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An Evaluation of Methods for the Reconstitution of Cytochromes P450 and NADPH P450 Reductase into Lipid Vesicles.*

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Abbreviations: BPC – bovine phosphatidylcholine; BPS- bovine phosphatidylserine; CD

– cholate dialysis method of reconstitution; DLPC - dilauroylphosphatidylcholine;

DMPC - dimyristoylphosphatidylcholine; GF – cholate gel filtration method of

reconstitution; SRS - standard reconstitution system;

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ABSTRACT

Two methods (cholate dialysis and cholate gel filtration) used to incorporate cytochromes P450 and reductase into unilamellar phospholipid vesicles were compared to a standard reconstituted system (SRS) where the proteins were reconstituted with preformed liposomes. Both cholate dialysis and gel filtration methods were comparable in their ability to physically incorporate reductase and either CYP2B4 or CYP1A2 into phospholipid, as determined by the elution of enzymes in the void volume using size exclusion chromatography (MW cutoff – 5,000,000 Da). Incorporation of these proteins was more efficient with both cholate methods than when reductase and P450 were mixed with preformed vesicles (SRS). Using either cholate method, greater than 85% of the P450 was physically incorporated into the phospholipids vesicles, whereas less than 40% of the P450 was physically incorporated into the phospholipids vesicles using the SRS. Catalytic activities of the vesicular preparations of reductase and either CYP1A2 or CYP2B4 also were significantly higher than those resulting from the SRS using dilaurylphosphatidylcholine. Although both cholate dialysis and gel filtration methods improved protein incorporation when compared to preincubation of proteins with preformed liposomes, reductase incorporation was dependent on the relative amount of reductase used. Reductase incorporation was complete at a 0.2:1 reductase to P450 ratio; however, the efficiency of incorporation decreased to <50% at equimolar reductase to P450. Interestingly, reductase incorporation was higher in the presence of CYP1A2 than with CYP2B4. Both cholate methods resulted in the loss of a proportion of spectrally detectable carbon monoxyferrous P450, resulting from incubation of the proteins with detergent.

INTRODUCTION

The cytochromes P450 constitute a superfamily of heme-containing enzymes that display enormous diversity with regards to substrate specificity and catalytic activity (Guengerich, 2001). Catalysis by the eukaryotic P450 enzymes involves a complicated, multi-step reaction cycle that includes two steps in which an electron is transferred from a redox partner. The diflavin protein, NADPH cytochrome P450 reductase (reductase) can transfer both electrons needed for the catalytic cycle (White and Coon, 1980). In some P450 reactions, the second electron of the reaction cycle also can be delivered by cytochrome b_5 (Schenkman and Jansson, 2003).

The P450 enzymes and their associated redox partners of the mammalian drug-metabolizing system are embedded in the membrane of the endoplasmic reticulum (Ortiz-De Montellano, 1995). Lipid plays an important role in the reconstitution of P450 dependent activities after protein purification (Lu *et al.*, 1969). Most *in vitro* studies for the reconstitution of P450 activities use dilaurylphosphatidylcholine (DLPC) as the lipid component. The reconstitution of enzymatic activity involves a concentrated incubation of P450, redox partners, and lipid followed by dilution into the final assay components. The reported preincubation conditions vary significantly from one laboratory to another (Causey *et al.*, 1990). For instance, the lipid is typically dispersed by sonication before mixing with the proteins. This step can involve the use of either a probe sonicator inserted directly into the lipid solution or a milder bath sonicator. Furthermore, the duration of the preincubation of proteins and lipid and the temperatures at which the preincubations are performed differ dramatically from one laboratory to the next. In spite of the lab to lab variability, these standard reconstituted systems (SRS) result in

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catalytically active preparations in which the enzymes are associated with but may not be anchored physically into the phospholipid liposomes (Ingelman-Sundberg and Glaumann, 1980d).

In previous studies, our lab has demonstrated that interactions can occur between different P450 enzymes and reductase when combined in a mixed reconstituted system (Backes *et al.*, 1998; Backes and Kelley, 2003). Furthermore, these interactions have been shown to affect metabolism of certain substrates. In order to more fully understand the effects of these protein-protein interactions, we have sought to utilize a reconstitution method that better simulates the lipid environment in the endoplasmic reticulum by having the proteins anchored in a monolamellar bilayer vesicle. The two most common ways of preparing these vesicular reconstituted systems are the cholate gel filtration (Ingelman-Sundberg and Glaumann, 1980c) and cholate dialysis (Taniguchi *et al.*, 1979) methods. Both treatments involve the solubilization of the P450, reductase, and lipids with sodium cholate followed by a detergent removal step. With the gel filtration method, the detergent is removed by size exclusion chromatography, whereas in the dialysis method, the concentration of detergent is reduced by dialyzing the detergent with several changes of a large volume of buffer. In the latter treatment, the dialysis steps encompass 1.5 to 2 days of incubation at 4°C. Upon removal of detergent, the lipids form unilamellar bilayer vesicles with the incorporated proteins (Ingelman-Sundberg and Glaumann, 1980b; Taniguchi *et al.*, 1979).

In this study, we examined the effectiveness of the cholate dialysis and gel filtration methods to incorporate reductase and either CYP2B4 or CYP1A2 into phospholipid membranes, and compare the characteristics of these vesicular reconstituted

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systems with those of the SRS. The methods are compared with respect to the proportion of enzymes physically incorporated into the vesicles and the catalytic activities of the preparations.

MATERIALS AND METHODS

Materials: Bovine liver phosphatidylcholine (BPC) was purchased from Avanti polar lipids (Alabaster, AL). Sodium cholate, 7-ethoxyresorufin, and Sephadex G50 were purchased from Sigma (St. Louis, MO). Benzphetamine was a gift from Upjohn (Kalamazoo, MI). Plasmid Mini, Midi, and Maxi kits were purchased from Qiagen Inc (Valencia, CA). C41 cells were purchased from Avidis SA, Biopole (Clermont-Limagne, France).

Enzyme sources: Rabbit NADPH cytochrome P450 reductase was expressed from a recombinant plasmid, containing the wild type cDNA insert in a vector using the T7 promoter, which was provided by Dr. Lucy Waskell (University of Michigan) and has been described previously (Kelley *et al.*, 2005a). Recombinant CYP2B4 was expressed and purified from *E. coli* as described previously (Kelley *et al.*, 2005b). CYP1A2 was isolated from β NF treated rabbit liver microsomes as previously described (Coon *et al.*, 1978). P450 levels were determined by measuring the carbon monoxy-ferrous complex (Omura and Sato, 1964a).

Enzymatic assays: The activity of CYP2B4 was assessed by measuring the rate of *N*-demethylation of benzphetamine. Formaldehyde production was quantified by measuring fluorescence after the reaction with a modified Nash reagent (de Andrade *et al.*, 1999). The spectrofluorometer was set at excitation and emission wavelengths of 410 nm and 510 nm, respectively. The reactions (1 mL) were incubated at 37°C for 10 min with 335 μ M benzphetamine in the presence of 25 mM potassium phosphate (pH 7.4). Reactions were initiated by the addition of NADPH to a final concentration of 0.8 mM

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and were stopped with 210 μ L of a 1:1:0.1 mixture of zinc sulfate: barium hydroxide: 50 mM semicarbazide as described previously (Hanna *et al.*, 2000).

