Shaw, J. O., Klusick, S. J., & Hanahan, D. J. (1981b) *Biochim. Biophys. Acta 663*, 222–229.
Smith, J. B. (1976) in *Prostaglandins and Hematology* (Silver, M. J., Kocsic, J. J., & Smith, J. I., Eds.) pp 227–244, Spectrum, New York.
St. Louis, P. J., & Sulakhe, P. V. (1976) *Int. J. Biochem. 7*, 547–558.
Tence, M., Coeffier, E., Heymans, F., Polonbsky, J., Godfroid, J.-J., & Benveniste, J. (1981) *Biochimie 63*, 723–727.
Tokumura, A., Fukuzawa, K., Isobe, J., & Tsukatani (1981) *Biochem. Biophys. Res. Commun. 99*, 391–398.
Tsung, P.-K., Kegeles, S. W., & Becker, E. L. (1978) *Biochim. Biophys. Acta 541*, 150–160.
Valone, F. H., Coles, E., Reinhold, V. R., & Goetzl, E. J. (1982) *J. Immunol. 129*, 1637–1641.
Vargaftig, B. B., Lefort, J., Chignard, M., & Benveniste, J. (1980) *Eur. J. Pharmacol. 65*, 185–192.
Williams, L. T., Snyderman, R., & Lefkowitz, R. J. (1976) *J. Clin. Invest. 57*, 149–155.
Williams, L. T., Snyderman, R., Pike, M. C., & Lefkowitz, R. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1204–1208.
Yavin, E. (1982) *Horiz. Biochem. Biophys. 6*, 67–81.

Effects of Physical States of Phospholipids on the Incorporation and Cytochalasin B Binding Activity of Human Erythrocyte Membrane Proteins in Reconstituted Vesicles†

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ABSTRACT: Proteoliposomes were reconstituted from a Triton extract of human erythrocyte membrane proteins and a mixture of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of varying ratios. With mixtures of egg PC and soybean PE, the protein/lipid ratio of the reconstituted vesicles was maximal at 25% PC and 75% PE, the composition which is known to have a maximum bilayer disruption (highest occurrence of lipidic particles seen by freeze–fracture electron microscopy). With mixtures of 1-palmitoyl-2-oleoyl-PC and dilinoleoyl-PE, which give vesicles with few isolated lipidic particles at room temperature, the effect was less pronounced. The specific activity of the cytochalasin B (CB) binding protein in the reconstituted vesicles, on the other hand, was increased monotonically up to severalfold as the PC content was increased in the egg PC/soybean PE mixture. A similar increase was observed when soybean PE was partially substituted by dimyristoyl-PC, cholesterol, or transphosphatidylated PE from egg PC. These findings indicate that preexisting defects in the lipid bilayer promote protein incorporation into the bilayer during reconstitution whereas reduction of the bilayer fluidity facilitates the CB binding activity in the reconstituted vesicles.

An ample body of evidence indicates that the compositions and the physical states of the lipids used in membrane reconstitution affect the incorporation of proteins and other molecules. The insertion of M-13 protein into model membranes with proper orientation depends on the phase transition of the lipids used (Wickner, 1977). Complex formation between serum apolipoproteins and lecithins (Pownall et al., 1979) and between glucagon and lecithins (Epand et al., 1981) was also found to depend on the phase states of the lipid. It has been proposed that at the phase transition many structural defects exist in the lipid bilayer, and these defects could affect the incorporation of proteins (Marsh et al., 1976). The composition and the physical state of lipids also affect the functions of reconstituted proteins. The mitochondrial ATPase incorporated into liposomes gives a well-defined maximum at a certain composition of the liposome (Kagawa et al., 1973), and this lipid composition coincides with the lipid compositions which give maximal bilayer instability (Hui et al., 1981a). A recent experiment by Woldegiorgis et al. (1982) confirmed this lipid dependency. When erythrocyte membrane proteins were reconstituted into liposomes, it was found that the composition of the exogenous lipid used in reconstitution had certain effects on the efficiency of the anion transport (Gerritsen et al., 1979; Wolosin, 1980). It is possible that the maximum amount of defects exists in the bilayer at the range of instability, and they either facilitate protein incorporation and/or affect the functions of the protein incorporated. Perhaps by the same token, this effect may also exist in natural membranes.

