Biochimica et Biophysica Acta, 601 (1980) 328—342

BBA 78917

DETERGENT REMOVAL DURING MEMBRANE RECONSTITUTION

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(Received January 3rd, 1980)

Key words: Liposome; Membrane protein; Reconstitution; Glycophorin; Detergent; Deoxycholate

Summary

Efficiency of detergent removal during the course of several different procedures for membrane protein reconstitution was examined. Reconstitution methods studied include ethanol injection-dialysis, detergent dialysis and detergent-gel filtration. In the ethanol injection-dialysis method, approx. 70 molecules of ethanol per 1000 molecules of phospholipid are retained even after extensive (150 h) dialysis. Efficiency of detergent removal by dialysis depends upon the detergent. However, even for sodium deoxycholate, a detergent possessing a large critical micelle concentration, there are approx. 7 molecules of deoxycholate per 1000 molecules of phospholipid retained by the bilayer even after extensive (310 h) dialysis. Detergent removal by gel filtration (Sephadex G-200 or G-50) of deoxycholate, cholic acid and Triton X-100 is more efficient than removal by dialysis; as few as 10 molecules of deoxycholate are retained per 1000 molecules of phospholipid after one column passage, taking only a few hours. Ethanol was less efficiently removed by one passage over a Sephadex column than by extensive dialysis. Removal of Triton X-100 by passage over, or dialysis against, Biobeads SM-2 resulted in a similar level of detergent retention to that found by passage over Sephadex G-200 or G-50. Utilizing gel-filtration techniques, we have examined the competition for the hydrophobic peptide of glycophorin, T(is), between sodium deoxycholate and a series of phospholipids as a possible means of obtaining a quanti-

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Abbreviation: CMC, critical micelle concentration.

tative measure of protein-lipid affinity. On the basis of these preliminary studies we conclude that the T(is) peptide has a relative lipid affinity of phosphatidylinositol > phosphatidylcholine > phosphatidylserine.

Introduction

One of the emerging techniques in membrane biochemistry is the reconstitution of membrane proteins into artificial lipid vesicles [1]. A number of the techniques used for reconstitution involve co-micellization of membrane proteins and phospholipids in detergent with the subsequent removal of the detergent [1—5]. Organic solvents, such as ethanol or trifluorethanol, have also been used for reconstitution in those situations where the membrane protein can be co-solubilized in the solvent with phospholipid [6,7]. Use of organic solvent reconstitution requires a subsequent removal of the organic solvent by dialysis [6—8] or vacuum drying [6].

In each reconstitution procedure involving detergents or solvents, the ability to remove these compounds from the protein-lipid recombinant can be critical. If reconstitution of biological properties such as transport is the end point, the presence of a few molecules of detergent per reconstituted proteolipid vesicle may be important as detergents have been shown to increase bilayer conductivities at concentrations well below their critical micelle concentrations [9,10]. If the purpose of the reconstitution is to perform physical studies such as nuclear magnetic resonance spectroscopy, fluorescence spectroscopy, or differential scanning calorimetry on the reconstituted proteolipid vesicles, the presence of a few molecules of detergent (or solvent) may be critical. Rice et al. [21], for example, have recently presented evidence that residual cholate in cytochrome oxidase-phospholipid complexes causes a significant disordering of hydrocarbon chain and head-group organization.

Another consideration is that when detergent is used to extract protein, a significant quantity of detergent may remain bound to the protein [11] and the ability of lipid to displace this detergent during reconstitution may be critical to the functioning of the protein. For this reason, we initially undertook experiments to determine the degree of detergent (or solvent) removal from proteolipid vesicles following reconstitution. During the course of these studies, described in the present communication, we made the observation that competition of sodium deoxycholate with phospholipid for binding of an integral membrane protein, the membrane-spanning hydrophobic T(is) peptide [6,7] obtained from tryptic digestion of MN-glycoprotein (glycophorin A), could be used as a quantitative measure of the relative affinity of the protein for specific phospholipids. We suggest, on the basis of this observation, that the procedure may offer a means of determining lipid-binding specificity for a variety of membrane proteins.

Materials and Methods

Preparation of MN-glycoprotein and its hydrophobic peptide T(is). MN-glycoprotein was isolated from erythrocyte membranes after the cells had

been disrupted with lithium diiodosalicylate [12]. T(is), the insoluble tryptic peptide, was prepared by trypsin digestion of pooled, intact MN-glycoprotein [6]. T(is) concentrations were determined by amino acid analyses on a Beckman 121-M amino acid analyzer with computing integrator.

Detergents and lipids. Sodium cholate (cholate) and sodium deoxycholate were purchased from the Sigma Chemical Company (St. Louis, MO). [^{14}C]-Cholic acid (45—60 mCi/mmol) was purchased from Research Products International Corporation (Elk Grove Village, IL), deoxy[^{14}C]cholic acid (2.98 mCi/mmol) was purchased from California Bionuclear Corporation (Sun Valley, CA), ^{3}H-labelled Triton X-100 (220 μCi/g) was obtained from Rohm and Haas Research Laboratories (Spring House, PA) and [^{14}C]ethanol (2—10 mCi/mmol) was purchased from New England Nuclear (Boston, MA). All radioactive samples were counted in Bray's solution or Scintiverse (Fisher) on a Searle Isocap/300 or in a Beckman LS-330 scintillation counter. Chromatographically pure phosphatidylcholine, phosphatidylserine and phosphatidylinositol were supplied by Avanti Biochemicals (Birmingham, AL). Phospholipid concentrations were determined by the Fiske-SubbaRow method for phosphate determination [13].

