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INCORPORATION OF PURIFIED COMPONENTS OF THE RABBIT LIVER MICROSOMAL HYDROXYLASE SYSTEM INTO PHOSPHOLIPID VESICLES

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Summary

Methods are described for incorporation of purified forms of rabbit liver microsomal NADPH-cytochrome *P*-450 reductase and cytochrome $P\text{-}450_{LM_2}$, $P\text{-}450_{LM_3}$ and $P\text{-}450_{LM_4}$ (LM, liver microsomes) into phospholipid vesicles. It was found that each cytochrome could individually be incorporated into preformed phospholipid vesicles in the absence of cholate. However, NADPH-cytochrome *P*-450 reductase prevented incorporation of *P*-450 by this method, a phenomenon possibly inherent in the formation of complexes between *P*-450 and the reductase in solution.

Using the cholate-gel filtration technique it was possible to prepare monolamellar phosphatidylcholine vesicles containing any of the cytochromes and *P*-450 reductase in good yields. It was found that $P\text{-}450_{LM_3}$-containing vesicles had a mean diameter of 47 nm, whereas vesicles formed under the same conditions but containing $P\text{-}450_{LM_4}$ were much smaller (mean diameter 33 nm). Vesicles formed with $P\text{-}450_{LM_2}$ were homogeneous in density (1.04 g/cm^3) according to isopycnic centrifugation in Ficoll but not in size (44—72 nm). These findings, taken together with results obtained from treatment of the cytochromes in soluble form and in reconstituted vesicles with the non-penetrating reagent, *p*-diazobenzene sulphonate, indicate a unidirectional, relatively peripheral orientation of $P\text{-}450_{LM4}$ with the major part localized on the outside of the vesicles. Experiments with trypsin and cytochrome *c*-reduction demonstrated a unidirectional orientation of *P*-450 reductase towards the outside of the vesicles.

Abbreviations: LM, liver microsomes; SDS, sodium dodecyl sulphate.

Introduction

The liver microsomal hydroxylase system was resolved more than 10 years ago by Lu and co-workers into three components: cytochrome *P*-450, NADPH-cytochrome *P*-450 reductase and phosphatidylcholine [1,2]. Reconstitution of cytochrome-*P*-450-dependent activities was performed by mixing the two protein components with NADPH and small amounts of phosphatidylcholine. The hydroxylase system has until recently been reconstituted in this way (cf. Ref. 3). Since the native environment of the hydroxylase system is the microsomal membrane bilayer, it is likely that complete reconstitution of the system will not be obtained unless the isolated protein components have been reincorporated into a membrane structure. It cannot be excluded that structure and function of the proteins, the type of protein-protein, protein-lipid or substrate-enzyme interactions and the efficiency of electron transport are different when the hydroxylase system has a lipophilic membrane environment as compared to a hydrophilic surrounding in water solution. Therefore it was considered of importance to work out efficient methods for incorporation of purified components of the liver microsomal hydroxylase system into liposomes.

Racker and co-workers have developed methods to assemble artificial membranes. These methods involve treatment of phospholipid and protein with cholate, with subsequent removal of cholate by dialysis [4] and also incubation of preformed phospholipid vesicles with proteins at 20°C [5].

We have previously shown in preliminary form [7] that by a slight modification of the cholate dialysis method, involving removal of cholate by gel filtration [6], it is possible to prepare monolamellar phospholipid vesicles containing partially purified components of the rabbit liver microsomal hydroxylase system in good yields. This method has also been successfully applied to the unidirectional incorporation of small intestinal sucrose-isomaltase into phosphatidylcholine vesicles [8] and, very recently, also to the reconstitution of the adrenal mitochondrial hydroxylase system into phosphatidylcholine liposomes [9].

In the present paper we report that micellar formation of NADPH-cytochrome *P*-450 reductase in solution and the formation of complexes between *P*-450 and the reductase prevent direct incorporation of the proteins into preformed vesicles. However, using the cholate-gel filtration technique, we show that it is possible to prepare monolamellar phosphatidylcholine vesicles containing purified preparations of NADPH-cytochrome *P*-450 reductase and cytochrome $P\text{-}450_{LM_2}$, $P\text{-}450_{LM_3}$ or $P\text{-}450_{LM_4}$ in good yields.

Materials and Methods

Materials

Sephadex G-50, medium, Sepharose 4B, DEAE-Sepharose CL-6B, CM-Sepharose CL-6B, Sephadex G-25, coarse, and 2′,5′-ADP-Sepharose were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Before use, the Sephadex G-50 and Sepharose 4B columns were pretreated in the following way: 20 nmol *P*-450, 20 mg egg yolk phosphatidylcholine and 20 mg sodium

cholate were mixed in 3 ml 50 mM potassium phosphate buffer, pH 7.4, and this solution was chromatographed on the column in question. The procedure was repeated until the recovery of protein and lipid exceeded 75% and it was found necessary in order to prevent nonspecific adsorption of protein and lipid onto the gel in the subsequent experiments. Egg yolk phosphatidylcholine (Sigma, type III E) was stored in batches of 10 mg in chloroform solution at −20°C in sealed tubes under nitrogen. Dilauroyl phosphatidylcholine was obtained from Sigma and stored as such at −20°C. Total microsomal phospholipids were extracted from liver microsomes of phenobarbital-treated rabbits according to Bligh and Dyer [10] and were stored as described for egg yolk phosphatidylcholine. Renex 690 was obtained from Atlas Chemical Company and sodium cholate from Merck. Ficoll 400 was obtained from Sigma. Hydroxyapatite and Bio-Gel HTP were purchased from Bio-Rad Laboratories. Reference cytochromes $P\text{-}450_{LM_2}$, $P\text{-}450_{LM_{3b}}$ and $P\text{-}450_{LM_4}$ were generously supplied by Professor M.J. Coon. *p*-Diazobenzene sulphonate was prepared immediately before use from sulphanilic acid and $NaNO_2$, as described by DePierre and Karnovsky [11].

Preparation of liver microsomes

Male rabbits, weighing 2—3 kg, were injected with 80 mg of phenobarbital per kg body weight in saline once daily for 5 days. Liver microsomes were prepared as described by Strobel and Dignam [12]. Immediately after death, the livers were homogenized in 1.14% (w/v) KCl containing 10 mM EDTA and freshly added phenylmethylsulphonyl fluoride to 0.25 mM concentration. The microsomal pellet (corresponding to approx. 3 g protein) was washed in the same medium and subsequently homogenized in 120 ml 10 mM Tris-HCl, pH 7.4, containing 20% glycerol and 1 mM EDTA.