The activity of CYP1A2 was determined fluorometrically by measuring the rate of formation of 7-hydroxyresorufin upon the *O*-dealkylation of 7-ethoxyresorufin (Lubet *et al.*, 1985). Excitation was set at 522 nm and emission at 586 nm. The samples (1 mL) were incubated at 37°C for 5 min with 7-ethoxyresorufin (2 μ M), 50 mM Hepes (pH 7.5), 0.1 mM EDTA, and 15 mM MgCl₂. Reactions were initiated with NADPH (final concentration of 0.8 mM and were stopped with 0.75 mL of ice cold methanol. Reactions were shown to be linear over this time range.

Reconstitution of reductase and P450 into preformed lipid vesicles: Standard reconstituted systems (SRS) were prepared by sonicating DLPC in a bath sonicator to clarity at a concentration of 8 mM in 50 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol, 0.1 M NaCl, and 5 mM EDTA. Preformed vesicles were also prepared from BPC by drying a chloroform solution containing 2 mg BPC under nitrogen. The dried lipid was mixed with 0.1 mL of 10% sodium cholate and 0.2 mL of 0.5 M potassium phosphate (pH 7.25) and then sonicated as described above. The sonicated lipid was combined with P450 and reductase in the desired amounts, and the volume was taken to 1 mL with water. The mixture was preincubated for 1.5-2 hr at room temperature, and the activities of the reconstituted systems were then measured (Causey *et al.*, 1990). All enzymatic assays were performed with 0.05 nmol P450 with the reductase concentrations indicated.

Preparation of lipid vesicles by the gel filtration method: For both of the reconstitution methods compared in this study (gel filtration (Ingelman-Sundberg and

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Glaumann, 1980a) and cholate dialysis (Taniguchi *et al.*, 1979)), the procedures described in previous publications were followed as closely as possible. Briefly, lipids, dissolved in chloroform and dried extensively (1.5 to 2 hours) under a stream of N₂. The dried lipid sample was suspended in 200 mM potassium phosphate (pH 7.2) containing 2% sodium cholate to a concentration ranging from 1 to 20 mg/ml of BPC. After clarification 0.5 ml of the lipid suspension was combined with 5 nmol of P450 and reductase (ranging from 1 to 10 nmol) and diluted to a final volume of 1 mL. The lipid/protein suspension was preincubated at 4°C for 1-2 h and was then injected on a Sephadex G50 size exclusion column (2.5 x 30 cm) that was run at a flow rate of 0.8 mL/min on an Akta FPLC system (Amersham Biosciences, Piscataway, NJ). The elution of the P450 heme was monitored by measuring its absorbance at 405 nm. Alternatively, in some cases when the eluent through the FPLC column was monitored nonspecifically at 254 nm, the elution of heme was determined by measuring the absorbance at 417 nm of 1.5 mL fractions collected during the chromatographic run. The void volume from this column was collected and used as the vesicular preparation of P450 and reductase.

Preparation of lipid vesicles by the cholate dialysis method: The lipid used in these reconstituted systems was dissolved in chloroform, dried, and sonicated in 200 mM potassium phosphate (pH 7.2) containing 2% sodium cholate solution as described above for the gel filtration method. After solubilization of lipid in 0.5 ml of sodium cholate, P450 (5 nmol) and reductase (ranging from 1-10 nmol) were added and diluted to 1 ml. The samples were then immediately transferred to 0.5 to 3 mL slide-a-lyzer cassettes (Pierce, Rockville, MD) and were dialyzed at 4°C for 36 hr with three changes (12 hr

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each) of 3 L of dialysis buffer containing 50 mM potassium phosphate (pH 7.25), 0.1 mM EDTA, and 0.1 mM dithiothreitol.

Characterization of vesicular preparations of P450 and reductase: The percentages of reductase and P450 actually incorporated in the vesicles were determined by injecting a fraction of the vesicular preparations derived from the different methods on a Tricorn Superose 6 column (Amersham Biosciences, Piscataway, NJ). Using this column, sample components with a molecular weight greater than 5,000,000 Da eluted in the void volume. Proteins that were detected in the void volume were considered to be physically incorporated in the lipid vesicle bilayer preparation.

The amounts of P450 reconstituted into the lipid vesicles were detected from the column eluates by monitoring the 405 nm absorbance of the fractions. The percentages of P450 in vesicles were quantified by measuring the P450 concentrations using the CO difference spectra (Omura and Sato, 1964b). CO difference spectra were also used to determine the amount of P450 recovered. The recoveries of reductase in lipid vesicles and after cholate treatment were determined by measuring cytochrome c reductase activity (Phillips and Langdon, 1962).

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RESULTS

Elution of individual components of the reconstituted system using Superose 6 chromatography: In order to provide a framework with which to evaluate the characteristics of the reconstituted systems on the Superose 6 columns, the individual components of the DLPC reconstituted systems were injected separately (Fig 1), and monitored at 254 nm. DLPC (40 nmol) itself eluted as a broad peak in the void volume of the column (Fig 1A). An injection of 5 nmol of CYP2B4 (Figure 1B) eluted after the void volume at approximately 15 mL, which corresponds to a molecular weight of approximately 250,000 Da. Also shown in Figure 1B are chromatograms showing either the 254 nm absorbance or the 417 nm absorbance of selected fractions (expressed as percentage of P450 eluted) after injection of an SRS with DLPC and CYP2B4. In the presence of DLPC, approximately 35% of the P450 incorporated in the vesicular fraction. The approximately 250,000 Da aggregate observed when P450 was injected without DLPC was also present in addition to a minor proportion of monomeric enzyme, eluting after 20 mL (Fig. 1B). The reductase (Fig 1C) eluted as a broad peak at approximately 12.3 mL, corresponding to a molecular weight of approximately 700,000 Da when absorption was measured at 254 nm. A peak at approximately 20 mL corresponding to monomeric enzyme was also observed. The reconstitution of reductase into DLPC resulted in incorporation of less than 10% of the reductase into lipid vesicles as indicated by cytochrome c reductase activity of selected fractions. The majority of the reductase remained in an aggregate of approximately 700,000 Da. Association with lipid also resulted in the loss of the monomeric sized peak and the appearance of a peak (at

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approximately 16 mL) that corresponds to a molecular weight of approximately 170,000Da.

Elution of the reconstituted systems by Superose 6 chromatography: The standard reconstituted system (SRS), where 2 μ M CYP2B4 and 2 μ M reductase were preincubated with 500 nmol of sonicated DLPC for 2 hours, was also injected on the column (Fig 2A). The elution of total lipid and protein were monitored at 254 nm, whereas reductase and P450 elution was monitored by cytochrome c reductase activity and absorbance at 417 nm, respectively. The chromatogram for 254 nm (detecting total protein and lipid) showed five peaks. The proportion of P450 and reductase in each peak are shown in Table 1. Some reductase (40 %) and P450 (27 %) were incorporated into DLPC vesicles. In addition, approximately 37 % of the reductase and 10% of the P450 were incorporated into 1,300 kDa-sized aggregates. The later-eluting peaks corresponding to smaller molecular weights were comprised of oligomers enriched with either reductase or P450. The large peak eluting at 17.4 mL is largely made up of lipid as indicated by the small absorbance at 417 nm (which detects predominantly P450) and the low cytochrome c activity. The predominant absorbance of the lipid in the reconstitutions at 254 nm is to be expected because of the nonspecific absorbances of the components at this wavelength and the excess lipid used in the SRS (lipid:P450 ratio is 250:1). The application of a spectrophotometric assay for phospholipids (Stewart, 1980) confirmed that the lipid component of the reconstitution systems was responsible for most of the absorbance at 254 nm (data not shown).