Separation of deoxy[^{14}C]cholic acid from higher molecular weight radioactive contaminant was effected by mixing it with 1.6% cold, carrier deoxycholate and dialysing it exhaustively against several changes of distilled water. The deoxycholate which passed through the dialysis membrane into the water was then lyophilized and used for some experiments.

Dialysis reconstitution. In each case, 5 mg of phosphatidylcholine in ethanol were rotary-evaporated to dryness and then lyophilized overnight. If proteoliposomes were to be reconstituted, 5.17 mg of lyophilized MN-glycoprotein were added to the lipid film and the entire contents of the tube were solubilized with 2.0 ml of a buffered solution of detergent-containing radioactively labeled detergent. If liposomes not containing protein were to be reconstituted, only the lipid was solubilized with detergent. Solubilization was allowed to proceed at room temperature for at least 2 h or until clarification, which was aided by mild vortexing.

Each 2.0 ml sample was dialyzed individually against 1 l of 10 mM Tris-HCl/0.02% NaN_3 (pH 8.2) at 4°C with a complete change of dialysate at every sampling interval. The dialysis bags were clamped at one end, rather than knotted, so that aliquots could be easily removed to determine the amount of detergent (deoxycholate, cholate or Triton X-100) or ethanol remaining inside as a functon of time.

Preparation of liposomes by the ethanol-injection method. The procedure of Batzri and Korn [8] was modified so that 5 mg of phosphatidylcholine were rotary-evaporated to dryness and then redissolved in 250 μl ethanol containing [^{14}C]ethanol. The ethanolic solution was then drawn up into a 250 μl syringe fitted with a 22 gauge needle and squirted into 4.75 ml of rapidly stirred 10 mM Tris-HCl/0.02% NaN_3 (pH 8.2). The mixture was then dialyzed as described above.

Removal of detergent by gel filtration. Samples of chromatographically pure phosphatidylcholine, phosphatidylserine or phosphatidylinositol (1 mg) were rotary evaporated to dryness and lyophilized overnight to remove any remain-

ing traces of solvent. The dried lipid was rehydrated in 1 ml 20 mM Hepes/150 mM NaCl buffer, pH 7.4, containing either 0.2 or 0.02% detergent, depending on the desired detergent-to-phospholipid molar ratio. Approx. 0.2 μCi of radioactively labeled detergent was added to each sample. All samples were chromatographed on 1 × 40 cm Sephadex G-200 or G-50 columns in 20 mM Hepes/150 mM NaCl, pH 7.4. Liposomes formed in the above manner were sized on a 1 × 40 cm Sepharose 4B or Sepharose 2B column in 20 mM Hepes/150 mM NaCl, pH 7.4. All columns were presaturated with phospholipid.

Removal of Triton X-100 by Biobead SM-2. 1 mg of chromatographically pure phosphatidylcholine was dried down, as above, and 1 ml of 0.2% Triton X-100 containing ^{3}H-labeled Triton X-100 was added to the dried lipid. The sample was passed over a small (0.5 × 15 cm) Biobead SM-2 column in 20 mM Hepes/150 mM NaCl buffer, pH 7.4. The eluate was analysed for ^{3}H-labeled Triton X-100 and for phospholipid. A similar sample was dialysed for 4 days against four changes of 1 l each of buffer containing suspended Biobeads SM-2 in amounts similar to that used for column chromatography.

Reconstitution of T(is) from deoxycholate by gel filtration. T(is) hydrophobic peptide from MN-glycoprotein was purified as described above. ^{125}I-labeled T(is) was prepared as follows. Purified T(is) in 0.1 M $NaHCO_3$ buffer, pH 7.4, containing 1 mg deoxycholate per mg protein was iodinated by the lactoperoxidase method according to the procedure of Kempner and Johnson [14]. The preparation had a specific activity of $2.21 \cdot 10^6$ cpm/mg protein. Then 1 mg of phosphatidylserine, phosphatidylcholine or phosphatidylinositol was taken to dryness under vacuum in a rotary evaporator. 50 μg of purified T(is) in trifluoroethanol were added to each sample which was again taken to dryness under vacuum, followed by several hours of lyophilization to remove any remaining traces of solvents. A small amount of ^{125}I-labeled T(is) in 20 mM Hepes buffer, pH 7.4, was added to each sample followed by 1 ml of 20 mM Hepes/150 mM NaCl buffer, pH 7.4, containing either 0.2 or 0.02% deoxycholate and 0.02% NaN_3. The samples were vortexed, sonicated and passed over G-200 columns as reported above. Duplicate samples containing 1.0 mg phosphatidylcholine, 50 μg cold T(is) (no ^{125}I-labeled T(is)), and a small amount of deoxy[^{14}C]cholate were also run as indicated above. ^{125}I was counted on a Searle 1197 gamma counter.

