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Reconstitution of Cytochrome P-450 and Cytochrome P-450 Reductase into Phosphatidylcholine-Phosphatidylethanolamine Bilayers: Characterization of Structure and Metabolic Activity

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SUMMARY

BÖSTERLING, B., J. H. DAWSON, A. G. HILDEBRANDT, A. STIER, AND J. R. TRUDELL: Reconstitution of cytochrome P-450 and cytochrome P-450 reductase into phosphatidylcholine-phosphatidylethanolamine bilayers: characterization of structure and metabolic activity. *Mol. Pharmacol.* **16**, 332-342, (1979).

Cytochrome P-450 LM-2 and NADPH-cytochrome P-450 reductase have been reconstituted into phosphatidylcholine-phosphatidylethanolamine vesicles using a cholate dialysis technique. The structure and composition of the reconstituted system has been characterized by gradient centrifugation, electron microscopy, phosphorous nuclear magnetic resonance, phosphate and protein analysis. The absence of conversion of cytochrome P-450 to cytochrome P-420 during reconstitution has been verified by visible spectroscopy of the reduced CO-bound form. The metabolic activity of the vesicle reconstitution and a nonvesicular preparation has been characterized by NADPH utilization and H_2O_2 production with and without substrates hexobarbital, cyclohexane, aminopyrine, and benzphetamine as well as by the metabolic production of formaldehyde from aminopyrine and benzphetamine. The reconstitution technique produces vesicles of sufficient diameter and protein content (33% by wt) that each may contain at least two reductase and 10 or more cytochrome P-450 proteins. This vesicle reconstituted system is similar to microsomes with regard to bilayer structure, phospholipid composition, surface charge, protein: lipid ratio, cytochrome P-450:NADPH-cytochrome P-450 reductase ratio, and enzymatic coupling. Therefore it may be an excellent system in which to study substrate specificity, electron transport, reductive versus oxidative metabolism, and protein-lipid interactions.

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INTRODUCTION

The preparation of pure forms of liver microsomal cytochrome P-450[4] and its associated reductase, followed by reconstitution of activity in a detergent-phospholipid dispersion, by Coon (1) and others (2, 3) has opened up a new area for the study of the mixed function oxidases. However, certain aspects of this detergent-phospholipid dispersion are unlike those of the endoplasmic reticulum. In particular, the ratio of reductase to P-450 used to obtain maximum activity in the detergent-phospholipid dispersion (1) is up to 100 times that which occurs in the endoplasmic reticulum. Furthermore, due to their aggregational state, it is possible that the cytochrome P-450 and NADPH-cytochrome P-450 reductase in the detergent-phospholipid dispersion do not exhibit the same range of protein-lipid and protein-protein interactions, due to lateral and rotational diffusion or transient cluster formation which are available to proteins in a phospholipid membrane.

These protein-protein and protein-lipid (4, 5) interactions may be important to an understanding of the normal hydroxylation function of the mixed function oxidase system as well as to the production of hydrogen peroxide (5–7), free radical intermediates (8), reactive carbanions (9–11) and activated carcinogens (12). In order to study these protein-protein and protein-lipid interactions, we have prepared a reconstituted system in which the proteins are introduced into the plane of a phospholipid bilayer vesicle and may be able to undergo lateral motion, transient or permanent cluster formation, and rapid vibration contacts.

Ingelman-Sundberg and Glaumann (13) described the reconstitution of cytochrome P-450 and P-450 reductase into phosphatidylcholine (PC) vesicles at a 1:40 protein: lipid ratio using the technique of rapidly removing sodium cholate from a protein-phospholipid-cholate suspension by passage through a gel filtration column. Taniguchi *et al.* have recently reconstituted cytochrome P-450 LM-2 and cytochrome P-450 reductase into egg lecithin vesicles with a protein:lipid ratio of 1:3 to 1:30 by application of a cholate dialysis technique (14). However, these techniques appear to be unsuitable for the preparation of nonaggregated reconstituted vesicles with natural phospholipid composition and very high protein-phospholipid ratios which we require for future studies with cytochrome P-450 and cytochrome P-450 reductase on electron coupling and protein-membrane interactions.

Based on knowledge of the structure and composition of liver microsomes (15) we consider the following characteristics to be required for these future studies: (a) A 1:5 ratio of reductase to P-450 similar to that achieved in microsomes (15). (b) A high enough protein to lipid ratio such that each vesicle may contain at least one P-450 reductase and five P-450 proteins. This condition will allow study of electron transfer between P-450 reductase and P-450 within the same vesicle. A 30 nm diameter vesicle with a 4 nm thick bilayer would require a protein to lipid ratio greater than 1:14 to contain six proteins totaling 3.5×10^5 daltons. The gel filtration system previously described (13) yielded a protein lipid ratio of only 1:40. (c) The vesicles should contain both PC and phosphatidylethanolamine (PE) because these phospholipids are the two most common in microsomes, where they occur in a 2:1 ratio (15). Mixtures of these phospholipids have previously been shown to be important for optimum protein reconstitution (16–19). (d) The vesicles should have a negative surface charge in order to mimic microsomal surface properties (15) as well as to prevent aggregation. (e) The reconstituted vesicles should exhibit an efficient coupling between NADPH oxidation and hydroxylation of substrate. (f) The production of hydrogen peroxide with and without substrate bound to P-450 should have the same rate constant as microsomes.