This experiment was repeated with dimyristoylphosphatidylcholine (DMPC), because of a previous report (French *et al.*, 1980) that proteins eluted with the void

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volume when reconstituted with DMPC, but not DLPC. In our hands, the use of DMPC did not improve incorporation of these proteins, but produced results similar to those observed with DLPC (data not shown).

Next, an equimolar ratio of reductase and either CYP1A2 or CYP2B4 were reconstituted with BPC using the cholate dialysis method as described in Materials and Methods. Representative chromatograms resulting from the elution of these systems from the Superose 6 column were obtained by measuring the absorbance at 405 nm and are shown in Fig 2B. A chromatogram of a reconstituted system prepared by adding CYP2B4 and reductase to sonicated BPC (SRS) also is shown. The profiles obtained from the cholate dialysis preparations with either CYP1A2 or CYP2B4 were very similar and showed that most of the absorbance at 405 nm (representing the elution of P450) was present in the void volume of the column. This finding indicates that the P450 is physically associated with the phospholipid vesicles. In contrast, when the standard reconstitution method was prepared with BPC instead of DLPC, less than a third of the P450 eluted at a size consistent with vesicle incorporation. The lower amount of P450 in the void volume of the SRS showed that simply mixing the proteins into preformed vesicles did not lead to efficient physical incorporation of proteins into the liposomes.

Although the 405 nm absorbance indicated that the P450 was successfully incorporated in vesicles using the cholate dialysis method, it was observed that the proportion of reductase incorporated in these liposomes was low when an equimolar ratio of P450 and reductase was used. Table 1 shows the percentages of P450 and reductase, respectively obtained in each of the three peaks shown for the three chromatograms of Fig 2B. Under these conditions for both P450 enzymes, only 40% and 65% of the

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reductase was incorporated in the void volume of the Superose 6 column with CYP2B4 and CYP1A2, respectively. The remaining reductase was recovered mainly in peak 2. It is interesting to note that reductase was more efficiently incorporated into phospholipid in the presence of CYP1A2 than CYP2B4.

We investigated whether the unincorporated reductase is actually the proteolyzed form of the enzyme (Black and Coon, 1982). Samples from peaks 1 and 2 from Fig 3 were subjected to polyacrylamide gel electrophoresis and stained with Coomassie Blue. No proteolytically cleaved reductase was eluted from the void volume of the size exclusion column. Although small amounts of the shortened form of the reductase were found in peak 2 from the column, this was present as a contaminant in the starting sample (data not shown). Thus, the incorporation of reductase into the liposomes is not solely a function of proteolysis. These results demonstrate that (1) only a portion of the full-length reductase is physically incorporated into the lipid vesicles, and (2) the proteolytically cleaved 71 kDa form of the reductase cannot incorporate into the void volume lipid. The eluant collected as peak 2 from the column contained both forms of the reductase; however the intensity of the band representing the shortened form of the reductase was much less than that of the full-length form contained in this fraction (approximately three times lower).

Comparison of the cholate gel filtration and cholate dialysis methods for reconstitution of the P450 monooxygenase system: Because of the incomplete incorporation of reductase in the P450-containing vesicles using the cholate dialysis (CD) method, we also prepared vesicles by the cholate gel filtration (GF) method. Figure 3 compares the two methods using identical conditions on the basis of the percent enzyme

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eluted in the void volume of the Superose 6 column, the percent recoveries of reductase activity and carbon monoxyferrous P450 after treatment with sodium cholate, and the rate of benzphetamine metabolism by the reconstituted systems.

Both CD and GF methods resulted in indistinguishable preparations with respect to protein incorporation when tested with identical lipid and detergent concentrations. Importantly, the preparations from both methods had comparable proportions of reductase eluting in the void volume at equimolar reductase to P450 ratios. Consistent with the results in Table 1 showing that both the CD and GF reconstituted systems were superior to a standard reconstitution with BPC in terms of the P450 recovered in the void volume, a similar result was observed when the catalytic activities of the three types of preparations were compared. The rates of benzphetamine metabolism by the preparations using cholate dialysis and gel filtration were almost equal, whereas that of the SRS with BPC resulted in negligible catalytic activity.

One shortcoming of both of the CD and GF methods was that a large proportion of the CO-reactive ferrous heme of P450 was lost upon treatment (Fig 3A). The amount lost in the cholate dialysis procedure was significantly greater than that lost using the gel filtration method. In an attempt to decrease the loss of CO-reactive, ferrous P450, lower concentrations of cholate (0.5% final) were tested (data not shown). Although less P450 was damaged, we also observed lower incorporation of the reductase into the phospholipid vesicles under these conditions. As a result, we chose 1% cholate as the optimal detergent concentration for these studies. Thus, adjustments to the starting enzyme concentrations are required to achieve a desired P450 concentration in these vesicle preparations.

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In an effort to determine if the loss of CO-reducible P450 was due to incubation of the proteins in the presence of cholate, experiments were conducted to examine the effect of prolonged cholate incubation with the P450 enzymes. Figure 4 compares the recoveries of ferrous carbon monoxy-CYP2B4 after various durations of incubation at 4°C in the presence and absence of 1% sodium cholate. The loss of the 450 nm-absorbing species was observed upon incubation with the cholate for the shortest time period (1.5 hr). In addition, the loss did not increase significantly with time of incubation with cholate. Thus, it appears a certain proportion of the CYP2B4 is susceptible to damage upon exposure to the cholate. Despite the loss of ferrous carbon-monoxo P450 after incubation with cholate, there did not appear to be a significant loss of heme from the enzyme because the absorbance at 417 nm was not significantly diminished (data not shown).

Incorporation of P450 and reductase into lipid vesicles of varying composition and proportions of enzymes: Reconstituted systems with lipid, reductase, and either CYP2B4 or CYP1A2 were prepared using different concentrations of BPC. Generally, reductase and P450 incorporation were not greatly affected by changes in BPC. The proportions of reductase and CYP2B4 incorporated into the BPC vesicles ranged from 35 to 55% and 87 to 98%, respectively, as the lipid:P450 ratio was varied from 250:1 to 2500:1 (data not shown). Even more consistency was observed with reconstitutions prepared from CYP1A2 and reductase, with varying amounts of BPC. As the BPC:P450 ratio was varied from 250:1 to 2500:1, the proportions of reductase and P450 incorporated into vesicles ranged from 55 to 63% and 85 to 98%, respectively (data not shown). Thus, the incorporation of reductase was more efficient when reconstitutions

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were prepared with CYP1A2 than with CYP2B4. When the catalytic activities of the reconstitutions with varying BPC were assessed by benzphetamine *N*-demethylation for CYP2B4 (Figure 5A) and 7-ethoxyresorufin *O*-dealkylation for CYP1A2 (Figure 5B), optimal lipid:P450 ratios were observed for each enzyme. At all of the lipid:P450 ratios tested, the reconstitutions in which the proteins were physically incorporated into the BPC lipid vesicles had significantly higher catalytic activities than SRS preparations using DLPC. The optimal lipid:CYP2B4 ratio was observed with the lowest concentration of lipid (250:1). In contrast, the optimal BPC:CYP1A2 ratio was 500:1, and there was a significant activation of the enzyme system at this ratio. With both P450 enzymes, the catalytic activities of reconstitutions with both DLPC and BPC decreased dramatically at the highest lipid:P450 ratios (2500:1). However, the CYP1A2 activity did not decrease as precipitously as the CYP2B4 activity at high concentrations of BPC.

