ROLE OF PHOSPHOLIPID IN THE RECONSTITUTED LIVER MICROSONAL MIXED FUNCTION OXIDASE SYSTEM CONTAINING CYTOCHROME P-450 AND NADPH-CYTOCHROME P-450 REDUCTASE

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As described elsewhere (1–3), studies in this laboratory have led to the solubilization of liver microsomal cytochrome P-450 by treatment with deoxycholate and to its separation by ion exchange chromatography from NADPH-cytochrome P-450 reductase and a heat-stable lipid fraction. All three components are required, as well as molecular oxygen and NADPH, for the hydroxylation of drugs (4, 5), fatty acids and paraffin hydrocarbons (1, 3), aniline (6), and polycyclic aromatic carcinogens (7, 8). The functional microsomal lipid has been identified as phosphatidylcholine and shown to be essential for rapid electron transfer from NADPH to cytochrome P-450 (9). Presumably phospholipids are functional components of this enzyme system in the endoplasmic reticulum as well as in the reconstituted enzyme preparations. Evidence pointing in this direction is provided by the finding of Chaplin and Mannering (10) that phospholipase C partially inhibits drug and aniline hydroxylation when added to liver microsomes. Eling and DiAugustine (11) have reported that the hydroxylation of aniline is unchanged by the pre-treatment of microsomes with phospholipase C but that both this enzyme and phospholipase D decrease the demethylation of benzphetamine.

The present paper is concerned with the problem of how phosphatidylcholine exerts its effect on this mixed function oxidase system. As shown in table 1, the phospholipid mixture, which may be replaced by lauroyl-GPC,3 is a necessary component not only of the reconstituted microsomal enzyme systems from rat and rabbit liver, but also that from human liver (13). Furthermore, a cytochrome P-450-containing enzyme system from yeast grown on tetradecane (14) shows a similar requirement (15). It seems unlikely that the phospholipid requirement is related to the use of deoxycholate, as proposed by Cater et al. (16), because it is observed in the yeast system after solubilization with a French pressure cell, whether or not deoxycholate is added. Furthermore, we have found that when cholate is added to the reconstituted liver microsomal system at levels which are inhibitory, the further addition of lauroyl-GPC does not restore the activity.

Effect of Phospholipid on Substrate Binding to Cytochrome P-450

The possibility was considered that the phospholipid might play a role in substrate binding, but the evidence so far available indicates this is not the case. Thus, the Kₐ of laurate (6.3 × 10⁻⁴ M) was the same in the presence or absence of the microsomal lipid fraction (3). Similarly,

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3 The abbreviations used are: lauroyl-GPC, a 5:1 mixture of di- and monolauroylglyceryl-3-phosphorylcholine; A.S. P-450, cytochrome P-450 prepared by a procedure including fractionation with ammonium sulfate (7, 12).
TABLE 1
Phospholipid requirement for cytochrome P-450-containing enzyme systems
from various species

The various cytochrome P-450 preparations were tested with the lipids indicated in the presence of NADPH and the deoxycholate-solubilized rat liver microsomal reductase. 1-¹⁴C-laurate was the substrate with human liver and yeast cytochrome P-450, and benzphetamine with the others. The assays were carried out at 30°C, and the lipid and protein fractions were mixed before the other components were added. The values given were determined at the optimal concentra-
tions of the lipid preparations and are expressed as activities relative to that with rat liver microsomal lipid as 100%.

The procedures and maximal rates were as described previously (3, 5, 13, 14).

| Source of Solubilized | Percent Maximal Activity with Various Lipids Added | Rat liver microsomal lipid fraction | Laurylethyl-
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Cytochrome P-450</td>
<td>% Maximal Activity</td>
<td>Yeast lipid fraction</td>
<td>None</td>
</tr>
<tr>
<td>----------------------</td>
<td>---------------------------------------------------</td>
<td>-----------------------</td>
<td>------</td>
</tr>
<tr>
<td>Rat liver microsomes</td>
<td>100</td>
<td>118</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit liver microsomes</td>
<td>100</td>
<td>110</td>
<td>7</td>
</tr>
<tr>
<td>Human liver microsomes</td>
<td>100</td>
<td>85</td>
<td>19</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>100</td>
<td>69</td>
<td>134</td>
</tr>
</tbody>
</table>

the Kᵢ of benzphetamine (1.8 x 10⁻⁴ M) was unaltered by the addition of the lipid fraction (5). We conclude, therefore, that the phospholipid has a function other than facilitating the binding of substrate to cytochrome P-450.