We have prepared the reconstituted system by a technique of solubilization of both proteins and phospholipids in sodium cholate followed by slow removal of the cholate

[4] The abbreviations used are: PC, egg phosphatidylcholine, PE, egg phosphatidylethanolamine, PA, dipalmitoyl phosphatidic acid; P-450, liver microsomal cytochrome P-450 LM-2; DLPC, dilauryl phosphatidylcholine; ML, microsomal lipid; ^{31}P-NMR, phosphorus-31 nuclear magnetic resonance.

by dialysis in the presence of glycerol. We have characterized the reconstituted system by gradient centrifugation, electron microscopy, and metabolic activity toward a variety of substrates.

MATERIALS AND METHODS

Cytochrome P-450 LM-2 was purified from microsomes of phenobarbital-pretreated rabbits (1–3, 6). Sixty grams of microsomal pellet were dissolved at 4° in 3 liters of 20 mM pH 7.7 Tris-HCl buffer containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.8% Renex 690 and 0.1% sodium cholate and were then applied on a 5 × 75 cm DEAE-cellulose column (Servacel, type 23 SS, Cap 0.62). The P-450 was eluted with the same buffer, collected, and then bound on top of a 5 × 30 cm column of 1:1 mixed Bio-Gel HTP and Sephadex G-75. The P-450 LM-2 was eluted with a linear potassium phosphate gradient from 0.02 to 0.25 M in a pH 7.3 buffer containing 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.2% Triton N-101 and 20% glycerol. The fractions containing the P-450 LM-2 were pooled and diluted with an aqueous solution of 20% glycerol. The protein was bound on a 2.5 × 20 cm Sephadex CM-50 column. Triton was removed with 10 column volumes of 0.02 M pH 7.3 potassium phosphate buffer containing 20% glycerol. The triton absorbance at 276 nm was at background after 2.5 column volumes. The P-450 LM-2 was then recovered in 12.5% overall yield by including 0.1% sodium cholate in the buffer and linearly increasing the phosphate concentration to 0.5 M. SDS electrophoresis with a modified Laemli method showed that over 90% of the protein was P-450 LM-2 with a molecular weight of 48,000. The major (5%) impurity was a protein with a molecular weight of 55,000. The specific activity was 18 μmoles P-450 per mg protein.

The cytochrome P-450 reductase was prepared essentially as described by Yasukochi and Masters (20). The starting material was the eluate of the DEAE-cellulose column from the P-450 preparation obtained when the buffer was made 0.3 M in KCl. The final reductase preparation had a specific activity of 40 μmole cytochrome C/min/mg at 30°.

Egg phosphatidylcholine and egg phosphatidylethanolamine, and dipalmityl phosphatidic acid (PA) were purchased from Lipid Products, Nutfield, England. The colorless material was checked for purity and identified by TLC on Kiesel gel F 254 eluted with a 65:25:4 mixture of $CHCl_3$:MeOH:H_2O. ^{31}P-NMR spectroscopy was used to test for the presence of minor phosphorus-containing impurities. Other chemicals were of reagent grade from Merck, Fluka, Boehringer and Serva, sodium cholate was recrystallized from water and ethanol before use. The "standard buffer" used in the reconstitution was 20 mM HEPES-Cl of pH indicated in the text containing 20% glycerol and 0.1 mM EDTA.

Preparation of reconstituted systems. A typical vesicle reconstituted system with a PC:PE:PA lipid ratio of 2:1:0.06 (w/w), a protein:lipid ratio of 1:5 (w/w) and a P-450 reductase:P-450 ratio of 1:5 (mole/mole) was prepared by a cholate dialysis technique. Twenty mg PC, 10 mg PE and 0.6 mg PA were mixed in $CHCl_3$/methanol 1:1, the solvent was removed with a stream of nitrogen and the lipids were maintained under vacuum for 5 hours. The phospholipids, 1.45 mg P-450 reductase and 4.5 mg P-450 LM-2 in 4 ml 0.1 M pH 7.5 potassium phosphate buffer containing 20% glycerol were sonicated in a bath under an atmosphere of air for one minute at room temperature. Very mild sonication was employed to prevent introduction of air into the solution which could result in lipid peroxidation. Then 0.4 ml of a clear 20% solution of sodium cholate was added and the mixture was allowed to stand for several hours at 4° until the lipid was completely dissolved. The solution was dialyzed under nitrogen against frequent changes of 250 ml portions of pH 8 standard buffer for 3 days at 4°. One g of prewashed (methanol, water) Amberlite XAD-2 was included in each change of buffer. The Na-cholate remaining in the vesicles after a similar reconstitution of rhodopsin was shown to be less than one cholate per 100 phospholipid molecules by measuring the residue of ^{14}C-cholate (17). The resulting vesicle solution was layered on top of a linear 5–50% glycerol gradient