In order to demonstrate that T(is) protein was associated with lipid bilayers and not present coincidentally in the void volume as protein aggregates, we performed density gradient ultracentrifugation on the void volume fractions from Sephadex G-200 chromatography. Isopycnic ultracentrifugation on 4% CsCl was performed at 350 000 × g for 16 h in a AH650 swinging-bucket rotor on a Sorvall OTD-2 ultracentrifuge. At the end of each run, tubes were punctured at the bottom and 17 0.3-ml fractions were collected and analysed for [^{14}C]phosphatidylcholine, ^{125}I-labeled T(is) and densities of each fraction were determined by refractometry.

Results

Reconstitution of liposomes by detergent dialysis

Phosphatidylcholine liposomes or proteoliposomes containing 1 : 40 (M/M)

MN-glycoprotein were prepared by solubilization of the lipid and protein in cholate, deoxycholate, or Triton X-100 followed by extensive dialysis to remove the detergent.

As shown in Fig. 1, cholate and deoxycholate are much more quickly and extensively removed from the liposomes than is Triton X-100. Initial and final ratios of mol detergent molecules : mol phosphatidylcholine are shown in Table I. Although the final moles of cholate and deoxycholate per mole phosphatidylcholine are essentially the same, cholate is removed by dialysis more quickly so that after 50 h dialysis the level of cholate is the same as that of deoxycholate after 100 h of dialysis, even though the initial level of cholate was twice that of deoxycholate.

The diminution of Triton X-100 in the liposome mixture is a much slower process and the level of this detergent never approaches those possible for the other detergents studied here (Fig. 1). In the case of cholate, after over 300 h dialysis, only 0.13% of the detergent initially present still remains while 7.7% of the Triton X-100 initially present still remains. Both of these values represent a considerable retention of detergent, however, over the theoretical values for complete equilibrium of the detergents with the dialysing solutions ($3 \cdot 10^{-24}$ mol cholate/mol phosphatidylcholine and $2 \cdot 10^{-23}$ mol Triton X-100/mol phosphatidylcholine).

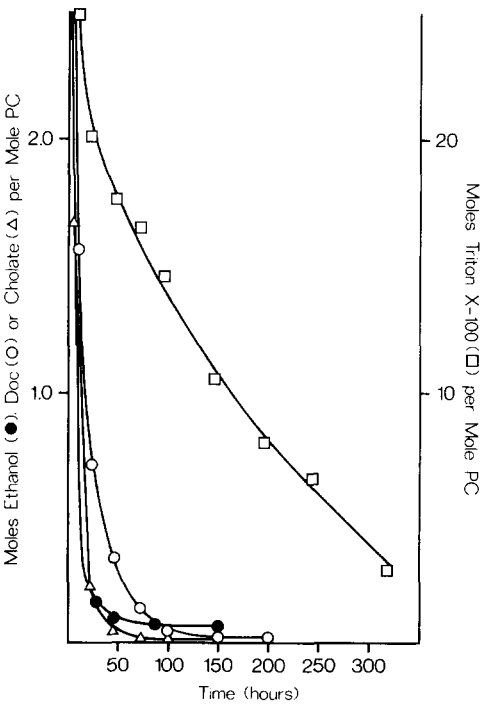

Fig. 1. Time course of removal of detergent or ethanol from liposomes by extensive dialysis. △———△, sodium cholate; ○———○, sodium deoxycholate; ●———●, ethanol; □———□, Triton X-100. Each 2.0 ml detergent sample was dialyzed against nine changes of 1 l each of 10 mM Tris-HCl, pH 8.2. The 5.0 ml ethanol sample was dialyzed against five changes of 1 l each of 10 mM Tris-HCl, pH 8.2. Doc, deoxycholate; PC, phosphatidylcholine.

TABLE I

REMOVAL OF DETERGENTS OR ETHANOL AFTER EXTENSIVE DIALYSIS

5 mg phosphatidylcholine (6.7 mol) in 2 ml of 10 mM Tris-HCl, pH 8.2, 0.02% NaN_3, were used in all detergent removal experiments. The samples were dialysed against nine changes of 1 l each of 10 mM Tris-HCl, pH 8.2. In those experiments performed in the presence of MN-glycoprotein, 5.17 mg (0.167 μM) of the glycoprotein were added as described in Materials and Methods. In experiments involved in the removal of ethanol, 5 mg of phosphatidylcholine in 250 μl ethanol were dispersed in 4.75 ml of 10 mM Tris-HCl buffer, pH 8.2, 0.02% NaN_3. The sample was dialysed against five changes of 1 l each of 10 mM Tris-HCl, pH 8.2. PC, phosphatidylcholine; GP, glycoprotein.