Reactions for Which Phospholipid Is Required

As already indicated, phosphatidylcholine is a necessary component of reaction mixtures for the overall hydroxylation of substrates to occur and also for electron transfer from NADPH to cytochrome P-450, catalyzed by the reductase. This step may be determined by the appearance of the reduced carbon monoxide difference spectrum under anaerobic conditions. Stopped flow measurements have shown that the lipid-dependent reduction of cytochrome P-450 has a first-order rate constant of about 100 min⁻¹, whereas in the absence of lipid the value is about 6 min⁻¹ (9, 17, 18). Clearly, only the lipid-dependent rate is great enough to support substrate hydroxylation, which has a turnover number of about 20 min⁻¹ (mol of benzphetamine hydroxylated per mol of cytochrome P-450 at 25°C). This turnover number is calculated on the basis of the total amount of cytochrome P-450 in the reaction mixture and would be somewhat greater if based only on that portion of the cytochrome P-450 which is rapidly reducible. Since 33-90% of this pigment appears to be reduced rapidly in various experiments, the corrected turnover number might be as great as 60 min⁻¹ but still less than the lipid-dependent rate constant for electron transfer. The detailed manner in which the lipid facilitates the electron transfer step is unknown at this time, but it seems much more likely that it aids in the formation of a functional complex rather than acts as an electron carrier.

The requirement for the lipid in electron transfer would fully account for its role in substrate hydroxylation but does not rule out an additional role, for example, at the stage of the reaction at which oxygen is inserted into the substrate. In more recent studies designed to investigate the mechanism of oxygen activation (6, 19, 20), superoxide-generating systems such as xanthine plus xanthine oxidase or light-irradiated riboflavin plus methionine were substituted for NADPH and the reductase in the usual reaction mixture, and drug demethylation still occurred, although at a somewhat reduced rate. Of particular interest is the fact that the phospholipid was still required for the demethylation of benzphetamine, or ethylmorphine, as judged by formaldehyde liberation, even in the absence of the usual reductase fraction. The interpretation of such experiments on the possible role of superoxide as an electron donor or source of activated oxygen is not yet clear, but it appears that the phospholipid may be required for all of the reactions in which the resolved cytochrome P-450 participates, rather than only for the reductase-catalyzed electron transfer to the cytochrome (21).

Effect of Phospholipid on Sedimentation and Other Properties of Resolved Cytochrome P-450

Since the resolved enzyme system is obtained from liver microsomal membranes, the possibility was also considered that the lipid might exert its effect by causing the solubilized cytochrome P-450 to form aggregates or membrane-like structures necessary for catalytic activity. For such studies cytochrome P-450 was solubilized from rat liver microsomes by a procedure developed in this laboratory involving sonication, treatment with cholate, and ammonium sulfate fractionation (7, 14). The A.S. P-450 was dialyzed
overnight against 0.1 M Tris buffer, pH 7.7, containing 20% glycerol and 1.0 \times 10^{-4} \text{ M} \text{ dithiothreitol}, and centrifuged at 100,000 \text{ g} at 5^\circ\text{C} for 1 hr. The supernatant fraction contained the cytochrome P-450 and was more consistently free of cytochrome P-420 than earlier preparations which were solubilized with deoxycholate and submitted to column chromatography on DEAE-cellulose (1). When the A.S. P-450 preparation was centrifuged at 105,000g for 1 hr at 5^\circ\text{C} in a Spinco preparative ultracentrifuge, the supernatant fraction contained 85–90% of the cytochrome P-450; the small pellet which had formed was resuspended and was found to contain the remainder, part of which had been converted to cytochrome P-420. When centrifuged under similar conditions, the reductase fraction yielded no pellet. Interestingly, when a mixture of the cytochrome P-450 fraction, reductase fraction, and lipid was centrifuged under similar conditions, the results were the same. These results indicate that the lipid does not cause aggregation of the cytochrome P-450, contrary to the proposal of Shoeman et al. (22). The effect of centrifugation on the hydroxylation activity of such preparations is shown in table 2. A complete reaction mixture containing NADPH and benzphetamine, as well as the three microsomal components, was centrifuged for 1 hr at 105,000g at 5^\circ\text{C}. Over 90% of the drug hydroxylation activity remained in the supernatant fraction, and none could be detected in the resuspended pellet supplemented with NADPH and benzphetamine. These results provide no indication that the addition of the lipid fraction causes the formation of a sedimentable lipoprotein complex with catalytic activity. Similar results were obtained when the centrifugation was carried out at 20^\circ\text{C}.