Purification of cytochromes $P\text{-}450_{LM_2}$ and $P\text{-}450_{LM_3}$ *

The microsomal suspension was solubilized with sodium cholate using a ratio of detergent to protein of 3 : 1 (w/w) as described by van der Hoeven and Coon [15]. Precipitation with poly(ethylene glycol) 6000 was performed as described [15] and the fractions precipitating between 6—8, 8—10 and 10—13% poly(ethylene glycol) were collected by centrifugation. The two fractions containing most *P*-450, usually the 6—8 and 8—10% fractions were homogenized with 40 ml 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 20% glycerol and dialyzed against 40 vols. of this buffer overnight. A 10% (w/v) solution of Renex 690 was added to a final concentration of 0.5% and the fractions were subjected to separate DEAE-Sepharose CL-6B columns (4 × 25 cm), equilibrated in the 10 mM Tris buffer. The columns were washed with approx. 1 l equilibration buffer and two separate fractions were usually eluted from each column; one slightly turbid fraction that was discarded and one clear fraction containing most of *P*-450 eluting afterwards. The latter fraction was applied

* This protein fraction was previously erroneously designated $P\text{-}450_{LM_2}$ [7]. The identification was based upon electrophoretic mobility in a SDS disc gel polyacrylamide system not capable of resolving $P\text{-}450_{LM_2}$ and $P\text{-}450_{LM_3}$ [13,14].

to a hydroxyapatite column (1.8 × 20 cm, with 20 ml Sephadex G-25, coarse, at the bottom) equilibrated in 10 mM potassium phosphate buffer, pH 7.4, containing 0.1% Renex 690 and 0.1 mM dithioerythritol. The column was washed with 50 ml equilibration buffer and thereafter with 300 ml of a similar buffer containing 50 mM phosphate. Cytochrome $P\text{-}450_{LM_2}$ was eluted from the column with approx. 1 l of a similar buffer containing 100 mM phosphate and the column was washed with buffer containing 150 mM phosphate until A_{416} of the eluate was less than 0.01. $P\text{-}450_{LM_3}$ was eluted in a total volume of approx. 200 ml with 300 mM phosphate buffer, pH 7.4, containing 0.1% Renex 690 and 0.1 mM dithioerythritol. The fractions containing $P\text{-}450_{LM_2}$ were pooled and dialyzed against 4 vols. 10% glycerol overnight. The preparations were concentrated and Renex was removed by treatment with calcium phosphate gel as described by van der Hoeven and Coon [15] including repeated washings of the gel by detergent-free buffer until A_{273} of the wash was less than 0.01. The fractions containing $P\text{-}450_{LM_3}$ were pooled and dialyzed against 20 vols. 10 mM Tris-HCl, pH 7.4, containing 20% glycerol, 0.5% Renex 690, 1 mM EDTA and 0.1 mM dithioerythritol and applied to a CM-Sepharose CL-6B column (2 × 5 cm) equilibrated in the dialysis buffer. The proteins were eluted from the column with a 500 ml linear gradient of KCl (0—0.3 M) in the same buffer. Fractions containing cytochrome $P\text{-}450_{LM_3}$ were pooled and treated with calcium phosphate as described for $P\text{-}450_{LM_2}$.

Purification of cytochrome $P\text{-}450_{LM_4}$

Cytochrome $P\text{-}450_{LM_4}$ was purified from livers of 3-methylcholantrene-treated rabbits (20 mg/kg for 3 days) as described by Haugen and Coon [16], except that DEAE-Sepharose CL-6B was used instead of DEAE-cellulose and the LM_4-fraction was eluted from the DEAE-Sepharose column with buffer containing 75 mM KCl.

Purification of NADPH-cytochrome P-450 reductase

The fraction not precipitated by 13% poly(ethylene glycol) (cf. purification of $P\text{-}450_{LM_3}$) was treated with ammonium sulphate as described [15] and applied to a DEAE-Sepharose CL-6B column (4 × 10 cm) previously equilibrated with 50 mM Tris-HCl, pH 7.4, containing 0.4% Renex 690, 10% glycerol, 0.1 mM EDTA and 1 mM dithioerythritol. The column was washed with 1 l of a similar buffer containing 0.1 M KCl and the reductase eluted with the Tris-HCl buffer containing 0.3 M KCl. The fractions containing the reductase were pooled and applied on a 2′5′-ADP-Sepharose column (1 × 10 cm) previously equilibrated in 10 mM potassium phosphate buffer, pH 7.4, containing 0.1% Renex 690, 20% glycerol, 0.1 mM EDTA and 0.1 mM dithioerythritol. The column was washed with 20 ml equilibration buffer and thereafter with 50 ml of a similar buffer containing 100 mM potassium phosphate. The column was washed with 10 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 0.1 mM dithioerythritol until the absorption at 273 nm of the eluant was less than 0.02 and subsequently equilibrated with 50 mM Tris-HCl pH 7.4, containing 0.1% sodium deoxycholate, 20% glycerol, 0.1 mM EDTA and 0.1 mM dithioerythritol. The *P*-450 reductase was eluted with approx. 200 ml of a similar buffer containing 4 mM 2′-AMP and

the preparation was concentrated to approx. 15 ml in an Amincon ultrafiltration cell, equipped with a PM-10 filter. The reductase preparation was subsequently dialyzed overnight against 40 vols. 100 mM potassium phosphate buffer, pH 7.7, containing 20% glycerol and 0.2 mM EDTA and stored in batches of 0.3 ml under nitrogen at −70°C.

Incorporation of the purified proteins into phosphatidylcholine vesicles

Two methods for incorporation of the enzymes into phosphatidylcholine vesicles were used; the cholate-gel filtration method previously described [7] with some modifications and a method developed by Racker and co-workers [5] involving incubation of the proteins with performed vesicles.

The cholate-gel filtration technique

10 mg of egg yolk phosphatidylcholine in chloroform solution was taken to absolute dryness under nitrogen at room temperature. The residue was suspended in 1 ml 10 mM Tris-HCl pH 7.2, containing 50 mM NaCl and 0.2 mM EDTA. Sodium cholate was added in a 10% water solution to 0.5% (w/v) concentration in the final preparation. When the phospholipid solution was completely clear, *P*-450 and/or *P*-450-reductase were added and the volume adjusted with the Tris-HCl buffer to 2.0 ml. After incubation at 4°C for 1 h, the preparation was subjected to chromatography on a Sephadex G-50 column (1 × 20 cm) equilibrated in the Tris-HCl buffer. The void volume was collected and used as the vesicular preparation. As shown previously [7], the gel filtration removes more than 99.7% of the cholate from the preparation. When vesicles containing $P\text{-}450_{LM_4}$ were prepared, the Tris-HCl buffer was replaced by 100 mM potassium phosphate buffer, pH 7.4, containing 50 mM NaCl and 0.2 mM EDTA. Egg yolk phosphatidylcholine vesicles were stable for 1 week at 4°C.

Incubation with preformed vesicles

10 mg egg yolk phosphatidylcholine or 10 mg microsomal phospholipids in chloroform were taken to absolute dryness under nitrogen. The residue was suspended in 1 ml 50 mM potassium phosphate buffer, pH 7.0, containing 0.2 mM EDTA and the suspension was sonicated under nitrogen at 22°C to clarity using an MSE 150 W disintegrator. Undispersed material was removed by centrifugation at $5000 \times g$ for 5 min. The protein fraction to be incorporated was dialyzed for 4 h against 50 mM phosphate buffer, pH 7.0, containing 0.2 mM EDTA (in the case of $P\text{-}450_{LM_4}$, 100 mM phosphate) and added to the vesicles. The volume was adjusted to 2.0 ml with the buffer and the preparation was incubated for 20 min at 37°C.

SDS-gel electrophoresis

Protein samples were treated with SDS (50 mg/mg protein) and β-mercaptoethanol (50 mg/mg protein) and subjected to slab gel electrophoresis at 4°C in the presence of SDS. The slab gels (130 × 80 × 1 mm) contained 8.5% acrylamide in the separation gel. Electrophoresis was performed at 5 mA per gel during stacking and 10 mA per gel during separation using the discontinuous buffer system described by Laemmli [17]. The gels were stained for protein at

60°C using 0.25% Coomassie brilliant blue R-250 in water/ethanol/acetic acid, 5 : 5 : 1 (v/v) for 20 min and destained overnight at the same temperature in acetic acid/ethanol/water, 1.5 : 1 : 17.5 (v/v).

Electron microscopy

The ultrastructural analyses of the vesicles were performed using negatively stained preparations. Bacitracin (50 μg/ml) was added to the sample as wetting agent and a droplet of the sample was subsequently transferred to Formvar carbon-coated grids. Enough liquid was added to cover the grid right to the edge. The grid was then placed under cover in a refrigerator for 10—20 min to allow the vesicles to attach to the grid. Most of the droplet was then removed with a torn edge of filter paper, leaving a thin film, and a droplet of 1% phosphotungstic acid was immediately applied. 'Staining' was performed for 15—30 s, excess liquid was removed and the film was allowed to dry at room temperature. Grids were examined in a JEOL 100B electron microscope operating at 60 kV, using liquid-nitrogen-cooled anti-contaminating device.