Surfactant	Lipid	mol surfactant/mol phosphatidylcholine				Theoretical value for complete equilibration
		0 h	50 h	150 h	310 h	
Sodium deoxycholate	PC	2.85	0.341	0.009	0.007	$1.4 \cdot 10^{-24}$
	PC-GP	2.85	0.250	0.014	0.006	
Sodium cholate	PC	5.80	0.041	0.010	0.008	$2.9 \cdot 10^{-24}$
	PC-GP	5.80	0.033	0.009	0.008	
Triton X-100	PC	19.07	10.72	5.2	1.47	$1.8 \cdot 10^{-23}$
	PC-GP	19.07	9.43	5.2	1.58	
Ethanol	PC	639	0.10	0.07	—	$4.0 \cdot 10^{-7}$

Reconstitution by the ethanol-injection method

Removal of ethanol by dialysis from the aqueous solution is rapid. After 150 h of dialysis, less than 0.15% of the ethanol initially present still remains. However, this represents 172 molecules of ethanol for a vesicle 250 Å in diameter, or 0.07 mol ethanol per mol lipid (Table I) which is again considerably higher than the theoretical equilibrium value of $4 \cdot 10^{-7}$ mol ethanol/mol phosphatidylcholine.

Detergent removal and liposome formation by column chromatography

Removal of either deoxy[^{14}C]cholate or deoxy[^{3}H]cholate from three different phospholipids, phosphatidylserine, phosphatidylcholine and phosphatidylinositol, was examined by G-200 column chromatography. The results are tabulated in Table II. No significant difference between phosphatidylserine and phosphatidylinositol was noted for retained detergent. Retained detergent by phosphatidylinosital was slightly lower than the other two. When the experiments were carried out using deoxy[^{3}H]cholate instead of deoxy-

TABLE II

REMOVAL OF DEOXYCHOLATE FROM PHOSPHOLIPID BY G-200 COLUMN CHROMATOGRAPHY

Phospholipid	mol deoxycholate/ mol phospholipid initial	mol deoxycholate/mol phospholipid after G-200 column chromatography using deoxy[^{14}C]cholate	mol deoxycholate/mol phosphatidylcholine after G-200 column chromatography using deoxy[^{3}H]cholate
Phosphatidylcholine	3.62	0.011	0.092
Phosphatidylserine	3.62	0.0097	0.100
Phosphatidylinositol	3.62	0.0073	—

[^{14}C]cholate, a significantly higher amount of detergent appeared to be retained by the liposomes, possibly due to ^{3}H exchange between the detergent and phospholipids, or to impurities present in higher amounts in the deoxy-[^{3}H]cholate. Controls containing detergent, but no phospholipid, were run on G-200 columns. No detergent migrated coincident with the void volume where the liposomes normally run. Therefore, detergent counts associated with the void volume peak are probably associated with the phospholipid vesicles, rather than being present coincidentally as micelles or other aggregated states or as high molecular weight impurities.

Peak phospholipid-containing fractions (liposomes) from each G-200 column were rechromatographed over Sepharose 4B. Little additional detergent was removed during this procedure (Fig. 2). Peak fractions of all three phospholipids ran in the void volume on Sepharose 4B, indicating that in all three cases the liposomes were larger than sonicated unilamellar liposomes. Typical results are shown in Fig. 2. Phosphatidylcholine liposomes were also made by chromatography over G-25, G-50, and G-100 columns. In all cases similar sized liposomes were formed, as demonstrated by sizing on Sepharose 4B, and similar amounts of detergent were retained. The size of the liposomes, in all cases, was larger than that described by Brunner et al. [15].

The removal of two other detergents ([^{14}C]cholate and ^{3}H-labeled Triton X-100) and ethanol removal was also examined by column chromatography. The results are tabulated in Table III. The lowest detergent retention was observed for deoxycholate. Cholate, surprisingly, showed a somewhat higher detergent retention than deoxycholate by column chromatography, although it was removed more quickly than deoxycholate by dialysis (Tables I and III). Passage of cholate-containing liposomes over a second column reduced the levels of cholate retention to levels similar to those found after 310 h of dialysis. Triton X-100 could be removed several-fold faster by column chroma-

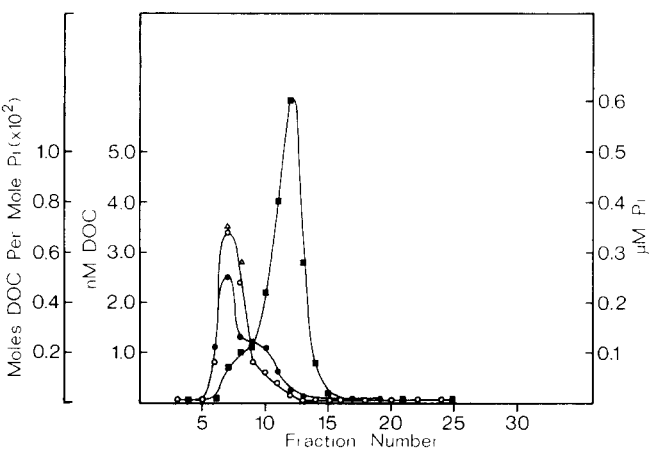

Fig. 2. Sepharose 4B column chromatography of phosphatidylcholine liposomes formed by deoxycholate detergent-gel filtration on a Sephadex G-200 column. The liposomes contained 0.0105 μM deoxycholate/μM phosphatidylcholine. (■———■) μM P_i of a control preparation of bath sonicated phosphatidylcholine liposomes. (○———○) μM P_i of liposomes formed on Sephadex G-200 column, (●———●) nM deoxycholate, (△) mol deoxycholate (DOC) per mol phospholipid.