These results demonstrate that although the fraction of protein incorporated into the vesicles is relatively insensitive to the alteration in the BPC:P450 ratio, the catalytic activities vary considerably with elevated lipid. These results are consistent with decreases in reductase:P450 complex formation when the proteins are 'diluted' into larger quantities of lipid. Furthermore, in the case of CYP1A2 but not CYP2B4, the physical incorporation of proteins into phospholipid vesicles (as by the CD and CGF methods) diminishes this dilution effect.

In other experiments (data not shown), we also compared the rate of benzphetamine *N*-demethylation by the cholate dialysate to that of the void volume from the Superose 6 column after injection of the dialysate. No significant differences in

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catalytic activities were observed for these samples. Thus, the presence of unincorporated reductase does not greatly influence metabolism of these substrates by the P450 enzymes. Previous studies have reported that P450 reactions catalyzed by reconstitutions in which the enzymes are physically anchored are stimulated by the inclusion of negatively charged phospholipids (Bosterling, *et al.*, 1981 and Blanck *et al.*, 1984). We suspected that these findings may have resulted from improved incorporation of reductase into the phospholipid vesicles and that the catalytic activities of these preparations were higher by virtue of a higher reductase:P450 ratio and not the intrinsic properties of the enzymes. Thus, cholate dialysis was used to prepare vesicular reconstitutions as described in Materials and Methods except the lipid used was a 3:1 mixture of BPC and bovine phosphatidylserine (BPS). A cholate dialysis reconstitution with BPC alone was prepared at the same time for comparison. In both of these preparations, a 1:5 reductase:CYP2B4 ratio was used because, as explained below, the reductase is efficiently incorporated into BPC vesicles under these conditions. When the reductase incorporation into the vesicles was measured by cytochrome c reductase activity (data not shown), we actually found a lower proportion of reductase in the vesicles containing the negatively charged BPS. However, despite this difference, the catalytic activity of the preparation, as determined by the rate of benzphetamine metabolism, was higher than that by the reconstitution containing only BPC (46 ± 2 and 34 ± 1 , respectively, $n=3$). Thus, negatively charged phospholipids appear to increase the catalytic efficiency of P450 reactions, but not the ability of reductase to incorporate into these vesicles.

Effect of the reductase to P450 ratio on characteristics of reconstituted systems:
CYP2B4 and various concentrations of reductase were reconstituted with PC using the

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CD method. Interestingly, higher proportions of reductase were incorporated in the vesicles at low reductase to P450 ratios (Fig 6A). Essentially all of the reductase was incorporated when the starting ratio of reductase to P450 was 1 to 5. This proportion decreased steadily as the ratio of reductase to P450 increased up to 2:1. As expected, benzphetamine demethylation was increased as a function of the reductase concentration using the vesicular reconstituted systems, approaching saturation at a reductase:P450 ratio between 1 and 2. Figure 6B represents the rate of benzphetamine demethylation as a function of reductase after correcting for efficiency of incorporation and cholate-dependent degradation. From these data, an apparent $K_m^{\text{Reductase}}$ of 33 nM and V_{max} of 154 nmol/min/nmol CYP2B4 were obtained. Figure 6C shows the rates of benzphetamine N-demethylation by SRS preparations with reductase, CYP2B4, and DLPC at comparable lipid to P450 ratios as those used in the cholate dialysis preparations shown in Figure 6B. The apparent $K_m^{\text{Reductase}}$ and V_{max} of the SRS preparations with DLPC were 42 nM and 87 nmol/min/nmol CYP2B4, respectively. Thus, the catalytic activity of the CYP2B4 monooxygenase system when the enzymes are physically incorporated into BPC vesicles is nearly double that observed with SRS preparations using an equivalent concentration of DLPC, whereas the affinity of binding for CYP2B4 and reductase was comparable with both lipid systems.

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DISCUSSION

In an effort to simulate a lipid environment that is more representative of the endoplasmic reticulum membrane, we have characterized two methods previously described for the incorporation of P450 and reductase into unilamellar bilayer lipid vesicles, the cholate dialysis method (Taniguchi *et al.*, 1979) and the gel filtration method (Ingelman-Sundberg and Glaumann, 1977). In our hands, both methods produced similar results by forming catalytically active phospholipid vesicles within which the enzymes appeared to be physically anchored. Furthermore, we could adjust conditions to obtain vesicles with varied lipid:P450 and reductase:P450 ratios.

Although reductase was efficiently incorporated into phospholipid vesicles when low reductase to P450 ratios were used (1 to 5), the proportion of incorporated reductase decreased at higher reductase to P450 ratios. Interestingly, the efficient incorporation of the reductase corresponded with the ratio of reductase: P450 observed *in vivo* (1:10-20) (Estabrook *et al.*, 1971; Peterson *et al.*, 1976). Both cholate methods caused a loss of spectrally detectable carbon monoxy-ferrous heme in the P450 enzyme. The source of the loss in detectable P450 is related to the incubation of P450 in the presence of sodium cholate. Consequently, in order to obtain vesicles with desired reductase to P450 ratios, the starting concentrations of enzymes need to be adjusted.

Interestingly, CYP1A2 appears to better facilitate the incorporation of reductase into the lipid membrane than did CYP2B4. Consistent with the higher incorporation of reductase, CYP1A2 was less susceptible than CYP2B4 to the dilution of catalytic activity when reconstituted into vesicles with a high molar ratio of BPC. Thus, it seems CYP1A2 may have a greater ability than CYP2B4 to “attract” reductase into vesicles and into a

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catalytically active complex. These findings are consistent with the model for heteromeric complex formation proposed by our lab in which the CYP1A2 moiety of a CYP1A2-CYP2B4 complex binds reductase more tightly in the presence of certain substrates (Backes *et al.*, 1998).

Our findings demonstrate that the P450 monooxygenase activities were about 12-fold greater when the enzymes were physically incorporated into the bilayer environment (about 100 nmol/min/nmol P450) than when they were preincubated with previously sonicated BPC (about 10 nmol/min/nmol P450). In addition, the activities of proteins incorporated by the gel filtration and cholate dialysis methods even exceeded those observed with DLPC prepared by the SRS. This finding was consistently displayed with a variety of lipid and reductase concentrations and with both CYP2B4 and CYP1A2. The maximal rate of benzphetamine N-demethylation by CYP2B4 in an SRS with DLPC has been reported to be approximately 50 nmol/min/nmol (Harris *et al.*, 2004; Cawley *et al.*, 1995). Our data suggest that the physical incorporation of reductase and P450 into phospholipid enhances the catalytic activity by bringing the proteins together and effectively increasing their relative concentrations. Thus, the physical incorporation of these enzymes into vesicles may provide a more favorable environment for catalysis.