Assay methods

Renex 690 was determined according to Goldstein and Blecher [18]. Protein was determined according to Lowry et al. [19] using bovine serum albumin as standard. Spectrophotometric assays were performed with a Cary 118 spectrophotometer. *P*-450 was measured according to Omura and Sato [20] using the absorption coefficient of 91 $mM^{-1} \cdot cm^{-1}$ for the absorbance difference between 450 and 490 nm.

Results

Purification of rabbit liver microsomal cytochromes P-450

The 100 mM phosphate fraction, eluted from the hydroxyapatite column in the absence of glycerol (cf. Methods) was found to have spectral characteristics and SDS-gel electrophoretic mobility in the Laemmli system [17] identical to reference cytochrome *P*-450_{LM_2} (supplied by Professor M.J. Coon). This pro-

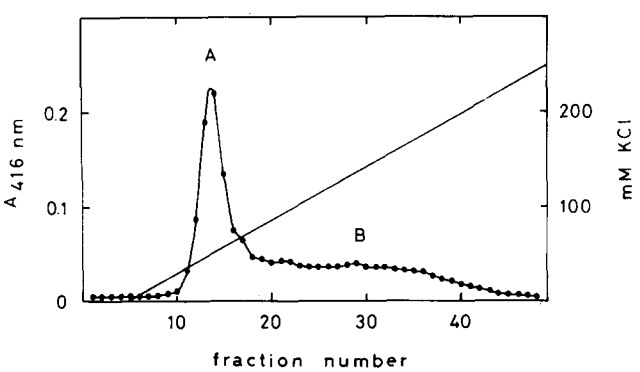

Fig. 1. Chromatography of the 300 mM phosphate fraction obtained from the hydroxyapatite column (cf. Methods) on a CM-Sepharose CL-6B column. The proteins were eluted with a linear gradient of KCl ranging from 0.0 to 0.3 M. Absorption of the different fractions at 416 nm was measured. Fractions A and B are indicated in the figure. Details are presented in Materials and Methods.

TABLE I

CHARACTERISTICS OF PURIFIED PREPARATIONS OF RABBIT LIVER MICROSOMAL CYTOCHROME *P*-450 USED IN THE PRESENT INVESTIGATION

In parentheses are quoted the respective treatments of the rabbits from which microsomes LM_2, LM_3 and LM_4 were isolated.

	LM_2 (phenobarbital)	LM_3 (phenobarbital)	LM_4 (3-methylcholanthrene)
Molecular weight	48 500	52 000	54 000
Specific content, nmol/mg	10.3—13.5	5.5—9.5	11.4
Absorption maximum, oxidized form	418	416	394
	535	530	645
	568	565	
Absorption maximum, CO-reduced form	451	449	448
	552	550	550

tein fraction had, furthermore, catalytic properties (cf. Ref. 21) identical to those of the LM_2-fraction isolated according to Haugen and Coon [16] and was therefore identified as cytochrome $P\text{-}450_{LM_2}$. Chromatography of the 300 mM phosphate fraction obtained from the hydroxyapatite column (cf. Methods) on CM-Sepharose CL-6B resolved two *P*-450-containing protein fractions, one major fraction (A) eluting at low salt concentration and one minor fraction (B) eluting at approx. 0.15 M KCl (Fig. 1). Fraction B had optical absorbance spectra and electrophoretic mobility identical to those of reference $P\text{-}450_{LM_4}$ or $P\text{-}450_{LM_4}$ purified from 3-methylcholantrene-treated rabbits, and was therefore identified as $P\text{-}450_{LM_4}$. Fraction A, on the other hand, had absorption spectra similar but not identical to those of $P\text{-}450_{LM_2}$ (cf. Table I) and, furthermore, a molecular weight, according to SDS-gel electrophoresis, of approx. 52 000, i.e. 4000 more than $P\text{-}450_{LM_2}$ (Ref. 13, cf. Fig. 2). The mobility of fraction A during SDS-slab gel electrophoresis in our Laemmli system was identical with that of $P\text{-}450_{LM3b}$ (supplied by Professor M.J. Coon). Due to limited reference material, no catalytic or spectral comparisons could be performed. Fraction A was therefore defined as $P\text{-}450_{LM_3}$.

Properties of the purified enzymes

Rabbit liver NADPH-cytochrome *P*-450 reductase was found to be homogeneous according to SDS-polyacrylamide slab gel electrophoresis (cf. Fig. 2) with an apparent molecular weight according to the electrophoresis of approx. 74 000, which is in agreement with the results of Coon et al. [22]. The preparation catalyzed the reduction of 30—35 μmol of cytochrome *c*/min per mg of protein in 0.3 M potassium phosphate buffer, pH 7.7, at 30°C. However, the activity measurements performed routinely were at 22°C in 50 mM phosphate buffer, pH 7.4, and the activity was then approximately one-third of that at the higher ionic strength and temperature. The specific contents of cytochrome $P\text{-}450_{LM_2}$ preparations purified according to Haugen and Coon [16] were 10.3—13.5 nmol/mg protein and of $P\text{-}450_{LM_2}$, purified as described in Methods,

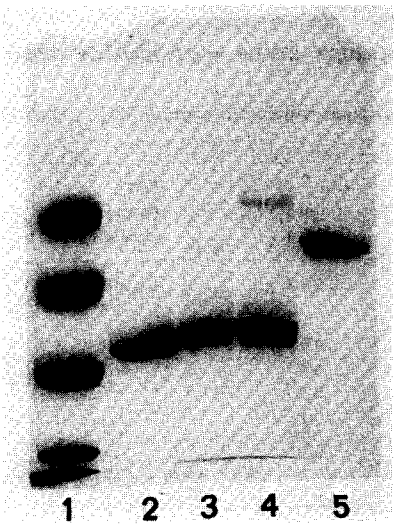

Fig. 2. SDS-polyacrylamide gel electrophoresis of different preparations of rabbit liver cytochromes *P*-450 and NADPH-cytochrome *P*-450 reductase. Electrophoresis was performed on slab gels containing 8.5% acrylamide at 4°C in a discontinuous buffer system according to Laemmli [17]. Migration was from top to bottom. Protein fractions in the different tracks are: (1) Marker proteins (phosphorylase *b*, M_r 94 000; bovine serum albumin, M_r 67 000; ovalbumin, M_r 43 000; carbonic anhydrase, M_r 30 000 and soybean trypsin inhibitor, M_r 20 100); (2) The 70 mM phosphate fraction obtained from hydroxyapatite eluted in the presence of glycerol = $P\text{-}450_{LM2}$, 5 μg protein; (3) Fraction A eluted from CM-Sepharose CL-6B (cf. Fig. 1) = $P\text{-}450_{LM3}$, 5 μg protein; (4) 300 mM phosphate fraction obtained from hydroxyapatite eluted in the presence of glycerol (from 3-methylcholantrene-induced liver microsomes) = $P\text{-}450_{LM4}$, 5 μg protein; (5) NADPH-cytochrome *P*-450 reductase fraction from the 2'5'-ADP Sepharose column, 5 μg protein.

8—10 nmol/mg protein. The amount of residual detergent in the LM_2-preparations was 0.03 mg/mg of protein according to the colorimetric assay method [10], i.e. approx. 2 nmol detergent/nmol cytochrome.