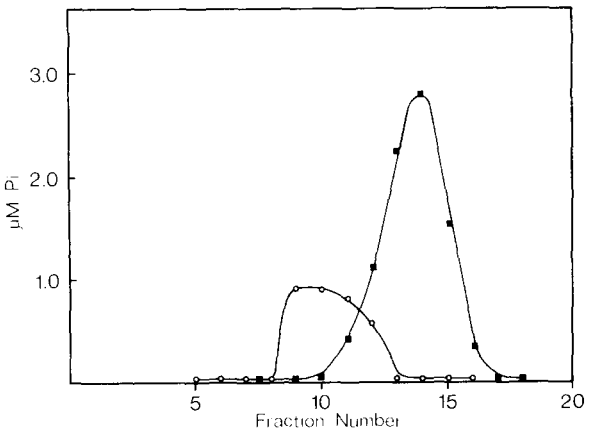

Fig. 3. Liposomes formed by deoxycholate: G-200 chromatography sized on Sepharose 2B. (○——○) μM P_i of liposomes formed by deoxycholate detergent-gel filtration on Sephadex G-200, (■——■) μM P_i of a control preparation of sonicated liposomes.

tography, and achived lower retention levels than those observed even after many hundreds of hours of dialysis. A typical separation of Triton X-100 is shown in Fig. 4. Ethanol was not efficiently removed by one pass over a G-200 column, possibly due to the large initial ratio of the solvent to phospholipid. When the samples were passed over G-50, rather than G-200, similar or slightly higher detergent retention was observed (Table III).

Removal of Triton X-100 by Biobeads SM-2

When 1 mg of phosphatidylcholine containing 2.4 mol Triton X-100/mol phosphatidylcholine was passed over a small (0.5 × 15 cm) Biobeads SM-2 column, 0.16 μmol Triton X-100/μmol phosphatidylcholine was retained by the liposomes. This is a similar value to that obtained when an identical sample was passed over a larger Sephadex G-50 column (Table III) and was obtained in a much shorter time period. When an identical sample was dialyzed against buffer containing an equal amount of suspended Biobeads SM-2 to that used for gel filtration, similar levels of Triton X-100 retention by liposomes were again found, but only after 4 days of dialysis.

TABLE III

REMOVAL OF DETERGENTS OR ETHANOL FROM PHOSPHATIDYLCHOLINE BY G-200 OR G-50 COLUMN CHROMATOGRAPHY

Detergent	mol detergent/mol phosphatidylcholine initial	mol detergent/mol phosphatidylcholine after G-200 column chromatography (n = 4)	mol detergent/mol phosphatidylcholine after G-50 column chromatography (n = 2)
Deoxycholate	3.7	0.012 ± 0.005	0.011 ± 0.003
Cholate	3.5	0.050 ± 0.013	0.080 ± 0.02
Triton X-100	2.4	0.10 ± 0.022	0.17 ± 0.07
Ethanol	2877	2.9 ± 0.37	5.4 ± 3.2

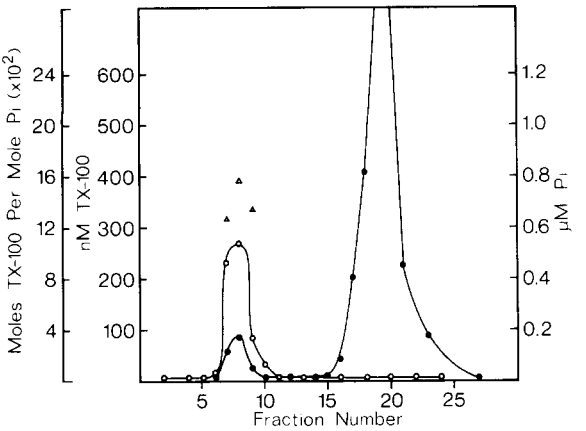

Fig. 4. Removal of ^{3}H-labeled Triton X-100 and formation of liposomes by G-200 column chromatography. (○———○) μM P_i of liposomes, (●———●) nM Triton X-100, (△) mol Triton X-100 (TX-100) per mol phospholipid.