prepared with standard buffer and centrifuged for 12–15 hours at 10^5 g. The resulting weakly opalescent vesicle band (density 1.060–1.075 g/ml) was concentrated with dry Sephadex G-50 and dialyzed against standard buffer at pH 7.5. The preparation was stable for at least two weeks at 4°.

In order to prepare a stock solution of P-450 LM-2 and P-450 reductase dispersed in dilauryl phosphatidylcholine (DLPC) and sodium cholate as a control for the vesicle reconstituted system; 300 μl of a 1% by weight sonicated suspension of DLPC in water was added to a solution of 1.5 mg P-450 reductase and 4.6 mg P-450 LM-2 in 3 ml pH 7.5 standard buffer containing 0.1% sodium cholate. For tests of activity toward substrates 20 to 100 μl aliquots of this stock suspension were diluted in 2 ml of pH 7.5 0.02 M HEPES-Cl buffer.

In an attempt to prepare a vesicle reconstituted system with a 1:5 protein:lipid ratio by gel exclusion chromatography (13), phospholipid, protein and cholate were mixed as described above for the cholate dialysis procedure. Two ml of this suspension were applied on a 1.5 × 25 cm Sephadex G-50 column and eluted at 4° with pH 7.8 standard buffer at 4 ml/min.

The oxidation of solutions of NADPH by the vesicle reconstituted and the DLPC-cholate control systems was measured at 340 nm with a Cary 14 spectrophotometer. Hydrogen peroxide was determined by the production of CH_2O during oxidation of methanol in the presence of catalase (7) in 0.05 M pH 7.5 Tris-HCl buffer.

Density gradient centrifugation of vesicle and control systems was performed in a linear 5–50% glycerol gradient containing pH 7.5 standard buffer with a SW 36 rotor at 30,000 rpm for 12–15 hours in a Beckman L5-65 ultracentrifuge. Visible bands in the gradient were analyzed for density via refractive index and for lipid and protein content.

In order to determine vesicle diameters by electron microscopy, a 20 μl portion of the vesicle suspension was diluted in 700 μl cold water, 100 μl 4% OsO_4 were added and the suspension was fixed at 4° for 30 min. The vesicles were negative stained with 1% uranyl acetate on formvar-coated copper grids and then examined with a JOEL 100 electron microscope.

Before and after the reconstitution procedures the phospholipids were extracted from the preparations and the ratio of PC to PE was determined by ^{31}P-nuclear magnetic resonance (^{31}P-NMR). The phospholipids were dissolved in 2 ml $CDCl_3$:methanol (1:1) and then proton decoupled ^{31}P-NMR spectra were recorded on a Bruker WH 270 pulse Fourier transform spectrometer operating at 109.32 MHz with a 10 μsec pulse width. The integrated spectra were compared with those taken from standard mixtures of PC and PE.

In a test of the thermal stability of the P-450 as a function of its membrane environment, a cholate-dialyzed reconstituted vesicle system, a microsome suspension, a control preparation of P-450 and P-450 reductase dispersed in DLPC-cholate aged at 4° for two hours and another control aged for six days at 4° were incubated in pH 7.5 standard buffer at 37°. Aliquots were taken at intervals and the remaining cytochrome P-450 was determined by difference spectra of the reduced P-450 LM-2 + CO complex versus reduced P-450 LM-2 (2).

RESULTS

We were able to reconstitute P-450 and P-450 reductase at a 5:1 mole ratio into PC:PE:PA (2:1:0.06 w/w) bilayer vesicles using a cholate dialysis technique (16–19). Vesicles with a protein:phospholipid ratio of either 1:5 or 1:2 (w/w) could be prepared as demonstrated by ultracentrifugation. The 1:5 protein:phospholipid vesicles banded between 1.062 and 1.078 g/ml in a 5–50% glycerol gradient (Fig. 1) and appeared to be single round closed vesicles of 40–100 nm diameter by electron microscopy. The vesicles with a 1:2 protein:phospholipid ratio banded between 1.090 and 1.097 g/ml and appeared to be slightly aggregated single vesicles of 40–60 nm diameter by electron microscopy.

An essential condition for preparation of protein-containing phospholipid vesicles was equilibration of protein and phospholipids in 1.5% sodium cholate in standard buffer for three hours at room temperature or 15 hours at 4° before dialysis was begun.