The specific contents of the $P\text{-}450_{LM_3}$-preparations obtained were 5.5—9.5 nmol/mg protein and that of $P\text{-}450_{LM_4}$ isolated from 3-methylcholantrene treated rabbits 11.4 nmol/mg. All three different *P*-450 preparations were essentially homogeneous according to SDS-slab gel electrophoresis (cf. Fig. 2).

Incubation of P-450 reductase and cytochrome $P\text{-}450_{LM_2}$ with preformed vesicles

Racker and co-workers [5] have described a method for incorporation of cytochrome oxidase into preformed phospholipid vesicles. This method, simply involving incubation of protein with vesicles, yields unidirectionally oriented cytochrome oxidase in the vesicles. This method was tried for incorporation of $P\text{-}450_{LM_2}$ and *P*-450 reductase into vesicles prepared from total microsomal lipids. After incubation, the preparations were characterized by chromatography on Sepharose 4B and by electron microscopy. As seen in Fig. 3A, Sepharose chromatography of vesicles incubated with $P\text{-}450_{LM_2}$ revealed that all protein had been incorporated into the vesicles which were eluted with the void volume of the column. Since empty vesicles are eluted at 1.7 void vols.

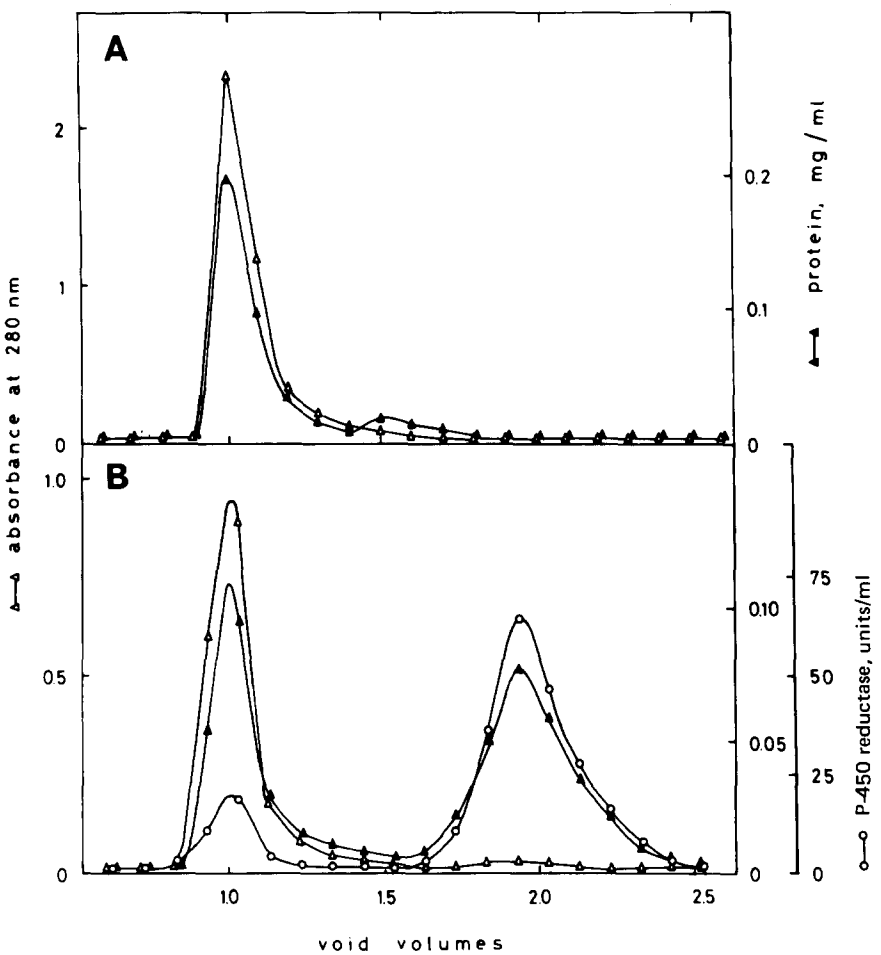

Fig. 3. Sepharose 4B chromatography of $P\text{-}450_{LM_2}$ (A) and $P\text{-}450_{LM_2}$ and NADPH-cytochrome P-450 reductase (B) incubated with preformed phospholipid vesicles. Vesicles of total microsomal lipids (10 mg) were prepared as described in Materials and Methods and incubated with either 5 nmol $P\text{-}450_{LM_2}$ or 5 nmol $P\text{-}450_{LM_2}$ and 3000 units P-450 reductase at 37°C for 20 min. The preparation was chromatographed on a Sepharose 4B column (1.3 × 60 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.4, containing 0.2 mM EDTA. The column was eluted at a rate of 6 ml/h and 3 ml fractions were collected. The void volume of the column was 35 ml. The vesicles were detected by absorbance at 280 nm, P-450 and P-450 reductase by protein determination according to Lowry et al. [19] or P-450 reductase, also specifically by measurement of cytochrome c reductase activity in 250 µl aliquots of the fractions.

(data not shown), fusion of the vesicles occurs during the incorporation. The diameter of the vesicles increases from approx. 25 to 60 nm as was evident from electron microscopy of negatively stained specimens (not shown in Figure). This fusion process could be followed by measurements of absorbance at 340 nm of the preparation upon addition of protein to empty vesicles. As evident from Fig. 4, the incorporation was complete after approx. 15 min at 37°C. When both $P\text{-}450_{LM_2}$ and P-450 reductase were incubated together with the preformed vesicles only limited incorporation of the proteins occurred,

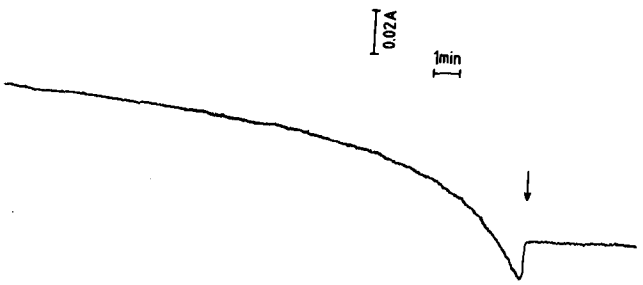

Fig. 4. Fusion of phospholipid vesicles induced by $P\text{-}450_{LM_2}$ as followed by increase of absorbance at 340 nm. Small vesicles were prepared by sonication of microsomal phospholipids as described in Materials and Methods. Base line was recorded at 340 nm and fusion of the vesicles was started by the addition of 2 nmol $P\text{-}450_{LM_2}$ in 250 µl 50 mM phosphate buffer, pH 7.4. The temperature was 37°C and the final concentration of lipid 2 mM. Arrow denotes addition of LM_2.

most of the reductase eluted from the Sepharose column (Fig. 3B) separated from the vesicles which were recovered in the void volume. Incubation of *P*-450 reductase alone with preformed vesicles resulted in no incorporation of the reductase. The reductase eluted from the Sepharose column at 1.9 void vols. corresponding to approx. 500 000 in molecular weight (cf. Fig. 3B) while the trypsinized form of the reductase eluted at 2.1 void vols. (see below). It may thus be concluded that the intact reductase in solution forms micelles with approx. 6—8 molecules per micelle. No monomers of the intact reductase could be detected, which indicates a very low critical micellar concentration. Obviously, this explains the inability of the reductase to be incorporated into the preformed vesicles.