Reconstitution of T(is) by column chromatography

When 0.01 mol T(is) per mol phospholipid was included in the samples, the T(is) protein partitioned between the void volume (phospholipid peak) and the free detergent (Fig. 5). Since the phospholipid and detergent could be almost totally separated from each other, even in the presence of hydrophobic peptide, the lipid to detergent partition coefficient could be measured for a variety of phospholipids. As seen from Table IV, T(is) appeared to partition with greatest preference into phosphatidylinositol liposomes followed by phosphatidylcholine and lastly phosphatidylserine. Density gradient centrifugation showed that the T(is) peptide was associated with the lipid bilayers and

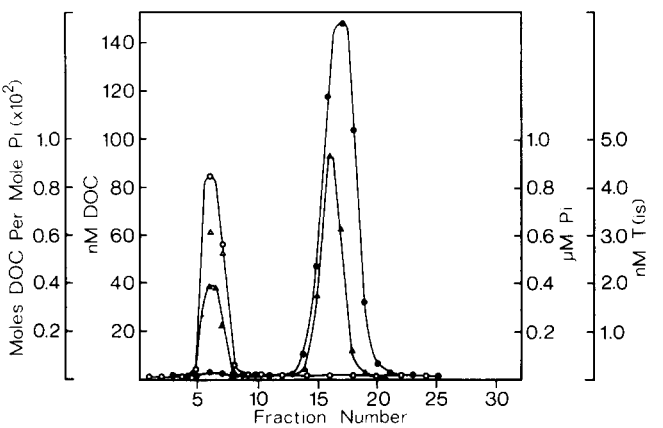

Fig. 5. Association of T(is) with phosphatidylcholine in the presence of detergent. 1 mg phosphatidylcholine (○———○), 1 ml of 0.02% deoxycholate (●———●), and 50 μg ^{125}I-labeled T(is) (▲———▲) were associated by the procedure described in the text and subjected to gel filtration on Sephadex G-200 in 20 nM Hepes/150 mM NaCl, pH 7.4. The ratio of deoxycholate/phosphatidylcholine (△) was determined for the void volume fractions. DOC, deoxycholate.

TABLE IV

DETERGENT REMOVAL AND RECONSTITUTION OF T(is) BY G-200 COLUMN CHROMATOGRAPHY

Phospholipid	mol deoxycholate/ mol phospholipid inititial	mol T(is)/mol phospholipid initial	mol deoxycholate/ mol phospholipid after G-200 column chromatography	mol T(is)/mol phospholipid after G-200 column chromatography
Phosphatidylcholine	3.62	0	0.0075	—
	3.62	0.01	0.0117	0.0021
	0.362	0	0.003	—
	0.362	0.01	0.0057	0.0028
Phosphatidylserine	3.62	0	0.0097	—
	3.62	0.01	—	0.0016
	0.362	0.01	—	0.0022
Phosphatidylinositol	3.62	0	0.0073	—
	3.62	0.01	—	0.0031
	0.362	0.01	—	0.0037

was not coincidentally present as protein aggregates (Fig. 6). The lipid and T(is) in co-chromatographed samples migrated at an intermediate density between that seen for T(is) alone (Fig. 6B) and phospholipid alone (Fig. 6A

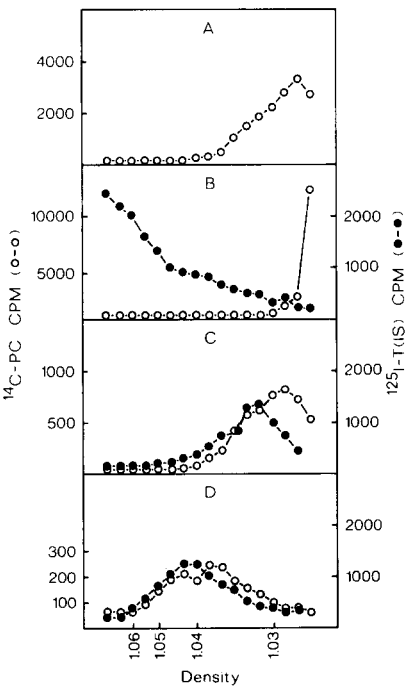

Fig. 6. Ultracentrifugation of T(is) sonicated phosphatidylcholine or T(is) associated with phosphatidylcholine by G-200 detergent-gel filtration. 1 mg phosphatidylcholine sonicated for 1 h at 0°C (A); 0.5 mg ^{125}I-labeled T(is) (●———●) or 1 mg phosphatidylcholine (○———○) subjected to G-200 detergent-gel filtration (B); 0.5 μg ^{125}I-labeled T(is) and 1 mg phosphatidylcholine in 0.2% deoxycholate after G-200 gel filtration (C); 50 μg T(is) and 1 mg phosphatidylcholine in 0.2% deoxycholate after G-200 gel filtration (D). Densities of the 4% CsCl gradient were determined by refractometry. PC, phosphatidylcholine.

and B). The possibility of using these preliminary observations as a basis for a method of measuring the affinity of proteins for different phospholipids will be explored in a future communication.

