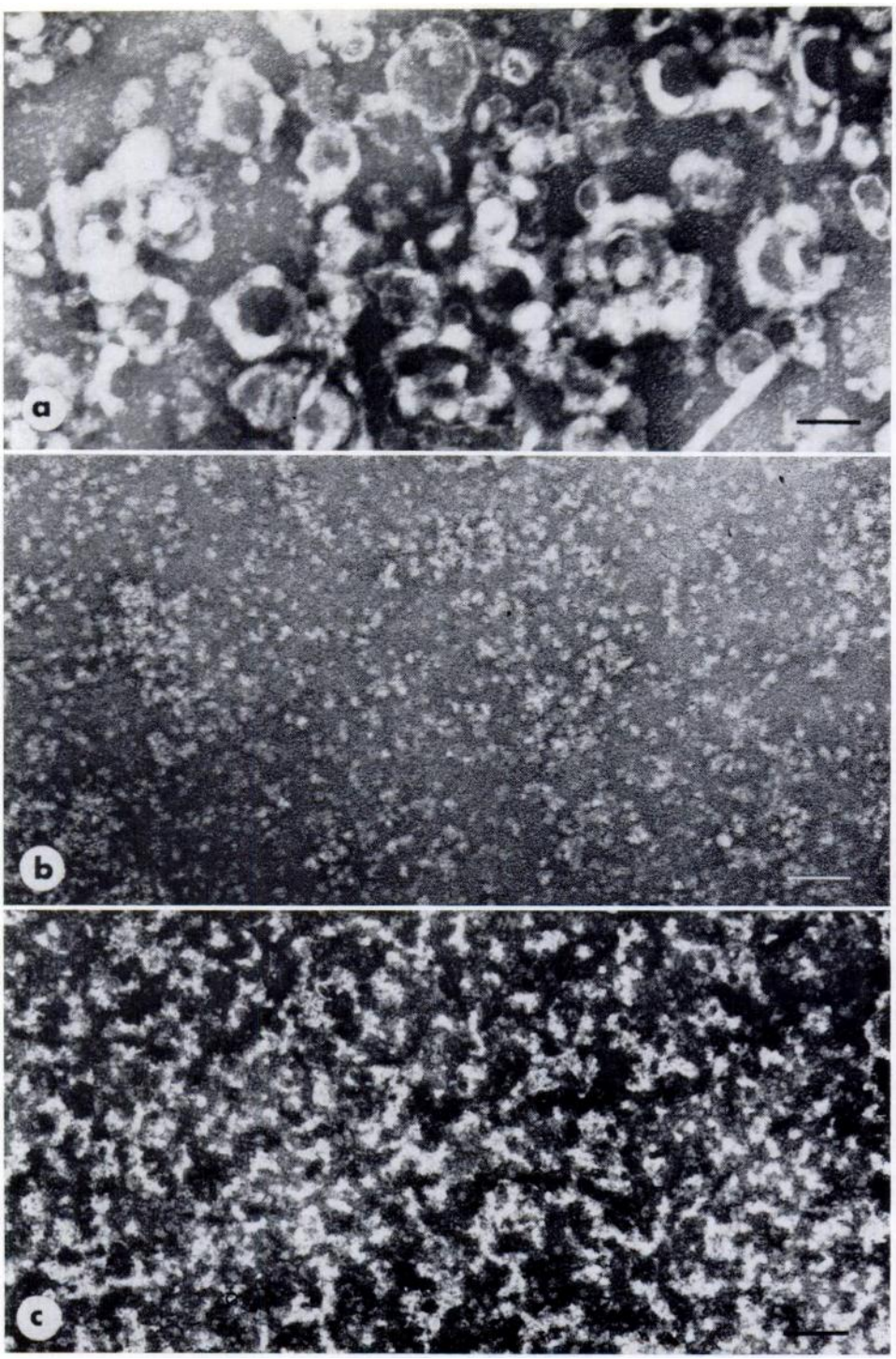

FIG. 5. *Electron micrographs of (a) microsomal suspension (4.1 mg of protein per milliliter of 0.25 M sucrose), (b) cytochrome P-450 fraction (1.3 nmoles or 1.0 mg of protein per milliliter of standard buffer mixture), and (c) a mixture of cytochrome P-450 fraction (0.6 nmole or 0.3 mg of protein), reductase fraction (0.04 mg of protein), and lauroyl-GPC (0.09 mg/ml of 0.1 M potassium phosphate buffer, pH 7.7).*

The final magnification was 90,000. The inserted bars in the photographs represent 10 pm.

hand, the existence of a dissociable complex of these components during catalysis is not ruled out by the evidence available. For example, such a complex containing 1 molecule each of cytochrome P-450 and reductase and 1 or more molecules of phospholipid would not have been detected by the methods so far applied, particularly if association-dissociation were a rapid process.

The sedimentation coefficients and the apparent molecular weight of the resolved cytochrome P-450, i.e., 350,000, must be considered provisional in view of the fact that we are dealing with a complex system and with heterogeneous enzyme fractions. The highly purified cytochrome P-450 of *Pseudomonas putida* has a molecular weight of 46,000 (19), but Triton N-101-solubilized adrenal mitochondrial cytochrome P-450 also has an apparent molecular weight in the 300,000 range, as judged by gel filtration.[3]

A number of enzyme systems are known to require lipid for catalytic activity. In some instances the requirement was demonstrated by depleting membranes of lipids (20), and in others the enzyme systems have been solubilized or resolved and shown to require one or more added lipids, usually phospholipids, for restoration of function. For example, Kagawa and Racker (21) have demonstrated a requirement for phospholipid as well as coupling factors in the restoration of P_i-ATP exchange activity in fragments of bovine heart mitochondria, which is accompanied by the formation of vesicular structures. Rothfield *et al.* (22) have shown that the galactosyl transferase system of *Salmonella typhimurium* forms a catalytically active monomolecular film in the presence of phosphatidylethanolamine and a purified lipopolysaccharide. In other cases, however, the lipid may exert its effect without causing aggregation, as shown by Cunningham and Hager (23) for the soluble *E. coli* pyruvate oxidase, which requires phospholipid as an allosteric effector. The reconstituted liver microsomal mixed-function oxidase, as shown in the present paper, is an unusual example in which an enzyme system originally bound to membranes exhibits a phospholipid requirement when solubilized but does not undergo reaggregation when the system is reconstituted.

Shoeman *et al.* (24) have reported that steapsin-solubilized cytochrome P-420 from rat liver microsomes forms aggregates when desalted and concentrated. The aggregates, which contained large numbers of tubular elements, gave characteristic difference spectra with drugs but apparently were not capable of catalyzing drug hydroxylation. More recently they have stated in a brief report (25) that in microsomal fractions prepared by our earlier procedure the lipid caused the cytochrome P-450 to form an aggregate and that upon centrifugation the level of *N*-demethylase activity was related to the cytochrome P-450 content of the pellet. The results given in the present paper indicate that, under the conditions described, aggregation is not necessary for hydroxylation activity.

Our earlier studies have shown that phosphatidylcholine is essential for electron transfer from TPNH to cytochrome P-450 (6) as well as for the hydroxylation of various substrates (1, 2, 4). The phospholipid is also required for hydroxylation activity when a photochemical or enzymatic system capable of generating superoxide is coupled with cytochrome P-450 in the absence of TPNH and the reductase (5, 26). Although the present studies indicate that phosphatidylcholine is essential for catalysis under conditions which do not lead to aggregation or membrane formation, the manner in which the phospholipid exerts this effect remains to be established.

[3] D. Y. Cooper, personal communication.

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