Incorporation of P-450 reductase and cytochromes P-450 into vesicles by the cholate-gel filtration method

Using the cholate-gel filtration technique previously described (Ref. 7, cf. Methods) all three types of *P*-450, and also *P*-450 reductase were quantitatively incorporated into phospholipid vesicles as determined by Sepharose 4B chromatography of the different preparations. As shown in Fig. 5A, chromatography of a vesicle preparation consisting of 5% (w/w) $P\text{-}450_{LM_2}$ in egg yolk phosphatidylcholine on Sepharose 4B resolved two populations of $P\text{-}450_{LM_2}$-containing vesicles, one eluting with the void volume and the other at 1.64 void vols., while a similar preparation containing 5% (w/w) $P\text{-}450_{LM_3}$ only eluted with the void vol. from the Sepharose column (Fig. 5B). In contrast, the same incorporation procedure applied to $P\text{-}450_{LM_4}$ (5% w/w) or *P*-450 reductase (6% w/w) and egg yolk phosphatidylcholine resulted preferentially in the formation of small vesicles eluting at 1.65 void vols. (Fig. 5C, D); i.e. vesicles with diameters of approx. 25 nm [23]. The results indicate that the various forms of *P*-450 interact with the membrane structure in different ways thereby forming egg yolk phosphatidylcholine vesicles with different diameters. One plausible explanation is that larger vesicles are formed if the protein is more deeply embedded in the membrane, thereby immobilizing more lipid and thus making a more rigid, less flexible membrane, than if the protein, as in the case of *P*-450

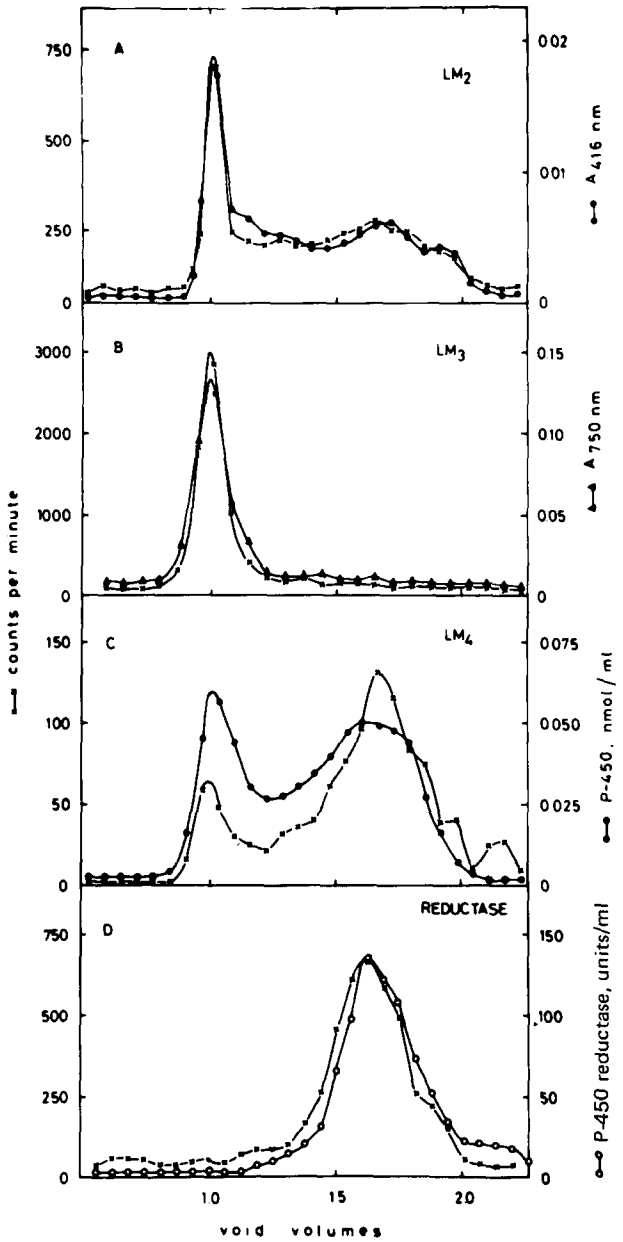

Fig. 5. Sepharose 4B chromatography of egg yolk phosphatidylcholine vesicles prepared by the cholate-gel filtration technique containing $P\text{-}450_{LM_2}$ (A), $P\text{-}450_{LM_3}$ (B), $P\text{-}450_{LM_4}$ (C) and NADPH-cytochrome *P*-450 reductase (D). The vesicles were prepared by taking 10 mg egg yolk phosphatidylcholine containing [^{14}C]phosphatidylcholine in chloroform solution to absolute dryness under nitrogen. The lipids were suspended in buffer and treated with cholate as described in Materials and Methods before addition of 1 mg of the protein indicated. After incubation for 1 h at 4°C, cholate was removed by Sephadex G-50 chromatography and an aliquot of the vesicles formed was applied to the Sepharose column. The Sepharose chromatography was performed as described in legend to Fig. 3. *P*-450 reductase was detected as cytochrome *c* reductase activity in 100 µl aliquots of the fractions and [^{14}C]phosphatidylcholine by counting 50 µl aliquots of the fractions in an Intertechnique SL30 scintillation spectrometer, with 3 ml of Lumagel® as scintillator.