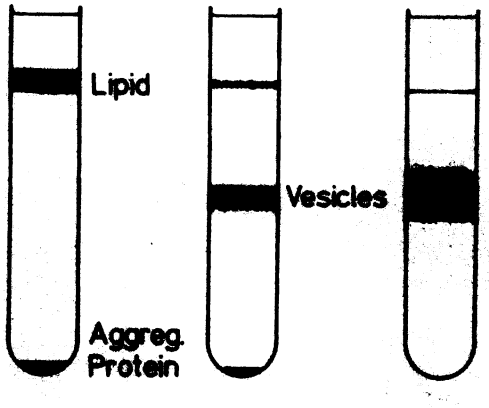

FIG. 1. *Linear 5 to 50% glycerol density gradients after 4.5 mg cytochrome P-450 LM-2, 1.45 mg P-450 reductase, 20 mg PC, 10 mg PE, 0.6 mg PA in 4 ml standard buffer were subjected to one of the following solubilization procedures*

Left) addition of either 16 mg sodium cholate or no cholate at all followed by sonication for 30 min in a bath at 25°; Middle) addition of 16 mg sodium cholate followed by sonication with a microtip for one minute at 20°; Right) addition of 32 mg cholate and treatment as in the middle vial; after which all three preparations were incubated for 14 hours at 4°, then dialyzed for three days at 4°, and finally evaluated by ultracentrifugation.

A pure lipid band at density 1.03 g/ml and a protein pellet are formed from any suspension containing less than 0.4% sodium cholate if dialysis is started immediately after mixing (Fig. 1, left). Electron microscopy showed that the lipid band consisted of large lipid aggregates and not of vesicles. Bands of protein-containing vesicles could be obtained with initial incubation mixtures varying from 0.5–5 mg protein and from 2–20 mg lipid per ml. The density of the band was controlled by the lipid to protein ratio but the width of the band and the diameter of the vesicles was dependent on the duration, temperature and concentration of cholate treatment at the given protein and lipid concentration (Fig. 1). The PC to PE ratio in the vesicles after reconstitution was determined by ^{31}P-NMR spectroscopy. The ratio was not changed during reconstitution. When sufficient cholate was used (1.5%) to dissolve all the lipid the initial ratio of protein to lipid was always maintained in the vesicles following the reconstitution procedure. After dialysis of the mixtures using PC:PE:PA ratios of 2:1:0.06 over 80% of the total lipid and protein are recovered. Up to 90% of these recovered components are found in a single band in the density gradient.

Further experiments demonstrated that PE is essential for the reconstitution of P-450 and P-450 reductase at high protein: phospholipid ratios (Fig. 2). P-450 may be reconstituted with PC:PA 1:0.02 (w/w) alone by the slow cholate dialysis technique only when a protein to lipid ratio of 1:10 (w/w) or less is used (Fig. 2e). When the protein to lipid ratio is increased from 1:10 to 1:5 with only PC + PA (Fig. 2f) at a phospholipid concentration of 5–15 mg/ml, a protein-lipid pellet is formed. Repeated attempts failed to form reconstituted protein-phospholipid vesicles at a 1:5 ratio (w/w) of P-450 to PC + PA (Fig. 2f). Use of other buffer substances (Tris-HCl, phosphate), higher or lower ionic strength, other pH values, different cholate concentrations, and the addition of $CaCl_2$ did not result in successful reconstitutions. Only rapid removal of cholate by dialysis at 30° at a rate that is comparable to that of the G-50 gel filtration method (13) resulted in a low yield of a thin band at density 1.08. The 1:10 protein:PC + PA preparations appeared to be slightly aggregated single vesicles of 40–100 nm diameter by electron microscopy. By comparison, control preparations of PC as well as extracted microsomal lipids (ML) sonicated in buffer gave dispersed vesicles of approximately 25 nm diameter.

In contrast to this unexpected behavior of the P-450 LM-2:PC + PA system, P-450 LM-2 is easily reconstituted with extracted microsomal lipids by the dialysis procedure (Fig. 2d). Therefore, a compound must exist in microsomal lipid that stabilizes vesicle membrane structure when P-450 is present at a protein to lipid ratio similar to microsomes (15). The inclusion of PE, the second largest component of microsomal phospholipids (15), along with PC and PA proved to be a satisfactory mixture for the formation of stable vesicles.