Discussion

Ethanol dialysis reconstitution

The major limitations of an organic solvent method of membrane reconstitution are that the procedure is limited to: (a) those membrane proteins soluble in an organic solvent, and (b) those membrane proteins either not denatured by the solvent or those that reversibly renature once the solvent is removed. There are severe limitations. If no enzymatic activity or well defined receptor function is associated with the membrane protein, it can be difficult to establish whether the reconstituted protein is native or denatured. In our studies, a significant number of ethanol molecules remain associated with the reconstituted lipid vesicles even after more than 6 days of dialysis. Over the last 100 h of ethanol dialysis (Table I) there is only a minimal decrease in the level of ethanol retained in the liposomes. This significant retention of ethanol in phospholipid vesicles over a period of time suggests that there is a highly unfavorable free energy of transfer of a small pool of ethanol molecules from phospholipid vesicles to water, i.e., $\mu_w^o - \mu_{PC}^o$ (PC, phosphatidylcholine) for the ethanol molecules is large and positive. The retained pool is not likely to be entrapped in the aqueous compartment because the leakage of a largely apolar molecule such as ethanol from a liposome is quite rapid (unpublished observation). This suggests that the retained ethanol pool is located within the phospholipid bilayer. This is a somewhat surprising conclusion since the water/hexane partition coefficient for ethanol is greater than 8 (unpublished observation).

Since ethanol is commonly used as a solvent for certain phospholipids, there is the possibility that methods commonly used to remove ethanol, such as rotary evaporation, may leave significant amounts of solvent present in hydrated liposomes if care is not taken to lyophilize the sample thoroughly. In one experiment where phosphatidylcholine in ethanol was rotary evaporated in the presence of [^{14}C]ethanol but not lyophilized, and subsequently made into liposomes, considerable (approx. 10 μmol ethanol/μmol phosphatidylcholine) ethanol was retained in association with the liposomes.

Detergent dialysis reconstitution

It is clear from the detergent dialysis experiments that the presence of an integral membrane protein, MN-glycoprotein, has relatively minor effects on the retention of detergents by reconstituted liposomes. For deoxycholate and cholate no difference can be detected at the end of 310 h. The difference in relative critical micelle concentration (CMC) between cholate (13—15 mM) and deoxycholate (4—6 mM) (Ref. 9) is reflected in the more rapid initial removal of cholate by dialysis compared to deoxycholate as seen in Fig. 1.

At the end of 310 h of dialysis all liposome preparations show a small retention of detergent (approx. 0.007 mol detergent per mol phosphatidylcholine). The presence of a radioactively labeled contaminant to explain the

retention seems unlikely because: (a) no contaminant was detected on thin-layer chromatography by autoradiography, and (b) both deoxycholate and cholate show identical molecular retention even though the radioactively labeled compounds are different and have different specific activities. The presumption then is that phosphatidylcholine vesicles contain a small number of high-affinity binding sites for deoxycholate and cholate, there being less than one site for every 100 phosphatidylcholine molecules. Although the possibility cannot be ruled out that these binding sites might be related to phosphatidylcholine degradation products or contaminants, the results suggest the need for caution when using either deoxycholate or cholate for dialysis reconstitution of membrane proteins for sensitive physical studies.

Triton X-100 has a CMC of 0.24 mM. This significantly higher CMC is most likely the reason for the 100-fold greater retention of Triton X-100 at the end of 310 h of dialysis compared with deoxycholate and cholate. Since the Triton X-100 concentration after this period of dialysis is greater than 1.0 mol per mol phosphatidylcholine, it is unlikely that the mixed product is in the form of liposomes or other lamellar structures but rather exists as isotropic mixed micelles [16].

Detergent removal by gel filtration

Gel filtration appears to be a better method than dialysis for removal of detergent during membrane reconstitution. After a single gel filtration passage on Sephadex G-200, the amount of deoxycholate retained is similar to that retained after almost 2 weeks of dialysis. Passage of the detergent-containing liposomes over a second column reduces the retention of deoxycholate, Triton X-100 and ethanol to a small degree.

Phosphatidylcholine and phosphatidylserine appear to retain deoxycholate to a similar degree while phosphatidylinositol retains it to a somewhat lesser degree. When a series of experiments was performed, substituting deoxy[^{3}H]-cholate for deoxy[^{14}C]cholate, a significant jump in the amount of retained detergent was observed. We attribute this increase to an exchange process for ^{3}H taking place between the detergent and phospholipid. Purification of deoxy[^{14}C]cholate did not result in a lesser retention of detergent by liposomes so the radioactivity retained by liposomes appeared not to be due to a high molecular weight radioactive contaminant.

Liposomes formed from all three phospholipids on Sephadex G-200 columns, as well as liposomes formed on G-25, G-50 and G-100 columns, all ran as a sharp peak in the void volume of Sepharose 4B, indicating that, in our hands, liposomes formed from several phospholipids using a variety of gel sizes are considerably larger than the small unilammellar vesicles reported to be formed by this method by Brunner et al. [15]. On Sepharose 2B, liposomes formed in the presence of Triton X-100 or cholate ran in the void volume while liposomes formed in the presence of deoxycholate or ethanol ran very slightly after the void volume (Fig. 3). The average size of the liposomes, based on their average capture volume of 3—4 μl/μmol phospholipid is 1000—1500 Å. Enoch and Strittmatter [17] have recently reported the formation of 1000 Å diameter single-bilayer phospholipid vesicles by a similar technique.