Other evidence that the resolved microsomal components behave as separate entities rather than as a tightly associated complex was obtained by gel exclusion chromatography. The three fractions were combined in proportions closely approximating those used in catalytic assay mixtures and were incubated at 30^\circ\text{C} for 30 min and then applied to a Sepharose 6B column at 5^\circ\text{C}. The void volume, representing very high molecular weight material, contained only a small amount of cytochrome P-450 and of the reductase and was somewhat turbid, whereas all subsequent samples were clear. Most of the cytochrome P-450 was then eluted, followed by the NADPH-cytochrome P-450 reductase. These two enzyme fractions, when combined, required supplementation by phospholipid for hydroxylation activity, thereby indicating that they behaved as individual entities upon exclusion chromatography.

Electron microscopy was also used to examine the state of aggregation of the enzyme system. The resolved cytochrome P-450 fraction was negatively stained (23) and examined by Dr. Robert H. Gray (School of Public Health, The University of Michigan) in an AEI EM-6B electron microscope. In contrast to the membranes seen in microsomal preparations, only much smaller structural features, primarily particles with a diameter of about 100 \text{ A}, were observed. The micrographs resembled those of other detergent-solubilized membrane proteins, such as the Na\textsuperscript{+}- and K\textsuperscript{+}-activated ATPase of brain microsomes (24). A mixture of phospholipid, reductase fraction, and cytochrome P-450 fraction showed essentially the same particle size as the cytochrome P-450 fraction alone, and similar results were obtained with cytochrome P-450 preparations made by DEAE-cellulose chromatography (1). Such results are in accord with the other evidence already presented that the phospholipid has no gross effect on the physical properties of the solu-

### Table 2

**Effect of centrifugation on the ability of the reconstituted microsomal enzyme system to hydroxylate benzphetamine**

<table>
<thead>
<tr>
<th>System</th>
<th>Hydroxylation Activity* (nmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system (before centrifugation)</td>
<td>4.0</td>
</tr>
<tr>
<td>Supernatant fraction</td>
<td>3.7</td>
</tr>
<tr>
<td>Resuspended pellet</td>
<td>0</td>
</tr>
<tr>
<td>Supernatant fraction + resuspended pellet</td>
<td>4.2</td>
</tr>
</tbody>
</table>

*Total in 1.5-ml reaction mixture.*


**Table 3**

*Estimated molecular weight of solubilized cytochrome P-450*

<table>
<thead>
<tr>
<th>Technique Used</th>
<th>Apparent Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentation in analytical ultracentrifuge</td>
<td>350,000</td>
</tr>
<tr>
<td>Sucrose density gradient centrifugation</td>
<td>330,000</td>
</tr>
<tr>
<td>Gel exclusion chromatography</td>
<td>360,000</td>
</tr>
</tbody>
</table>

...bilized protein components of this enzyme system.

**Estimated Molecular Weight of Solubilized Cytochrome P-450**

A broad peak with an $S_{30,w}$ value of about 12 was observed when the cytochrome P-450 fraction was submitted to preliminary sedimentation velocity studies in the analytical ultracentrifuge; this value was not changed upon the addition of the reductase and lipid components (25). For determination of the apparent molecular weight, as shown in Table 3, the A.S. P-450 fraction was dialyzed for two periods of 20 hr each at 5°C against 0.1 M Tris buffer, pH 7.7, containing 10% glycerol and $1.0 \times 10^{-4}$ M dithiothreitol, before ultracentrifugation. A single broad peak was observed with $S_{30,w} = 18$. The diffusion coefficient, $D_{30,w}$, was determined to be $4.6 \times 10^{-7}$ cm$^2$ sec$^{-1}$. With these values and an assumed partial specific volume of 0.72 ml g$^{-1}$, an apparent molecular weight of 350,000 was calculated. It should be noted that the same sedimentation constant ($S_{30,w} = 18$) was determined after the cytochrome P-450 fraction had been mixed with the reductase fraction and the phospholipid. Gel filtration in the presence of other proteins of known molecular weight gave a broad peak having a similar molecular weight, as did sucrose density gradient centrifugation in the presence of marker proteins. Although these apparent values agree well, it must be emphasized that the cytochrome P-450 preparation is contaminated by lipids as well as by other proteins and that, in all three techniques used, a broad protein peak was observed. Therefore, the molecular weight must be considered provisional.