In all vesicle reconstituted systems, 2% by weight of the total lipid was phosphatidic acid. This was included not only to

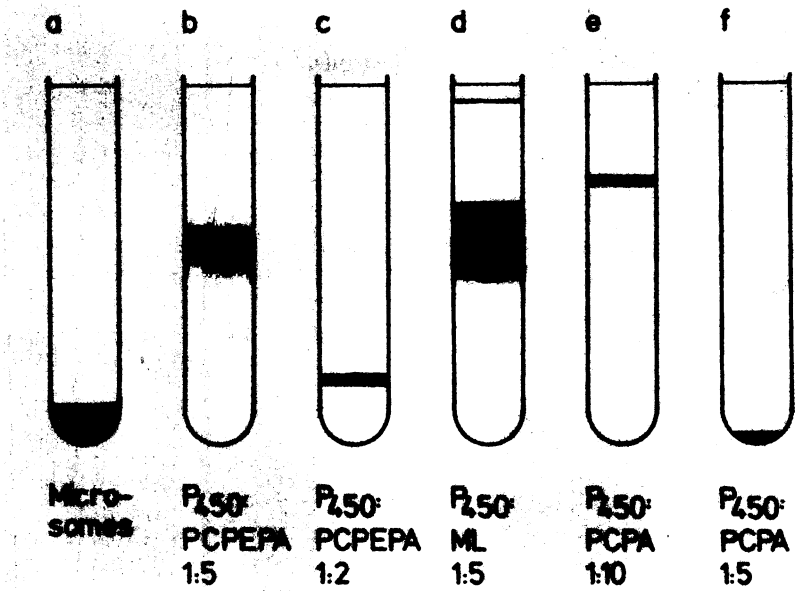

FIG. 2. *Comparison of linear 5 to 50% glycerol density gradients in reconstitution of P-450 and P-450 reductase*

(a) microsomes; (b) a vesicle reconstituted system of P-450 LM-2 + P-450 reductase:PC + PE + PA (1:5 w/w); (c) same as b (1:2 w/w); (d) P-450 LM-2 + P-450 reductase:extracted microsomal lipid (1:5 w/w); (e) P-450 LM-2 + P-450 reductase:PC (1:10 w/w); and (f) same as e except (1:5 w/w).

have a negative surface charge on the vesicles, as do microsomes, but to reduce the aggregation tendency of the vesicles.

The addition of a preformed sonicated suspension of phospholipids to a solution of P-450 LM-2 followed by centrifugation resulted in the formation of a separate pure lipid band and a pellet consisting of all of the P-450 LM-2 protein and some phospholipid (Fig. 1a). Intensive microtip sonication of a suspension of P-450 LM-2 and preformed phospholipid vesicles for three minutes at 20° did not result in the incorporation or attachment of P-450 LM-2 to the vesicles. Isolated P-450 LM-2 centrifuged without lipid or detergent banded on top of the gradient. When the protein plus vesicle mixture was made 0.4% in cholate no change was observed, not even when it was sonicated in a bath for 30 min and allowed to stand overnight (Fig. 1a). Intensive microtip sonication of the mixture containing 0.4% cholate resulted in the partial formation of vesicles (Fig. 1b). When the incubation mixture was made in 0.8% cholate and was sonicated by microtip as above, all the protein in the mixture was incorporated into vesicles although not all the lipid was reconstituted (Fig. 1c).

The P-450 LM-2 and P-450 reductase dispersed in DLPC-cholate that was used as a control for a comparison of the enzymatic activity of the vesicle-reconstituted system was also analyzed by density gradient centrifugation (Fig. 3). P-450 reductase and P-450 LM-2 1:5 were mixed in pH 7.5 standard buffer with sodium cholate and a sonicated vesicle suspension of DLPC at a protein:lipid:cholate ratio of 2:1:1 (w/w/w). Density gradient centrifugation revealed that this system has at least three components after an incubation of 30 min at 25° (Fig. 3a). There was a lipid band and a lipid-protein pellet as was found for the lipid-protein and lipid-protein-cholate mixtures that were analyzed (Fig. 1) before cholate dialysis. After prolonged incubation of this mixture for six days at 4° these two components disappeared and a broad band appeared that was similar in density to the vesicles reconstituted with PC + PE + PA by slow cholate dialysis (Fig. 3b).

We attempted to use the technique of rapid cholate removal by gel filtration de-

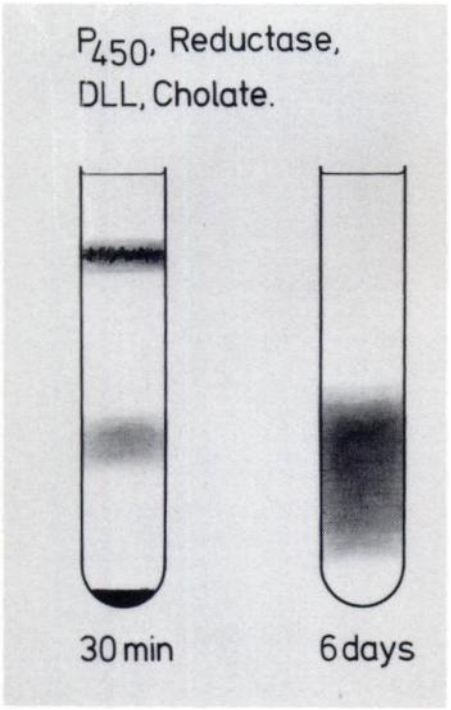

FIG. 3. *Dispersal of P-450 LM-2 and P-450 reductase in a DLPC-cholate system*

Linear 5 to 50% glycerol density gradients of solubilized control preparations consisting of mixtures of 4.6 mg P-450 and 1.5 mg P-450 reductase in 3 ml pH 7.5 standard buffer containing 0.1% sodium cholate and 3 mg DLPC were subjected to one of the following incubations prior to centrifugation: (a) for half an hour at 25° or (b) for six days at 4°. The enzymatic activity of (b) increased as it became more homogeneous.