Cholate and Triton X-100 also could quickly be reduced to low levels in liposomes after gel filtration. In the case of cholate, the levels of detergent retained by liposomes after one pass over G-200 were equivalent to those observed after 50 h of dialysis. Further dialysis, however, reduced the level of retained cholate significantly below this level. It is puzzling why cholate with its larger CMC should not be removed by column chromatography more efficiently than deoxycholate with its somewhat smaller CMC. Perhaps the explanation lies in the presence of undetected radioactively labeled impurities present in the [^{14}C]cholic acid. However, passage of the liposome fractions containing retained cholate over a second column resulted in reduction of cholate remaining associated with liposomes to levels similar to those found after 310 h of dialysis.

Triton X-100 was removed to the same extent as seen with gel filtration over G-50 or G-200 when it was passed over a small Biobead SM-2 column. Holloway [18] has reported that Triton X-100 concentrations can be reduced from 1% to approx. 0.01% after a 2 h exposure to Biobeads SM-2, so it would appear that gel filtration over Biobeads SM-2 is the most rapid and efficient way of removing Triton X-100 from liposomes.

The incorporation of the tryptic hydrophobic peptide from glycophorin, T(is), into liposomes by means of gel filtration did consistently lead to a very slightly increased retention of detergent by the liposomes (Table IV). This is in contrast with our observation that incorporation of glycophorin into liposomes by the dialysis method did not lead to increased detergent retention. Perhaps the explanation lies in the increased hydrophobic character of the T(is) peptide as compared to glycophorin.

Lipid-detergent partitioning as a means of determining lipid specificity for membrane proteins

From Table IV, differences are seen in percent retention of T(is) by different phospholipids. This suggests that gel filtration of mixed micelles of phospholipid and deoxycholate may be useful as a qualitative indicator of the relative affinity of an integral membrane protein or peptide for various phospholipids. The rationale for this approach is as follows. If the T(is) peptide has a higher attraction for one phospholipid than for another, then a proportionately greater amount of the peptide would chromatograph with the peak of the preferred lipid compared to the less preferred lipid and the T(is) to phospholipid ratio of the former would be higher.

The single most important criterion required for a realistic estimate of relative lipid affinities is that T(is) should be at equilibrium (or near equilibrium) with the phospholipid vesicles and deoxycholate micelles. There seem to be at least two factors that can affect the rate of attainment of equilibrium in a phospholipid-deoxycholate gel filtration system.

First, the concentration of deoxycholate is important. It is well known that mixed micellization of two amphipaths results in a decrease in the free monomer concentration of each amphipath relative to the free monomer concentration in a pure micellar solution of either amphipath [16]. The effective CMC of the amphipath with the higher CMC can be lowered markedly depending upon the molar ratios of the two amphipaths. Since the rate of collisional

equilibration of any mixed micelle solution will depend to some extent upon the free monomer concentration of the amphipath with the highest CMC, it is important that this amphipath, i.e., deoxycholate, should be present in a higher molar concentration than the other. This criterion is probably achieved only in the 3.62 molar ratio sample.

A second factor to consider is that if equilibrium of T(is) is to occur between separating phospholipid vesicles and deoxycholate micelles during gel filtration, it must occur along the narrow zone of overlap between the separating zones. Attainment of equilibrium will be enhanced by optimizing the lifetime of this zone. It would appear to us that this can be done in two ways. The mixed micelles can be eluted at a very slow rate; or the mixed micelles can be applied to the column in a large volume to produce broad elution bands which do not completely separate. In our experiments the columns were run at a very slow flow rate (approx. 2 ml/h). However, even given this precaution, it is likely that absolute equilibrium may not have been obtained in the present studies.

Our preliminary experiments suggest that T(is) has a more favorable association with phosphatidylinositol vesicles than with either phosphatidylcholine or phosphatidylserine vesicles. Based on ^{31}P-NMR studies, Armitage et al. [19] suggest that MN-glycoprotein isolated by the lithium diiodosalicylate-phenol procedure [12] contains diphosphoinositide bound specifically to the hydrophobic region of the protein. On the other hand, van Zoelen et al. [20] report that MN-glycoprotein isolated by the same procedure contains a significant amount of bound phospholipid, predominantly phosphatidylserine. In our studies, T(is) associates more favorably with phosphatidylinositol than phosphatidylserine and thus is compatible more with the findings of Armitage et al. [19]. However, since T(is) represents only a portion of the intact MN-glycoprotein, it may be that complete lipid-binding specificity depends upon portions of MN-glycoprotein in addition to T(is). For larger proteins such as MN-glycoprotein, a gel with a large exclusion weight such as Sepharose 4B may be required for these studies. Because many membrane proteins have been successfully reconstituted using the deoxycholate dialysis procedure, we are optimistic that the detergent-lipid partition technique will also prove useful as a general membrane technique.

Acknowledgements

We thank Drs. Gary K. Ackers, Mark Poznansky and Robert E. Lenkinski for helpful discussions. We would also like to thank Mr. John Wood and Ms. Elli McCullough for excellent technical assistance. This work was supported in part by HEW grants GM-23177, CA-13148 and DE-02670; NSF grant PCM 76-11952; and MRC of Canada grant MA-6487. A.Y.R. was an NIH Postdoctoral Fellow GM-07561.

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