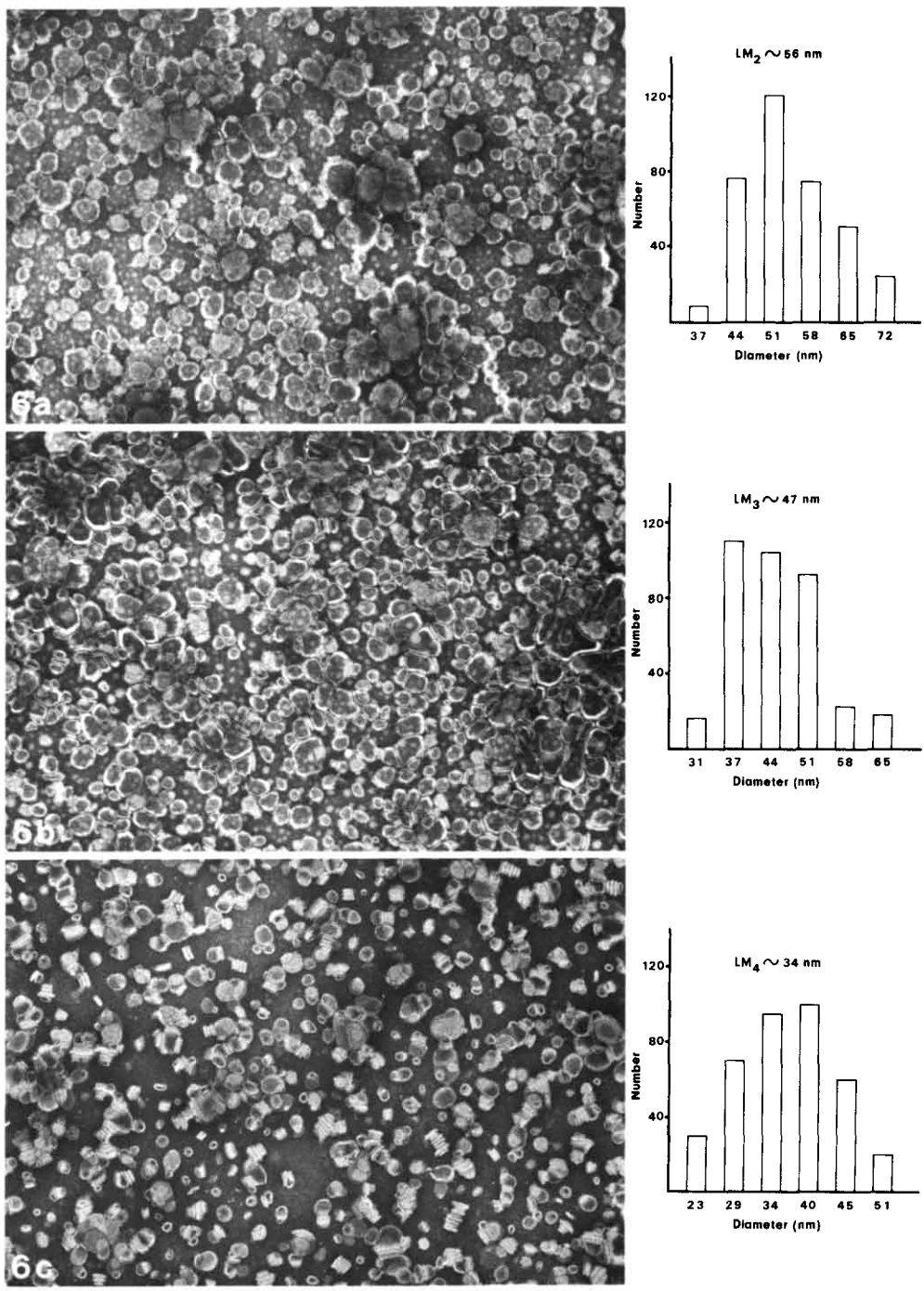

Fig. 6. Electron micrographs of negatively stained phospholipid vesicles containing NADPH-cytochrome *P*-450 reductase and *P*-450_{LM_2} (a), NADPH-cytochrome *P*-450 reductase and *P*-450_{LM_3} (b) or NADPH-cytochrome *P*-450 reductase and *P*-450_{LM_4} (c). The vesicles, containing approx. 0.3 mg *P*-450 reductase and 0.9 mg *P*-450/10 mg phospholipid were prepared by the cholate-gel filtration technique as described in Materials and Methods. Vesicles containing *P*-450_{LM_2} and *P*-450_{LM_3} (a and b) are unilamellar and have diameters of 40—70 nm while the *P*-450_{LM_4}-containing vesicles are also unilamellar but have diameters of only 23—51 nm. Magnification. X 43 800.

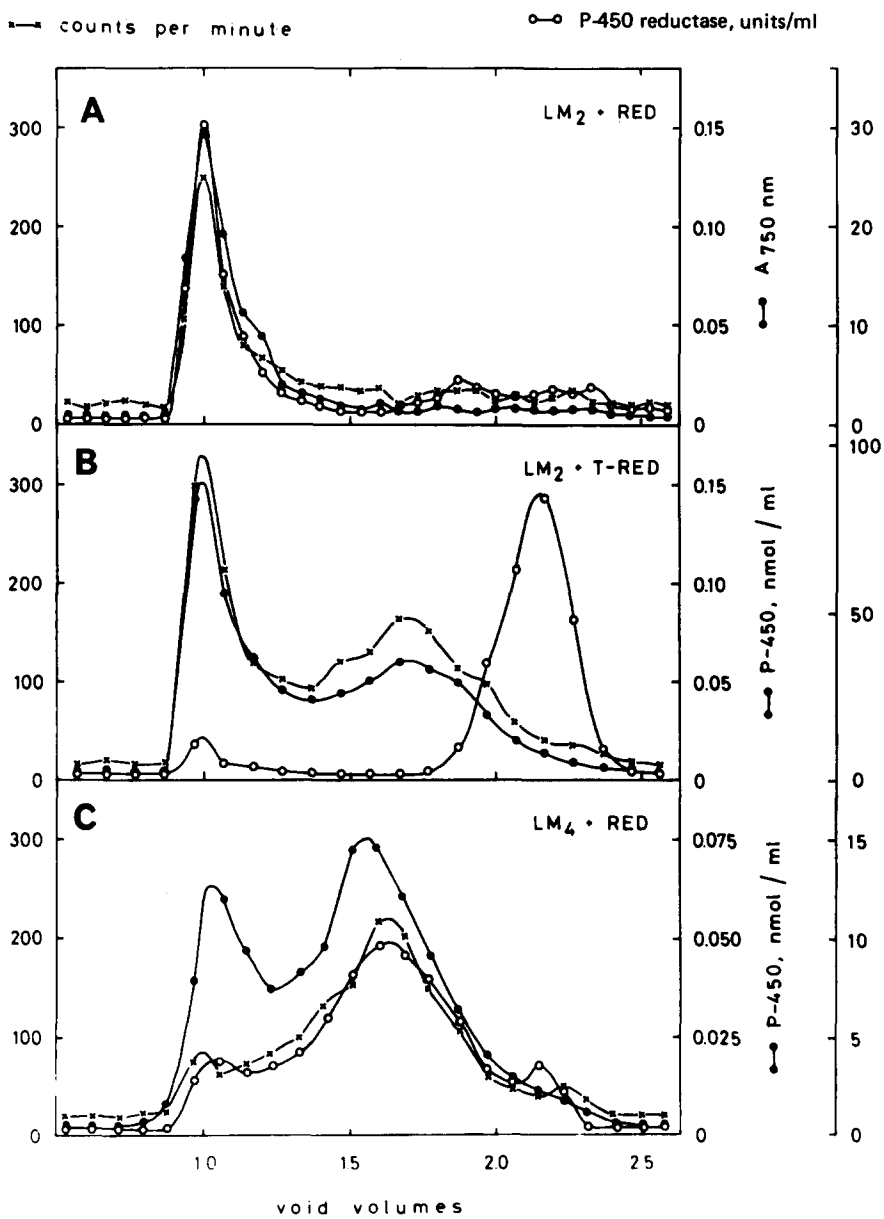

Fig. 7. Sepharose 4B chromatography of vesicles prepared by the cholate gel filtration technique from egg yolk phosphatidylcholine and: A, NADPH cytochrome *P*-450 reductase and *P*-450$_{LM_2}$; B, the trypsinized form of *P*-450 reductase and *P*-450$_{LM_2}$ and C, *P*-450 reductase and *P*-450$_{LM_4}$. The vesicles were prepared as described in Materials and Methods from 10 mg phosphatidylcholine, 0.3 mg *P*-450 reductase and 0.9 mg cytochrome *P*-450. The void volume from the Sephadex G-50 columns was collected and subjected to chromatography on Sepharose 4B. This chromatography was performed as described in the legend to Fig. 3. The components were detected as described in the legend to Fig. 5.

reductase, only has a small part of the molecule embedded in the membrane [22,24], which probably could make the formation of smaller vesicles possible.

Treatment by 0.5% (w/v) cholate of a solution consisting of 5 nmol *P*-450, 2000 units *P*-450 reductase and 10 mg egg yolk phosphatidylcholine with subsequent chromatography on Sephadex G-50 resulted in the formation of vesicles with diameters of approx. 50 nm when *P*-450_{LM_2} or *P*-450_{LM_3} was used, but only 33 nm in the case of *P*-450_{LM_4} (Fig. 6). The vesicles were relatively homogeneous in size in the different preparations and monolamellar as determined by electron microscopy (Fig. 6). When these preparations were examined by chromatography on Sepharose 4B, the results from electron microscopy were confirmed with respect to size distribution of the different types of vesicle (Fig. 7A, C and Ref. 7). Furthermore, the Sepharose chromatograms show that 100% incorporation of the proteins had been obtained in all cases, i.e. vesicles containing approx. 12% (w/v) protein had been formed. In contrast, when 6000 units *P*-450 reductase was treated with 25 μg trypsin and subsequently incubated with 10 mg egg yolk phosphatidylcholine, 6 nmol *P*-450_{LM_2} and 0.5% cholate, Sepharose chromatography of the vesicles obtained from the Sephadex G-50 column revealed that no incorporation of the reductase had been obtained (Fig. 7B); the trypsinized reductase eluted separated from *P*-450 and lipid at 2.1 void vols. SDS-polyacrylamide gel electrophoresis of the reductase preparation after treatment with trypsin showed that 95% of the protein had been trypsinized, i.e. had a molecular weight of approx. 69 000. It may thus be concluded that the trypsinized form of *P*-450 reductase cannot bind to the phosphatidylcholine vesicles.