The cholate dialysis and gel filtration methods are effective techniques for physically incorporating reductase and P450 enzymes into phospholipid vesicles. However, these methods have several shortcomings. First, both methods are more time consuming than standard reconstitution procedures. Both the gel filtration and cholate dialysis methods require at least 1 day to be completed, as compared with 2 h for the SRS. Second, they lead to a generalized destruction of a fraction of the P450, due

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primarily to the incubation of the enzymes in cholate. Third, the efficiency of reductase incorporation is dependent on the relative amount of reductase in the incubation, being completely incorporated at 0.2:1 reductase to P450, but decreasing to less than 60% at a 1:1 ratio. The cholate gel filtration method also suffers in that the final preparation is diluted with respect to the enzyme concentration (P450 concentrations are generally less than 1 μM), limiting the types of studies that can be readily performed. Furthermore, the cholate gel filtration method limits the ability to perform direct comparisons of different reconstitution conditions because samples can only be prepared one at a time. The cholate dialysis method allows for several reconstituted systems to be prepared at once, and the resulting preparations can be made at a relatively high concentration of enzyme (P450 concentrations of approximately 5 μM). However, the method requires 3 to 4 days to prepare reconstitutions involving changes of large volumes of dialysate. This seemed to result in a greater loss of carbon monoxy-reactive ferrous P450. Although none of the drawbacks associated with the methods are insurmountable, they do need to be considered when these techniques are used for reconstitution of these membrane proteins.

We are presently investigating whether we can improve the methods for reconstitution of P450 enzymes and reductase by increasing the proportion of reductase physically incorporated into the vesicles and limiting the loss of ferrous carbon monoxide P450 complex while decreasing both the time required for sample preparation and the sample dilution associated with the preparation method.

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FIGURE LEGENDS

Figure 1 – Interactions of reductase or P450 with DLPC in standard reconstituted

systems determined by gel filtration – Standard reconstitutions with DLPC were prepared and injected on the Superose 6 column as described in Materials and Methods.

(A) Elution of 625 nmol of DLPC as monitored by absorbance at 254 nm. The approximate molecular weights of the peaks are shown at the top of the figure.

(B) Elution of either 5 nmol of CYP2B4 monitored by absorbance at 405 nm or 1 nmol of CYP2B4 in an SRS with 250 nmol of DLPC as monitored at 254 nm. The elution of P450 from the preparation with DLPC is expressed as the percentage of total P450 injected and was determined by measuring the absorbance of selected fractions at 417 nm. The points on the chromatogram show where fractions were taken.

(C) Elution of either 5 nmol of reductase monitored at 254 nm or 1 nmol of reductase in an SRS with 250 nmol of DLPC as monitored at 254 nm or by cytochrome c reduction. The elution of reductase in the mixed system with DLPC is expressed as the percentage of total reductase injected as determined from the relative cytochrome c reductase activities of the different fractions.

Points on the chromatogram indicate where fractions were taken.

Figure 2 – Gel filtration eluates of reconstituted systems combining reductase,

CYP2B4 and lipid– (A) CYP2B4 and reductase (1 nmol of each) were reconstituted with previously sonicated DLPC (250 nmol) as described in Materials and Methods and injected on the Superose 6 size exclusion column. Elution of the components of the reconstituted system was followed by monitoring absorbance at 254 nm. The molecular weights of the peaks are indicated at the top of the figure. (B) Each reconstitution, containing 5 nmol of P450 (CYP1A2 or CYP2B4 as indicated), 5 nmol of reductase, and 2500 nmol BPC, was prepared by the cholate dialysis (CD) or by a standard procedure

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(SRS) involving sonication of BPC in the absence of detergent prior to addition of the proteins. The elution profiles of the reconstituted systems were monitored at 405 nm. The molecular weights of the eluted peaks are indicated at the top of the figure.

Figure 3 – Comparison of CYP2B4-containing reconstituted systems using the cholate

dialysis, gel filtration, and SRS methods – Preparations using 5 nmol each of P450

(either CYP2B4 or CYP1A2 as indicated) and reductase with 1% cholate and 2500 nmol of PC were prepared by the cholate dialysis and gel filtration methods as described in

Materials and Methods. (A) Comparison of the methods on the basis of the percentages

of P450 and reductase eluting in the void volume from the Superose 6 FPLC column, and

the percentages of reductase activity and carbon monoxy-ferrous P450 that were

recovered from the original preparation following treatment with sodium cholate. (B)

Rates of benzphetamine N-demethylation by the different types of reconstituted systems.

Also shown in this panel are results using the “standard” method of reconstitution with

2500 nmol of BPC that was sonicated before adding to 5 nmol each of P450 and

reductase (BPC SRS). The BPC SRS was assayed for benzphetamine N-demethylation

before the sample was run on the Superose 6 FPLC column. The numbers represent the

averages and standard errors of triplicate analyses. * indicates that the average is

statistically different from that of the corresponding gel filtration preparation using an

unpaired student t-test ($P < 0.001$).

Figure 4 – Effect of cholate on the stability of the ferrous carbon-monoxo CYP2B4

complex. CYP2B4 (5 μ M) in 100 mM potassium phosphate (pH 7.4) was incubated at

4°C in the presence or absence of 1 % sodium cholate. The ferrous carbon-monoxo

complex was monitored with time as described in Materials and Methods, and the

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numbers represent the averages and standard errors of three experiments performed on three separate days.

Figure 5 – Effects of lipid concentration on the catalytic activities of different types of reconstitutions of lipid , reductase , and P450 – Vesicular preparations of reductase and PC with either CYP2B4 (A) or CYP1A2 (B) were prepared by the cholate dialysis method. In order to better validate the comparison, the results shown in each panel were derived from reconstitutions that were performed on the same day and with the same batches of lipid and enzyme. (A) Averages and standard errors of triplicate assays of benzphetamine demethylation of the reconstituted preparations with CYP2B4. (B) Averages and standard errors of triplicate assays of 7-Ethoxyresorufin-O-dealkylation of the reconstituted systems containing reductase and CYP1A2. The catalytic activities of SRS preparations made with the indicated DLPC:P450 ratios are also shown for comparison. The catalytic activities of samples collected from the void volume of the Superose 6 FPLC column after injection of the reconstitutions prepared by cholate dialysis are indicated as “Superose void”. “N.D.” indicates that activity was not detected at the 1000:1 lipid:P450 ratio. Thus, a DLPC SRS reconstitution was not prepared with CYP1A2, reductase and lipid at the 2500:1 lipid to P450 ratio. * indicates that the average is statistically different from that of the corresponding “Superose void” preparation using an unpaired student t-test ($P < 0.001$). ** indicates that the average is statistically different from that of the corresponding “Superose void” preparation using an unpaired student t-test ($P < 0.01$).