scribed by Ingelman-Sundberg and Glaumann (13) for reconstitution of P-450 into vesicles. These authors were able to reconstitute P-450 and P-450 reductase into phosphatidylcholine vesicles with a 1:40 protein:lipid ratio. However, in our hands all attempts to reconstitute P-450 at a 1:5 protein:phosphatidylcholine ratio resulted in aggregation on the column. Substitution of our PC + PE + PA 2:1:0.06 mixture for PC in the gel filtration system resulted in a very low yield of vesicles with properties like those prepared by slow cholate dialysis. As discussed in the INTRODUCTION, a 1:14 protein:phospholipid ratio is necessary in order to have at least one P-450 reductase and five P-450 proteins in each vesicle. Therefore we did not study the gel filtration technique further.

In Figure 4 the thermal stability at 37° of P-450 LM-2 in various preparations is shown in order to assess the importance of the lipid environment of the protein. The increase of the thermal stability of P-450 in the DLPC-cholate dispersion paralleled its increase in activity as the dispersion was aged for six days. The P-450 reductase + P-450:PC + PE + PA (1:5 w/w) vesicle system proved to be stable for at least two weeks at 4° in pH 7.5 standard buffer without loss of benzphetamine N-demethylation activity. Both the P-450:PC + PA (1:10 w/w)

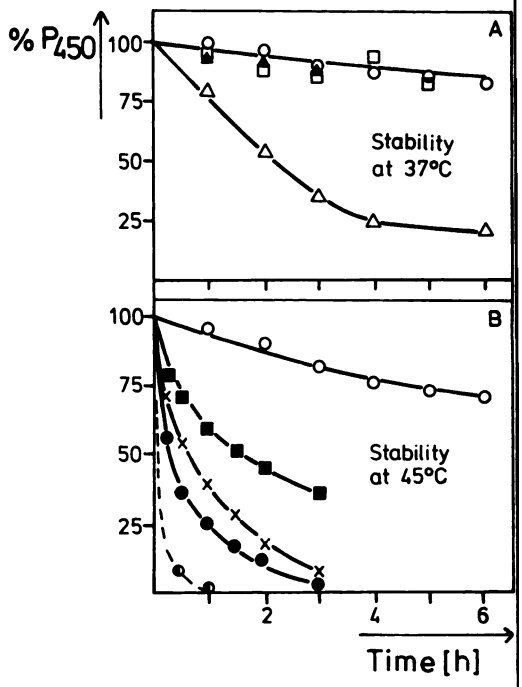

FIG. 4. *Thermal stability of P-450 LM-2*

(A) The percentage of P-450 LM-2 remaining after incubation at 37° in 0.1 M pH 7.5 potassium phosphate buffer containing 20% glycerol in: (a) ○ a suspension of microsomes; (b) □ P-450 reconstituted into vesicles of extracted microsomal lipid; and P-450 LM-2 solubilized in DLPC sodium cholate solutions that were allowed to age at 4° for (c) △ two hours or (d) ▲ six days. P-450 in reconstituted vesicles is as thermally stable as microsomes whereas P-450 LM-2 in the DLPC-cholate dispersion is initially labile and becomes stable with aging. The increase in stability is coincident with a large increase in metabolic activity. In (B) the thermal stability of P-450 LM-2 in buffer ■ and in microsomes ○ is compared to its stability in 1:1 (w/w) mixtures of: 0.1% Renex 690 ◐, extracted microsomal lipid ● and DLPC ×.

nd the P-450:PC + PE + PA (1:2 w/w) esicles aggregated strongly at concentra- ions above 15 mg lipid/ml. Without glyc- rol in the buffers, the aggregation was aster and cytochrome P-420 was formed.

The enzymatic activity of the vesicle re- onstituted system containing P-450 LM-2: -450 reductase 5:1 mole/mole, and pro- ein:PC + PE + PA (1:5 w/w) was meas- red by several methods and compared ith microsomes and the control of P-450 nd P-450 reductase dispersed in DLPC- holate. Over 80% of the dithionite reduci- le P-450 in the vesicle system was reduced nzymatically with NADPH and no cyto- hrome P-420 was detected. The benzphet- mine demethylation activity of the vesicle reparations was used to test systems with -450 to reductase ratios of 5:1 and 50:1 mole/mole) at a constant protein to lipid atio of 1:5 w/w. The two P-450:P-450 re- uctase ratios were chosen to span the nat- rally-occurring ratio (15). The consump- ion of NADPH was compared to the mount of formaldehyde formed; the activ- ties of the two systems are given in Table . It is noteworthy that by decreasing the -450 reductase by a factor of 10 both the NADPH consumption and the product for- nation go down by about a factor of 10. Therefore, the efficiency of the system is onstant and the rate depends on reductase ontent as in microsomes (21).