In experiments where the protein-to-lipid ratio was increased and 20% (w/w) protein (*P*-450_{LM_2} and *P*-450 reductase) was incubated with egg yolk phosphatidylcholine and cholate no complete incorporation of the proteins into the vesicles was obtained as evident from Sepharose 4B chromatography of the vesicles. However, vesicles containing approx. 15% (w/w) protein could be prepared in this way.

Generally the recoveries were 70—90% for *P*-450 and 60—75% for *P*-450 reductase in the vesicles irrespective of which type of *P*-450 was used.

Characterization of P-450_{LM_2} vesicles on Ficoll gradients

It has been reported that cytochrome *c* oxidase, upon incorporation into phospholipid vesicles by either the cholate dialysis technique or by incubation with preformed liposomes, is only incorporated into one subpopulation of the vesicles [25,26]. Using Ficoll gradient centrifugation, it was possible to separate empty vesicles from protein-containing vesicles [25,26].

P-450_{LM_2} vesicles containing 10% (w/w) protein with a molar ratio of *P*-450 to reductase of 2 were layered on Ficoll gradients (5—20% w/v). After centrifugation, fractions were collected from the bottom of the tubes and the fractions were assayed for *P*-450, *P*-450 reductase and phospholipid. As seen in Fig. 8, the vesicles concentrated as a narrow band which corresponded to a density of approx. 1.04 g/ml; all *P*-450 and *P*-450 reductase activity was associated with the vesicular fraction. It may thus be concluded that all vesicles have the same density and furthermore contain both *P*-450 and *P*-450 reductase, since, upon Sepharose chromatography of the preparation, small vesicles containing only the reductase were not obtained (cf. Fig. 7A).

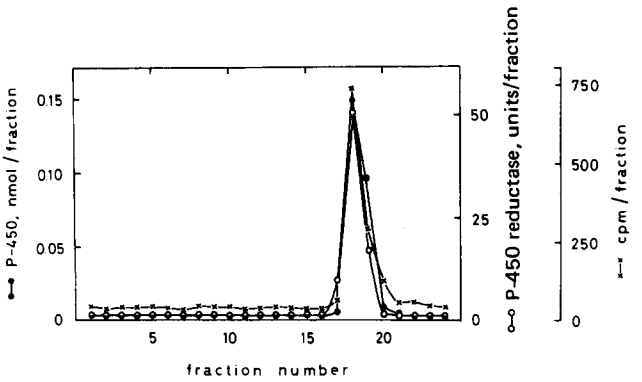

Fig. 8. Ficoll continuous gradient centrifugation of egg yolk phosphatidylcholine vesicles containing NADPH-cytochrome *P*-450 reductase and *P*-450$_{LM_2}$. Vesicles were prepared by the cholate-gel filtration technique described in Materials and Methods from 10 mg phosphatidylcholine containing 100 000 cpm [^{14}C]phosphatidylcholine, 0.3 mg *P*-450 reductase and 0.9 mg *P*-450$_{LM_2}$. From the void fraction isolated from the Sephadex G-50 column, 0.5 ml aliquots were applied on 5 ml 5—20% (w/v) continuous gradients of Ficoll prepared in 50 mM potassium phosphate buffer, pH 7.4. After centrifugation at 150 000 × *g* for 14 h at 4°C, 0.2 ml fractions were collected from the bottom of the tubes and assayed for radioactivity, *P*-450 and *P*-450 reductase (see legend to Fig. 5). The concentration of Ficoll was determined by an Abbe refractometer.

Orientation of the proteins in the vesicles

It is well known that *P*-450 reductase is readily solubilized from microsomes with trypsin [24]; treatment of microsomes with 50 μg trypsin/mg protein completely releases the hydrophillic part of *P*-450 reductase without disrupting the membrane [27]. Vesicles containing 10% (w/w) *P*-450$_{LM_2}$ and *P*-450 reductase were treated with increasing amounts of trypsin, and after addition of a 20-fold excess of trypsin inhibitor the preparations were analyzed by SDS-polyacrylamide gel electrophoresis. As seen in Fig. 9, nearly complete solubilization of the reductase was obtained at a concentration of trypsin of 14 μg/mg protein, indicating that *P*-450 reductase was almost exclusively oriented towards the outside of the vesicles. In contrast, *P*-450$_{LM_2}$ was unaffected by the trypsin treatment (Fig. 9). The unidirectional orientation of *P*-450 reductase is further indicated by the relatively high recovery (50—70%) of *P*-450 reductase in the vesicles as measured by NADPH-cytochrome *c* reductase activity and only 20% increase in the reductase activity upon solubilization of the vesicles with 0.5% Renex 690. This increase could be attributed to facilitated interactions between the solubilized reductase and cytochrome *c*. Renex was without effect on reduction of cytochrome *c* catalyzed by soluble NADPH-cytochrome *P*-450 reductase.

Diazobenzene sulphonate is an hydrophilic azo compound that does not penetrate membranes but reacts with a number of groups on proteins such as sulphydryl, amino, and hydroxyl groups [27—29]. Cytochromes *P*-450$_{LM_2}$, *P*-450$_{LM_3}$ and *P*-450$_{LM_4}$ were treated with increasing amounts of diazobenzene sulphonate in both the solubilized form and in reconstituted phospholipid vesicles also containing *P*-450 reductase. As evident from Fig. 10, the vesicular preparations of *P*-450$_{LM_4}$ showed identical sensitivity for denaturation by diazobenzene sulphonate in both the solubilized and membrane-bound form, indicating that *P*-450$_{LM_4}$ is almost completely oriented towards the outside of

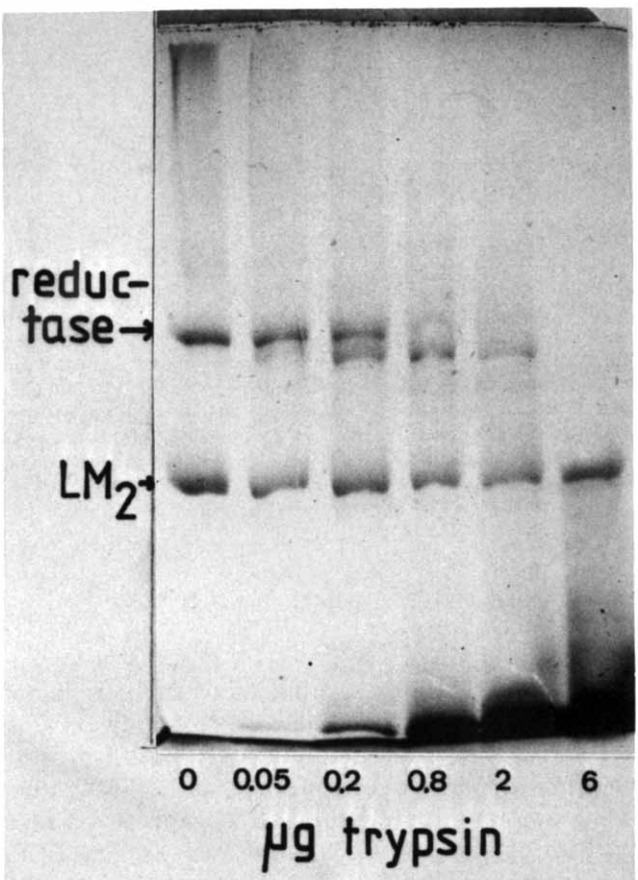

Fig. 9. Treatment of egg yolk phosphatidylcholine vesicles containing *P*-450 reductase and $P\text{-}450_{LM_2}$ with increasing amounts of trypsin. Vesicles were prepared from 0.5 mg $P\text{-}450_{LM_2}$, 0.3 mg *P*-450 reductase and 10 mg egg yolk phosphatidylcholine as described in Materials and Methods. Vesicles corresponding to 14 µg protein in 50 µl 50 mM phosphate buffer were distributed in 6 tubes, and 0, 0.05, 0.2, 0.8, 2 or 6 µg trypsin in 10 µl water was subsequently added. The tubes were incubated for 1 h at 4°C before addition of trypsin inhibitor (20 times the amount of trypsin) in 10 µl water. Subsequently, 5 µl 20% (w/w) SDS, 5 µl 20% (w/v) β-mercaptoethanol and 10 µl 40% (w/v) sucrose were added and the samples were subjected to SDS slab gel electrophoresis as described in Materials and Methods.