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Figure 6 – Characteristics of vesicular reconstituted systems with BPC, CYP2B4, and reductase at different reductase to P450 ratios using the cholate dialysis method –

Reconstituted systems with 2500 nmol BPC, reductase (at the concentrations indicated), and 5 nmol CYP2B4 were prepared using the cholate dialysis method as described in Materials and Methods. (A) The reductase and P450 concentrations in the void volume from the Superose 6 column were determined as described in Materials and Methods. The reconstitutions represented in the figure were prepared on the same day with the same stock solutions of enzyme and lipid. The ratios are expressed as the “actual” ratios determined by calculating the proportions of enzymes not incorporated into vesicles and not recovered after treatment with sodium cholate. The ratios of the enzymes added at the start of the cholate dialysis are shown in parentheses on the abscissa. The reductase and P450 concentrations were determined from cytochrome c reductase activities and the absorbencies at 417 nm, respectively. (B) Benzphetamine N-demethylation of reconstituted systems prepared by cholate dialysis using BPC (panel B) as a function of reductase concentration. Each assay included 0.05 nmol of P450 as described in Materials and Methods. (C) Benzphetamine N-demethylation of SRS made with 2500 nmol of DLPC and 5 nmol of P450 as a function of reductase concentration. Each assay contained 0.05 nmol of P450.

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Table 1. Percentages of the total reductase and P450 in the peaks from each chromatogram shown in Figure 2.

Peak #*	1		2		3		4		5	
Condition	% CPR	% P450	% CPR	% P450	% CPR	% P450	% CPR	% P450	% CPR	% P450
DLPC SRS 2B4	40	27	36	11	3	18	6	21	14	3
CD BPC 2B4	40	96	60	2	N.D.	2	Peaks not observed			
CD BPC 1A2	64	98	34	2	N.D.	N.D.				
BPC SRS 2B4	13	37	84	28	3	35				

*The peak numbers refer to those indicated in the chromatogram of figures 2A and 2B. Abbreviations: cpr – NADPH cytochrome P450 reductase