The heterogeneity of the preparations is emphasized by the analytical data in Table 4. The presence of cytochrome $b_5$ as well as additional heme and also of the non-heme iron is apparent in all of the preparations, including those purified to an average content of 5.5 nmol of cytochrome P-450 per mg of protein. The best preparation so far obtained had 6.7 nmol of cytochrome P-450 per mg of protein. Preliminary experiments have shown that, upon titration of the resolved A.S. P-450 preparation by dithionite under anaerobic conditions, up to 3 times as many electrons are consumed as would be predicted from the cytochrome P-450 content. Whether electron carriers other than cytochrome P-450 and the reductase are functional in substrate hydroxylation remains to be established.

In summary, phospholipid is required for the hydroxylation of various substrates in reaction mixtures containing cytochrome P-450 solubilized from human liver microsomes, rat liver microsomes, rabbit liver microsomes, and yeast. Studies with the reconstituted enzyme system from rat liver microsomes have shown that phosphatidylethanolamine has no effect on the binding of substrates to cytochrome P-450, nor does it cause the cytochrome P-450 to form large aggregates or membrane-like structures, as judged by sedimentation measurements, gel exclusion chromatography, or electron microscopy. The results obtained are compatible with the formation of a

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**Table 4**

*Analysis of cytochrome P-450 preparations*

<table>
<thead>
<tr>
<th>Cytochrome P-450 Preparation</th>
<th>Components Present (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytochrome P-450</td>
</tr>
<tr>
<td>Rat liver column P-450</td>
<td>1.1*</td>
</tr>
<tr>
<td>Rat liver A.S. P-450</td>
<td>1.7</td>
</tr>
<tr>
<td>Rabbit liver A.S. P-450</td>
<td>2.5</td>
</tr>
<tr>
<td>Partially purified P-450</td>
<td>5.5</td>
</tr>
<tr>
<td>(rabbit liver)</td>
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</table>

*Includes cytochrome P-420.

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dissociable complex containing cytochrome P-450, NADPH-cytochrome P-450 reductase, and phosphatidylcholine during catalysis.

References

Discussion

Dr. Masters, U. Texas Southwestern Med. Sch.: I would like to ask Dr. Autor or Dr. Coon how they reconcile the lack of inhibition by supernoxide dismutase of their reconstituted system, which on the one hand suggests a tightly coupled system, with the ultracentrifuge and gel filtration results, and on the other hand suggests a loosely coupled system. The allusion I am making is related to the fact that other reconstitution systems, including highly purified preparations of reductase [B. S. S. Masters, C. H. Williams, Jr., and H. Kamin, in "Methods in Enzymology, vol. X (R. W. Estabrook and M. E. Pullman, eds.), p. 555, Academic Press, New York, 1967; S. D. Aust, Bioch. Biophys. Res. Commun. 47, 1133 (1972)] or xanthine plus xanthine oxidase, are inhibited by supernoxide dismutase, but a reconstituted system (M. J. Coon, H. W. Strobel, and R. F. Boyer, this Symposium) utilizing the Lu and Coon preparation of detergent-solubilized reductase is not.

Dr. Coon: Effective coupling refers to the ability of various reducing systems to transfer electrons to different cytochrome P-450 preparations. The dismutase apparently can't get to the active site in an efficiently coupled system. As an alternative explanation, there might be an inhibitor in the A.S. P-450 or an activator in the column P-450.

Dr. Estabrook, U. Texas Southwestern Med. Sch.: You showed us a number of criteria indicating that your cytochrome P-450 preparation is homogeneous with a molecular weight of about 350,000. Could you indicate for us what polyacrylamide gel electrophoresis of this preparation shows? How many components are present?