the vesicles. In contrast, $P\text{-}450_{LM_3}$ was not susceptible to diazobenzene sulphonate in the membrane-bound state, which could indicate that all the $P\text{-}450_{LM_3}$ molecules are oriented towards the inside of the vesicles or, alternatively, that amino acid residues sensitive to diazobenzene sulphonate are embedded in the membrane in this case. Since the electron transport flow from *P*-450 reductase to $P\text{-}450_{LM_3}$ in the presence of androstenedione takes place at a rate 4 times faster in the reconstituted vesicles than with the proteins in a solubilized form with dilauryl phosphatidylcholine [21], the latter possibility seems to be more plausible.

$P\text{-}450_{LM_2}$ was not affected by the diazobenzene sulphonate treatment, as was evident from the CO spectra, in either the vesicles or in the soluble form and no

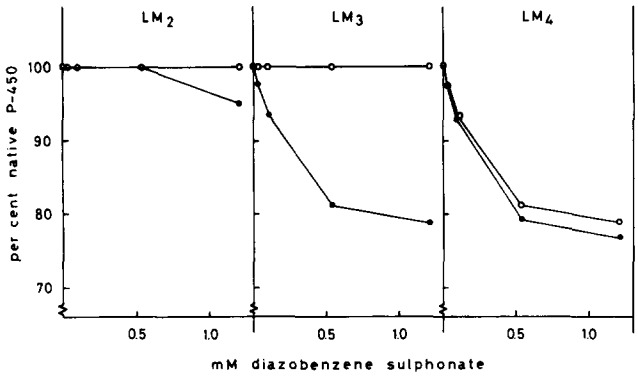

Fig. 10. Treatment of cytochromes $P\text{-}450_{LM_2}$, $P\text{-}450_{LM_3}$ and $P\text{-}450_{LM_4}$ in soluble form (●———●) and in reconstituted egg yolk phosphatidylcholine vesicles (○———○) also containing NADPH-cytochrome *P*-450 reductase with the non-penetrating reagent diazobenzene sulphonate. Vesicles were prepared by the cholate-gel filtration technique as described in Materials and Methods from 10 mg phospholipid, 0.3 mg *P*-450 reductase and 0.9 mg *P*-450. 100 µl of each vesicle preparation, containing approx. 0.7 µM *P*-450, was diluted with 50 mM potassium phosphate buffer, pH 7.4, (100 mM phosphate in the case of $P\text{-}450_{LM_4}$) to 1.0 ml. The solution was bubbled with CO for 1 min and reduced with a few grains of $Na_2S_2O_4$. Absorption spectrum from 500—400 nm was recorded. Subsequently, 5 mM diazobenzene sulphonate in water was added to 0.025 mM concentration and a new spectrum was recorded after 2 min. The procedure was repeated after further additions of diazobenzene sulphonate. The concentrations of *P*-450 indicated in the figures are compensated for dilution by the addition of the reagent. The same procedure was applied for 0.7 µM *P*-450 in the soluble form.

definite conclusion could therefore be drawn about the orientation of this protein in the vesicles.

Calculation of the number of protein molecules in each vesicle

Assuming a cross-sectional area of each phospholipid molecule in the vesicles of 60 Å^2 and a bilayer thickness of 40 Å [30], a vesicle with a diameter of 60 nm should contain 33 000 phospholipid molecules, i.e. 10 mg egg yolk phosphatidylcholine (mean M_r = 770) will give $2.37 \cdot 10^{14}$ vesicles. Incorporation of 5 nmol of a protein into 10 mg phospholipid vesicles will give, if homogeneously distributed, approx. 13 molecules protein per vesicle.

Discussion

In the present paper it has been shown that, using the cholate-gel filtration technique previously described [7], it is possible to incorporate purified preparations of one of the cytochromes $P\text{-}450_{LM_2}$, $P\text{-}450_{LM_3}$ or $P\text{-}450_{LM_4}$, together with NADPH-cytochrome *P*-450 reductase, into unilamellar vesicles of egg yolk phosphatidylcholine. Complete incorporation is obtained as evident from Sepharose 4B chromatography of the vesicles unless the protein-to-lipid ratio (by weight) exceeds 0.15. Omission of cholate in the procedure results in no incorporation of *P*-450 reductase and only partial incorporation of *P*-450 if incubated together with *P*-450 reductase, a phenomenon probably inherent in micellar formation of the reductase molecules and formation of complexes between *P*-450 and *P*-450 reductase [31] with subsequent nonaccessibility of

the hydrophobic, membrane-binding part of the *P*-450 reductase to the vesicles. Cholate thus seems to affect the incorporation procedure in two ways: (i) by breaking up the *P*-450 reductase micelles and the *P*-450-*P*-450 reductase complexes and (ii) by solubilizing the phosphatidylcholine membrane and binding to the proteins, thereby facilitating the self-assembly of the membrane upon removal of cholate. Similar results have been obtained by Strittmatter et al. (cf. Ref. 32) with the amphipatic proteins cytochrome b_5 and NADH-cytochrome b_5 reductase. Both proteins have small hydrophobic membrane-binding parts [33,34] which induce micellar formation of the proteins in solution and make spontaneous incorporation of these proteins into egg yolk phosphatidylcholine vesicles difficult.

Other laboratories [35,36] have recently described the incorpration of rabbit liver microsomal cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase into phospholipid vesicles by the cholate dialysis method. The disadvantages of this method are the long time needed for preparation of vesicles (36—48 h [35] compared to 1.5 h for the present method) and the heterogeneity in size of the vesicles formed (30—200 nm [35] compared to 30—70 nm for the present method).

Using the cholate-gel filtration technique, we found that $P\text{-}450_{LM_3}$ generally formed vesicles with larger diameters than $P\text{-}450_{LM_4}$ (especially), even though the vesicles were prepared under the same conditions. A possible explanation for this phenomenon could be a different type of membrane association between the two proteins. The experiments with the non-penetrating reagent diazobenzene sulphonate support this view. Amino acid residues on $P\text{-}450_{LM_4}$, which were sensitive to binding of the diazo-reagent in such a way as to result in denaturation of the protein, were affected by the reagent whether or not the protein was bound to the membrane. In contrast, such amino acid residues on $P\text{-}450_{LM_3}$ were inaccessible for the reagent when the protein was in the membrane-bound but not in the soluble state, probably because of their localization within the membrane. Equal sensitivity of $P\text{-}450_{LM_4}$ towards treatment with diazobenzene sulphonate in membrane-bound and soluble state, indicates an unidirectional orientation of the protein towards the outside of the vesicles. Furthermore, NADPH-cytochrome *P*-450 reductase seems to have an unidirectional orientation in the reconstituted membranes according to the results obtained from treatment of the vesicles with small amounts of trypsin and comparing the rate of NADPH-supported reduction of cytochrome *c* before and after solubilization of the vesicles. Such a unidirectional orientation of proteins in vesicles prepared by the cholate-gel filtration technique has recently been described also for Sucrose-Isomaltase by Brunner et al. [8].

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