Figure 1

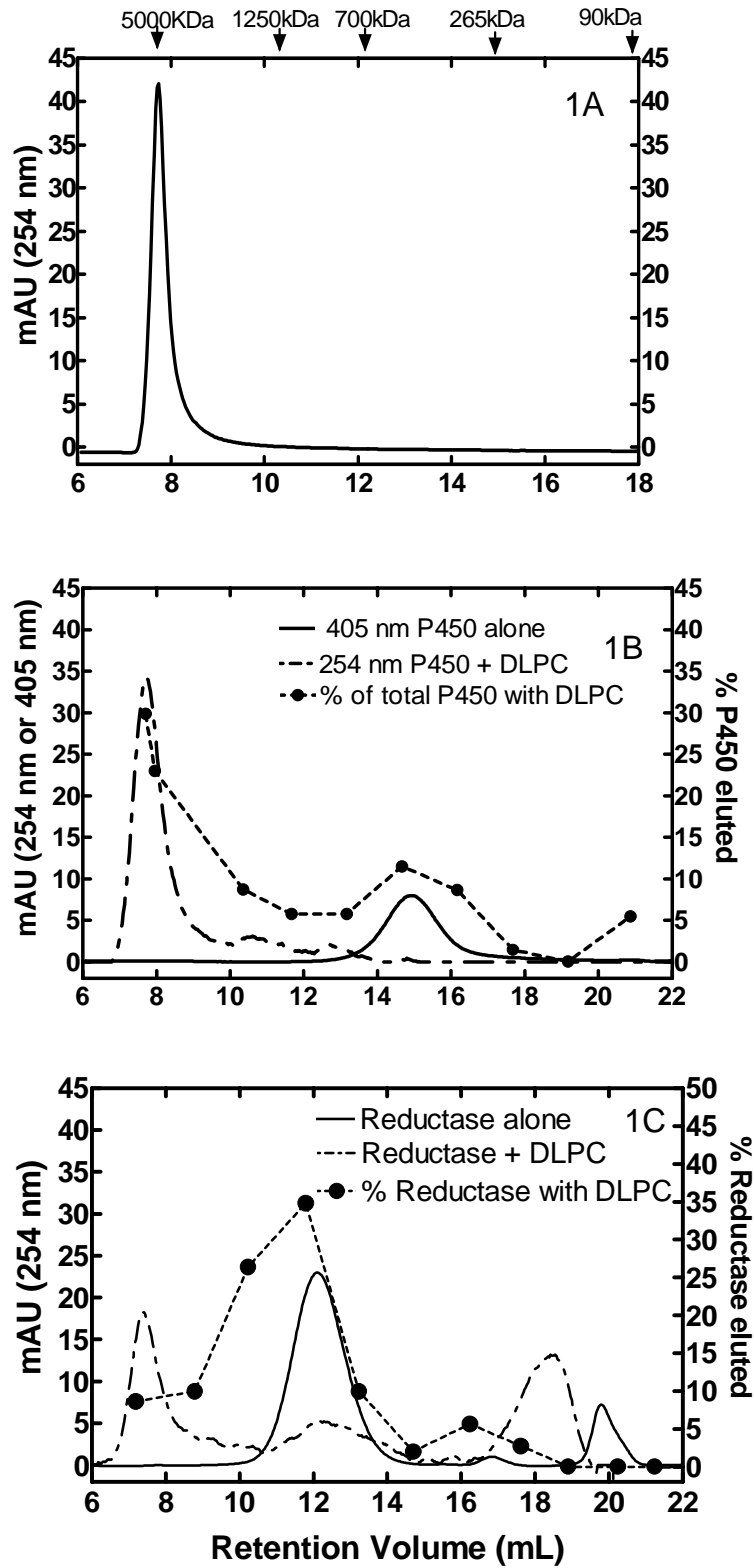


Figure 2

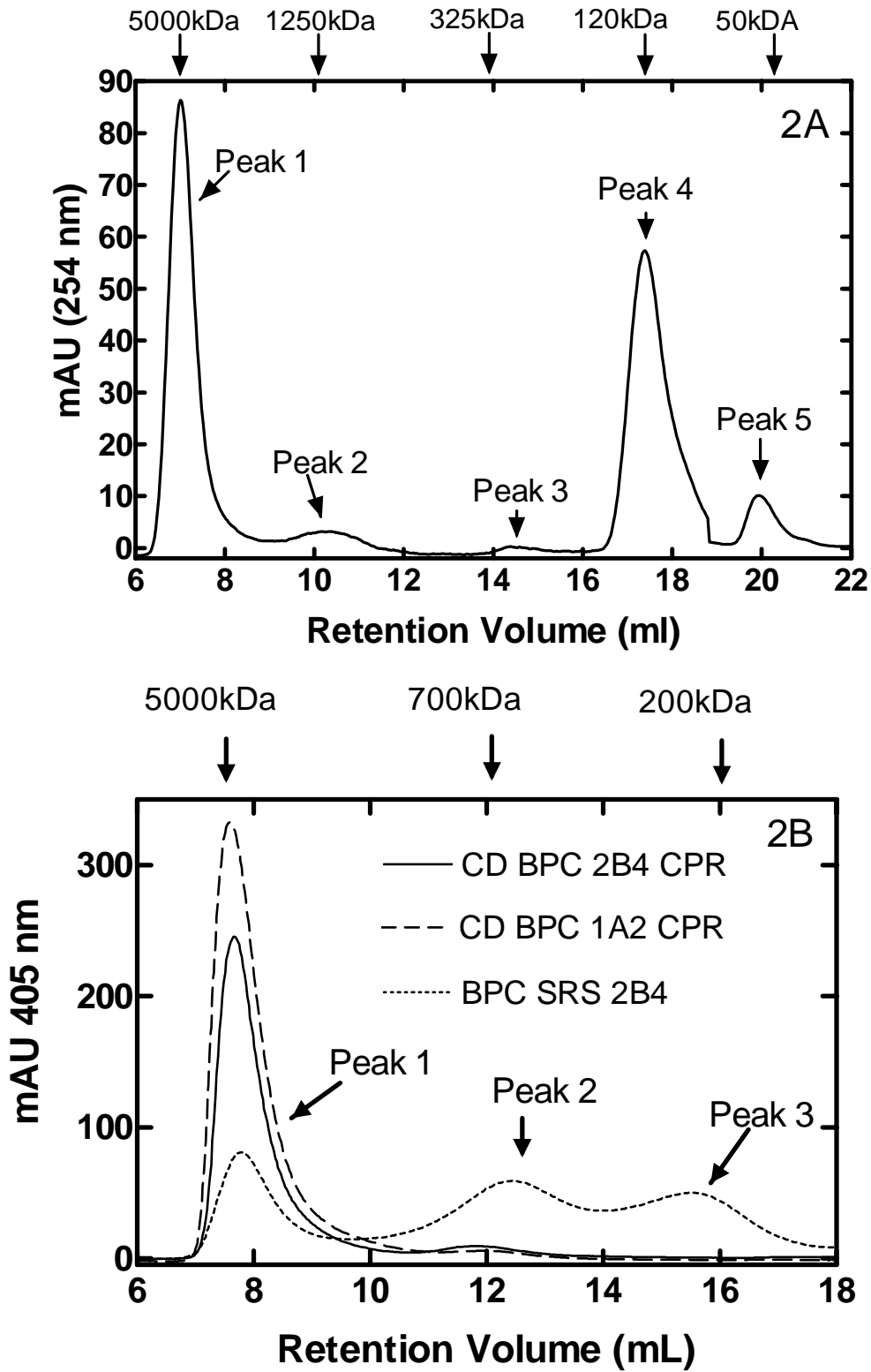


Figure 3

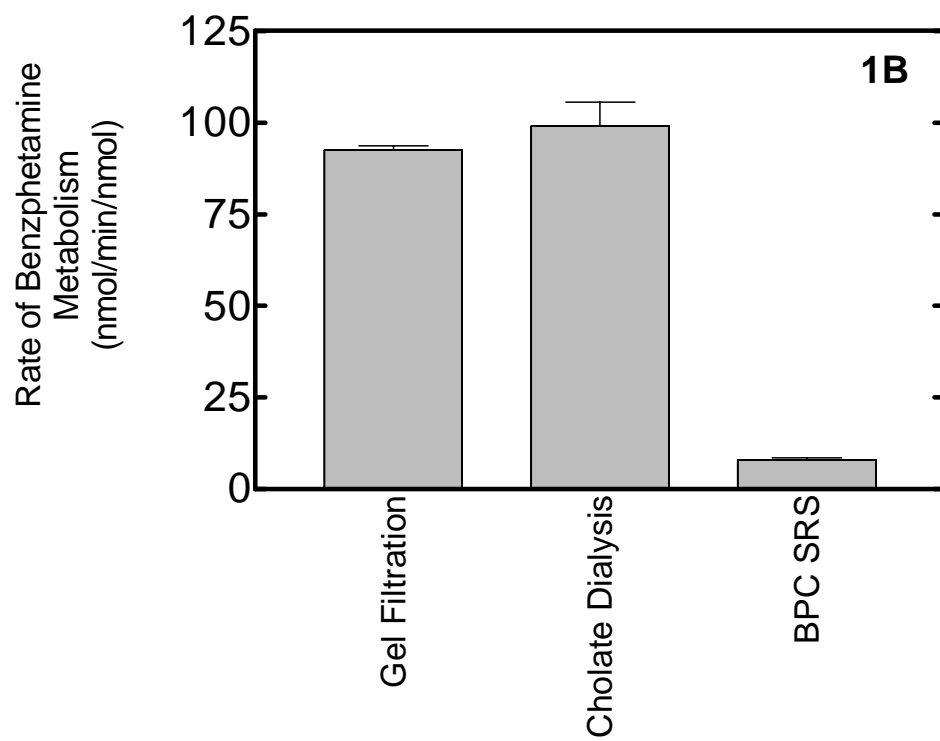
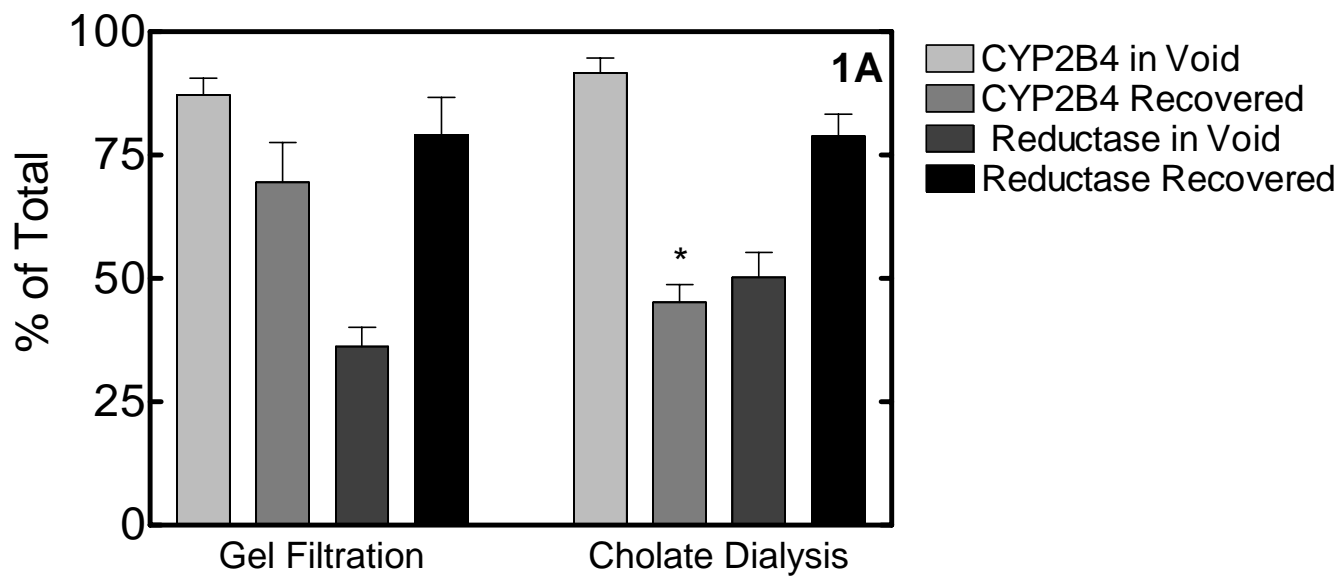