The enzymatic coupling of the mono-ox- genase function of these systems was measured for the substrates aminopyrine, hexobarbital, cyclohexane and benzphetamine (Table 2). A typical vesicle system formed 13.3 ± 0.3 μmole CH_2O per minute per μmol P-450 from benzphetamine compared to 4.1 ± 0.3 in the control.

For the compounds tested in Table 2 the vesicle system produced more product and less H_2O_2 than the DLPC-cholate control system. Little product is formed from aminopyrine by both systems, in contrast to microsomes. It may be that aminopyrine is a poor substrate for P-450 LM-2. In both systems aminopyrine produces a slight stimulation of H_2O_2 production over the P-450 LM-2 without substrate. Cyclohexane was a good substrate for the vesicle system, it is noteworthy that the increased NADPH consumption of the vesicle system was used for product formation and not hydrogen peroxide formation. With benzphetamine, the vesicle system was also more efficient than the control dispersion. The vesicle system behaved like microsomes relative to the H_2O_2 production in the presence of cyclohexane and relative to the efficiency of benzphetamine demethylation. However, microsomal activity is not a clear reference because it is a sum of several different forms of P-450 and direct comparison with P-450 LM-2 is difficult.

As might be expected from its behavior on density gradient centrifugation (Fig. 3), the enzymatic activity of the DLPC-cholate control system increased appreciably with incubation time allowed after mixing components but before adding substrate. After 15 min of initial incubation time 15 μmole CH_2O/per/min/μmole P-450 LM-2 were formed when the P-450 reductase:P-450 ratio was 12:1, only 0.9 μmole/min/μmol P-450 when the ratio was 1:1 and undetectable when the ratio was 1:5. In order to compare the reconstituted vesicle system with the most active DLPC-cholate dispersion, we studied the protein:DLPC:cholate mixture after it had been equilibrated for three days at 4° to obtain the results shown in Table 2.

TABLE 1

Consumption of NADPH and formation of formaldehyde by P-450 + reductase

The benzphetamine demethylation activity of the tandard slow cholate dialysis reconstituted vesicles escribed in METHODS with a constant 1:5 (w/w) pro- ein:phospholipid ratio (PC:PE:PA = 2:1:0.06 w/w) at -450 LM-2:P-450 reductase mole ratios of 50:1 and 5: . The efficiency of the system is constant and the ctivity is reductase-dependent.

P-450:Reductase	Mol NADPH / Min × Mol P-450	Mol CH_2O / Min × Mol P-450	Mol CH_2O / Mol NADPH × 100
50:1	3.4 ± 0.3	1.7 ± 0.4	50.0 ± 17%
5:1	38.5 ± 0.3	18.5 ± 0.4	48.0 ± 2%

DISCUSSION

The criteria proposed in the INTRODUCTION for a vesicle reconstituted system with

TABLE 2

Metabolic activity of vesicle system, cholate-DLL system, and microsomes

A comparison of the enzymatic activity toward several substrates at 30° in 0.05 M pH 7.5 standard buffer of the slow cholate dialysis reconstituted vesicles containing 5:1 mole ratio of P-450 LM-2:P-450 reductase and 1 5 w/w protein:lipid (PC:PE:PA = 2:1:0.06) with a control preparation of the same total protein content and P-450 LM-2:P-450 reductase ratio dispersed in DLPC:sodium cholate (total protein:lipid:cholate) = 2:1:1 w/w/w and equilibrated for three days at 4°. The consumption of NADPH is compared to the production of H_2O_2 and CH_2O. Values are mean ± S.D. from three experiments. The values for microsomes were determined for a single suspension and are shown in parentheses because of known variation between preparations. All substrate concentrations were 12 times the K_M determined in microsomes.