Dr. Autor: There are many components.
Dr. Estabrook: Do you presume that your preparation is composed of a large number of components all of molecular weight 350,000?  
Dr. Autor: No, I don't think we can say that. It is probably an indication of either an average molecular weight of the preparation consisting of a number of proteins of fairly similar molecular weight or a nonspecific binding of smaller protein to the cytochrome P-450. The results from both sedimentation velocity experiments and gel column chromatography show a single but very broad peak. If one protein were homogeneous and not subject to dissociation or aggregation, one would expect to see a single sharp peak.  
Dr. Kaschnitz: We have seen a broad band of protein upon electrophoresis using 4% polyacrylamide gel, corresponding only to the heme-containing band. Furthermore, in SDS gel electrophoresis, about 15 bands were seen; the major protein band was seen at a molecular weight position of 50,000–60,000, which is possibly the subunit of cytochrome P-450. I would like to show electron micrographs of negatively stained preparations of microsomes, a resolved cytochrome P-450 preparation, and the reconstituted system. In the latter two micrographs no membranous structure can be seen. The particle size of these preparations is approximately 100 Å.  
[Editors' note: Prints of figures were not supplied for publication.]  
Dr. Raisfeld, New York U.: Do you have these electron micrographs with you? The diameter of the microsomal membrane has been described to be about 70 Å and you are describing a particle of 100 Å diameter. It is a tight fit.  
Dr. Estabrook: Yes, this is a good point. This question has also been raised in connection with mitochondria.  
Dr. Kaschnitz: This depends on the type of membrane model you are thinking of. If you agree with S. J. Singer's model, it is possible to visualize such a large protein in a fluid lipid matrix.  
Dr. Raisfeld: You are suggesting then, that the diameter of the microsomal membrane would be entirely comprised by a fairly large particle. The diameter of the microsomal membrane has been described to be about 70 Å, so a particle in the range of 100 Å would occupy the entire diameter of the membrane. What was your protein concentration used in your preparations for the electron micrographs?  
Dr. Kaschnitz: About 1 mg/ml.  
Dr. Raisfeld: I believe your solutions are too concentrated for clear structural definition using the negative staining technique. I suggest that you dilute your samples to a protein concentration of 10–100 μg/ml. You should see aggregates of particles and aggregates of subunits in different combinations. This is what we have found with the Lu and Coon preparation in our laboratory.  
Dr. Kaschnitz: We have two reasons for using this protein concentration: first, it corresponds to that used in the assay mixture for catalytic activity; second, electron micrographs taken at the lower protein concentration gave the same results but the quality was not good. In addition, I repeat, we were not attempting to show details of protein structure in these heterogeneous preparations but only to demonstrate that the resolved preparations do not contain membrane-like structure.  
Dr. Raisfeld: Are you using carbon-coated grids?  
Dr. Kaschnitz: Yes, of course.  
Dr. Gillette, NHLI: I am a little bit confused. What is the amount of protein per P-450 heme in these preparations? Is it consistent with a 350,000 molecular weight?  
Dr. Kaschnitz: We did not calculate the data in this way since the preparations were not homogeneous.  
Dr. Raisfeld: What was the heme to protein ratio in your preparations?  
Dr. Autor: The ratios varied for different preparations.  
Dr. Kaschnitz: The preparations contained between 2 and 6 nmol of cytochrome P-450 per mg of protein. The content of heme is always greater than the cytochrome P-450 content, and the heme/cytochrome P-450 ratio varies from 1 to 2. There is also always extra iron present which cannot be accounted for by the heme analysis and is therefore called non-heme iron.  
Dr. Estabrook: I would like to go back to the gel electrophoresis studies. If there is a subunit of 50,000 molecular weight which contains the heme, this means then that there are a number of other subunits associated with cytochrome P-450 which do not contain heme. This is necessary to account for your 350,000 molecular weight. One of the subunits may be the substrate-binding protein associated with cytochrome P-450.  
Dr. Raisfeld: Have you determined the heme to protein ratio of your preparations shown in the electron micrographs?  
Dr. Autor: Yes, that has been done. We know that our cytochrome P-450 preparation is not pure. However, we have demonstrated that it has the properties of a soluble protein after the resolution procedure has been carried out. We were interested in determining whether the addition of phospholipid facilitated large changes in the apparent molecular weight of the preparation to produce, for instance, a membrane-like structure. We are not attempting to read into the data on the apparent molecular weight any more meaning than this.  
Dr. Brodie, NHLI: Turning the question around, it is difficult to see how a reaction can occur between a nonpolar enzyme and a lipid-soluble substrate without something to bring them together. A phospholipid would be an ideal bed on which a polar enzyme could become acquainted with its nonpolar substrate. Is the problem really so difficult?  
Dr. Raisfeld: I think Dr. Coon's lab is going to have a great time solving the rest of this problem!