Figure 4

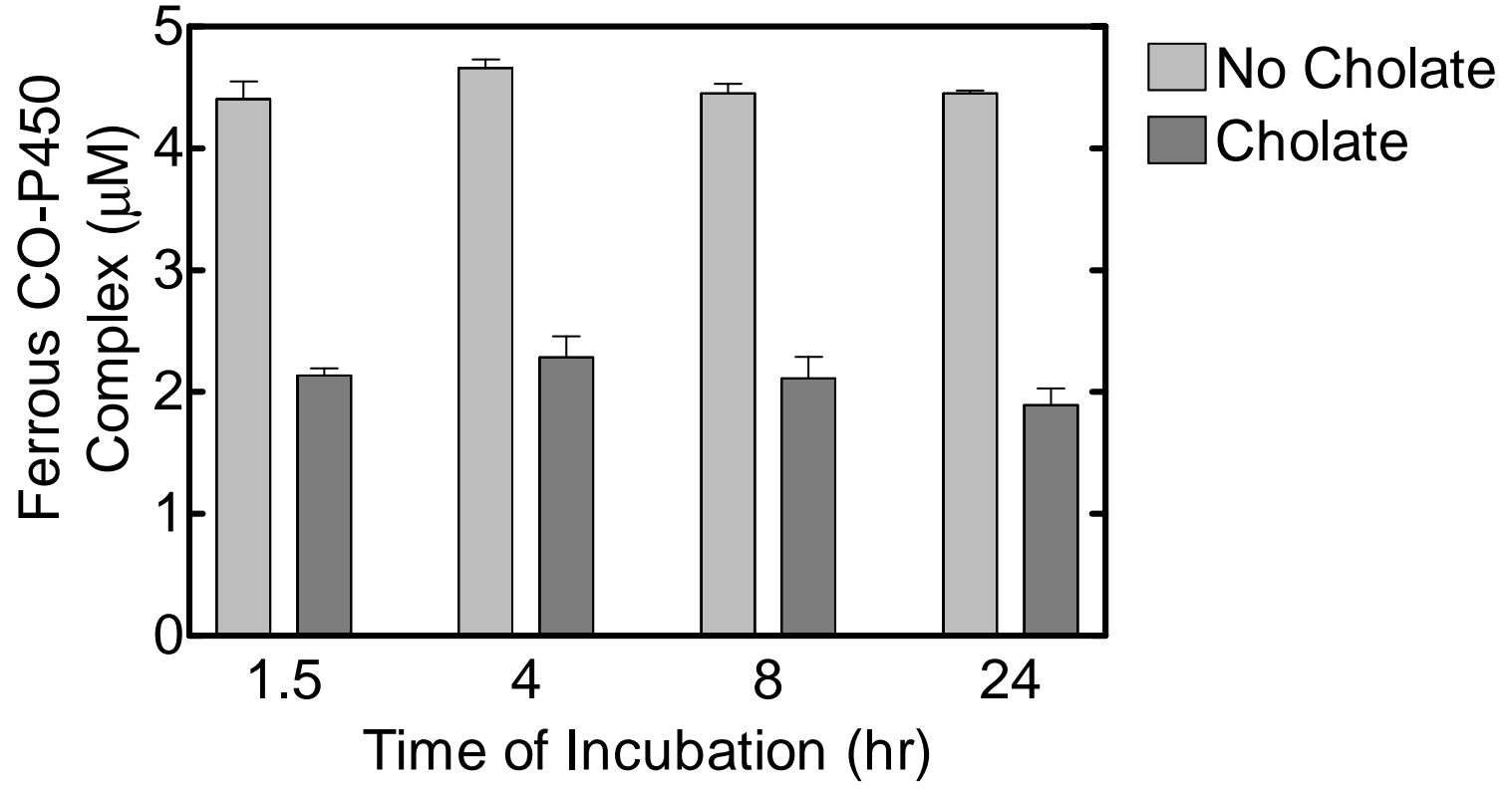


Figure 5

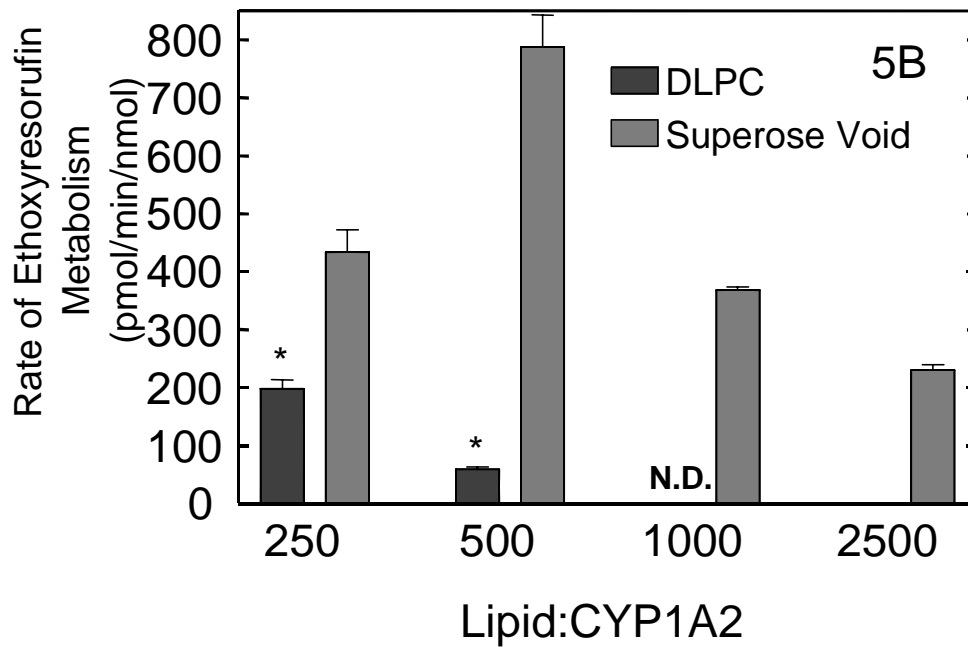
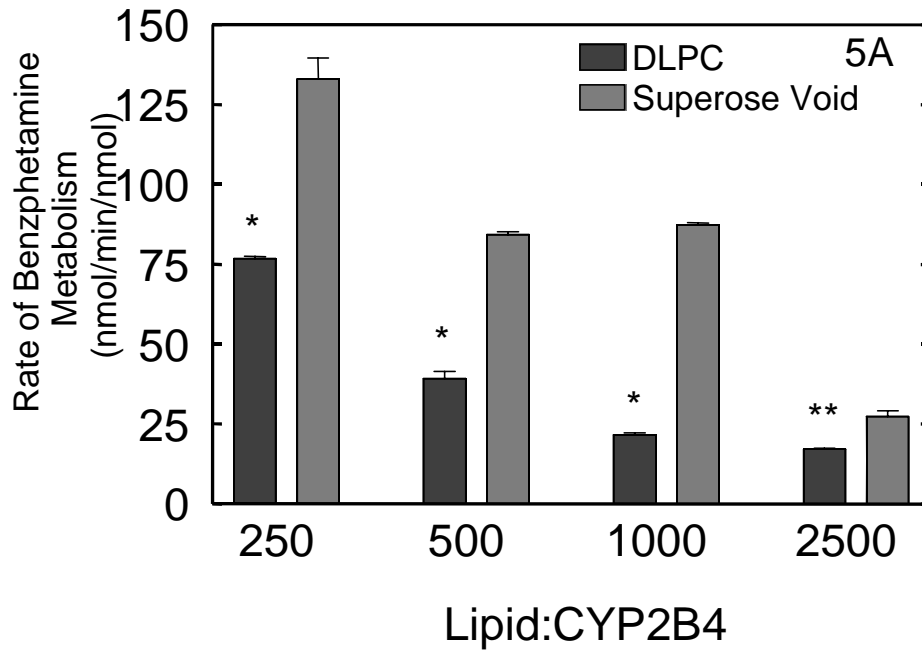


Figure 6