Mol / Min × Mol P-450 Control	Aminopyrine	Hexobarbital	Cyclohexane	Benzphetamine	
NADPH					
Vesicle System	4.1 ± 0.2	6.1 ± 0.2	10.4 ± 0.6	15.6 ± 0.6	30.3 ± 1.2
Cholate-DLL System	5.3 ± 0.2	7.7 ± 0.4	15.6 ± 0.8	20.0 ± 0.4	27.8 ± 1.5
Microsomes (±10%)	4.0	4.7	6.1	5.5	8.4
H_2O_2					
Vesicle System	4.7 ± 0.5	5.2 ± 0.5	8.5 ± 0.3	4.4 ± 0.6	16.9 ± 0.5
Cholate-DLL System	5.5 ± 0.5	7.6 ± 0.4	15.0 ± 0.5	13.6 ± 0.4	23.0 ± 0.4
Microsomes (±5%)	0.5	0.2	1.7	0.6	1.0
CH_2O					
Vesicle System		1.0 ± 0.3			13.3 ± 0.3
Cholate-DLL System		0.6 ± 0.3			4.1 ± 0.3
Microsomes (±5%)		0.8			1.3
$\frac{H_2O_2}{NADPH}$					
Vesicle System	1.15 ± 0.18	0.85 ± 0.12	0.82 ± 0.08	0.28 ± 0.05	0.56 ± 0.04
Cholate-DLL System	1.04 ± 0.14	0.99 ± 0.10	0.96 ± 0.09	0.68 ± 0.03	0.83 ± 0.06

structure and composition like microsomes have been met. (a) We were able to prepare vesicles with a 1:5 ratio of cytochrome P-450 reductase:cytochrome P-450 LM-2. (b) We could incorporate these proteins at 1:5 or even 1:2 protein:phospholipid ratios. Therefore, each vesicle should contain at least one cytochrome P-450 reductase and five cytochrome P-450 proteins that could exhibit many of the possibilities of electron transfer and protein cluster formation that may occur in the endoplasmic reticulum. (c) These high protein:phospholipid ratios were only possible when both PC and PE were present.

A very important aspect of our PC:PE:PA reconstituted vesicle system is that it does not aggregate, even when prepared in a total concentration of 25 mg/ml. This property will be of great benefit in using the preparation in metabolism studies. The strong aggregation tendency observed by Taniguchi *et al.* (14) was also seen in our attempts at reconstitution using pure PC. We suggest that the PC:PE:PA vesicles do not aggregate because of the structural stability conferred by the PC:PE mixture and the negative surface charge due to the inclusion of ionized PA.

We suggest that fulfillment of these criteria in the vesicles system allows a spatial arrangement between cytochrome P-450 and cytochrome P-450 reductase in the plane of the vesicle bilayer that is similar to that in the endoplasmic reticulum (21). In particular, they may be able to form cluster structures of varying lifetimes, or alternatively, participate in rapid interactions between monomers by lateral and rotational diffusion or transfer electrons within the hydrophobic region of the bilayer by means of small carrier molecules.

The requirement for a PC + PE + PA mixture which approximates the lipid ratios in the endoplasmic reticulum suggests that cytochrome P-450 has a requirement for a specific lipid environment. A similar requirement for both PC and PE for optimum reconstitution was described by Kagawa *et al.* for mitochondrial ATPase (16) and by Hubbell *et al.* for rhodopsin (17). The possible importance of the PC:PE ratio for regulation of intrinsic membrane proteins has been suggested by Hirata and Axelrod (22) for the activity of adenylate cyclase and by Natsuki *et al.* in studies of morphine-dependent rats (23). Further suggestive evidence for an influence of PE:PC ratio on metabolic rate is that rats have a sex difference in their microsomal PC:PE ratio as well as a sex difference with regard to rate of drug metabolism. In contrast, rabbits have neither a sex difference in PC:PE ratio nor in rate of drug metabolism (24, 25).

The empirical findings of the requirement for PE in reconstitution and activity of proteins may be interpreted in structural terms: Stier *et al.*, in a ^{31}P-NMR study of rabbit liver microsomal membranes, as well as of the reconstituted cytochrome P-450 vesicle system described here, suggested that part of the lipids exist in an inverted micellar state which may be indispensible for the structural integration of cytochrome P-450 into the bilayer membrane (5).

The importance of fulfilling our criteria for optimum reconstitution is borne out by characterization of the enzymatic activity of our vesicle reconstituted system in comparison with microsomes and the DLPC-cholate dispersed system. The vesicle reconstituted system has excellent efficiency in terms of moles of NADPH oxidized per mole of hydroxylated substrate. At a given cytochrome P-450 concentration, the activity of the vesicle system is limited by the reductase concentration; reductase-limited activity has recently been reported for benzphetamine *N*-demethylation in liver microsomes of phenobarbital stimulated rats (21). The production of hydrogen peroxide with and without substrate is similar to that in microsomes. Hydrogen peroxide production has been suggested to be a measure of uncoupling of mixed function oxygenation (26) or as an intermediate in substrate hydroxylation (27). The rate of hydrogen peroxide formation appears to depend critically on the disposition of P-450 in a membrane as well as on interaction with a particular substrate. Therefore, we propose that our vesicle reconstituted system may be particularly appropriate for studying the production of hydrogen peroxide as a by-product of P-450 activity (26, 27), as well as electron transport, the activation of reductase by substrate binding to cytochrome P-450, the production of free radicals (8, 28), carbenes (8) or carbanions (9, 10) by the mixed function oxidase system, and for spectroscopic studies of membrane-protein interactions (5, 29).

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