Glucose transport across the human erythrocyte membrane is known to be mediated by a specific protein, and cytochalasin B (CB)[1] inhibits this transport function by binding to this protein (Jung & Rampal, 1977). Successful effort has been made to isolate this transport protein, namely, band 4.5, and to reconstitute it into liposomes (Kasahara & Hinkle, 1977; Phutrakul & Jones, 1979; Sogin & Hinkle, 1980; Froman et al., 1980, 1981; Lukacovic et al., 1981). It has been shown that cytochalasin B binds specifically to this protein (Carter-Su

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[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DLPE, 1,2-dilinoleoylphosphatidylethanolamine; IMP, intramembranous particle; LIP, lipidic particle; TPE, transphosphatidylated product of egg PC in the presence of ethanolamine; DMPC, 1,2-dimyristoylphosphatidylcholine; SDS, sodium dodecyl sulfate; CB, cytochalasin B; Tris, tris(hydroxymethyl)aminomethane.

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et al., 1982). These studies have shown that the reconstituted proteoliposomes mimic many aspects of glucose transport in intact erythrocytes, including a CB-sensitive, stereospecific transport of D-glucose and a glucose-sensitive binding of CB.

In this paper, we studied the incorporation of Triton-extracted human erythrocyte membrane proteins into lipid mixtures of varying composition with known bilayer instabilities. The extract includes the glucose transport protein. We also measured the CB binding activity of the reconstituted vesicles, a parameter known to represent the carrier-mediated glucose transport function of the human erythrocyte (Jung & Rampal, 1977). PE and PC, the two major phospholipids found in the human erythrocyte membrane, were used as lipid mixtures. The phase diagrams of these lipid mixtures have previously been determined in our laboratory (Hui et al., 1981a). Based on these findings and the existing knowledge of the phase structure of these lipids, we conclude that while instability or structural defects in the lipid bilayer promote protein incorporation, the activity of CB binding protein in the reconstituted system requires bilayer stability.

Materials and Methods

Chemicals. Lipids (egg PC, DMPC, POPC, soybean PE, TPE, and DLPE) were purchased from Avanti Polar-Lipids Inc., Birmingham, AL. Triton X-100 was from J. T. Baker, Phillipsburg, NJ. Bio-Beads (SM-2) was from Bio-Rad Laboratories, Richmond, CA. CB was obtained from Sigma Chemical Co., Saint Louis, MO, and ^{3}H-labeled cytochalasin B and water were purchased from New England Nuclear Co., Boston, MA.

Protein Extraction. Essentially hemoglobin-free human erythrocyte membranes were prepared from outdated units of whole blood by the method of Dodge et al. (1963). The membranes were treated with 0.5% Triton X-100 in 10 mM Tris buffer (pH 7.4) for 30 min at room temperature. The ratio of protein to detergent was kept at 1:5 (mass/mass) throughout the extraction procedure. After centrifugation at 100000*g* for 60 min, the resulting supernatant (Triton X-100 extract) was collected.

Reconstitution Procedures. A mixture of PC and PE in chloroform at varying ratios containing 0, 15, 25, 50, and 75% by weight of PC with the total 20 mg of phospholipids was dried under a nitrogen stream with constant rotation to form a thin film in a 40-mL Pyrex glass test tube. An aliquot of 30 mL of Triton X-100 extract containing 15–20 mg of protein was added to each of these phospholipid mixtures which were vigorously shaken with a Vortex shaker for 1 min and then incubated for 30 min with gentle stirring at 4 °C. The mixtures were subjected to a successive treatment of Bio-Beads to remove the detergent as follows: 3 g of the moist copolymer beads was added to 30 mL of the mixture containing 0.5% Triton X-100 (Bio-Beads:Triton X-100 20:1, mass/mass). The mixture was gently agitated at 4 °C on a blood tube rotator (Bodine Electric Co., Chicago, IL) for 1 h and was filtered through glass wool. This treatment with beads was repeated twice with the filtrates, agitating for 2 and 4 h, respectively. After centrifugation at 100000*g* for 60 min, the pellets were suspended in 1/10 balanced salt solution (BSS) (about 6 mL). Vesicles were separable into two populations with a density gradient of 0–40% sucrose by centrifugation at 60000*g* for 18 h using an SW 27 rotor in a Beckman L5-50 centrifuge. In subsequent steps, both populations were mixed, repelleted, freeze–thawed, and washed twice before use.

Lipid Analysis. In order to determine the lipid composition, an aliquot (0.2 mL) of the membrane suspension was applied to a thin-layer chromatograph. The phosphatidylcholine and phosphatidylethanolamine spots were indicated by charring at 180 °C for 10 min after spraying with a sulfuric acid/ethanol mixture (1:1). The charred spots were scraped out, and the phosphate composition was determined by the method of Bartlett (1959).

Protein Analysis. The disc polyacrylamide gels and the suspension of protein were prepared for electrophoresis by the method of Fairbanks et al. (1971). Normally, electrophoresis was performed with the voltage gradient at about 7–8 V/cm and the current at 8 mA/tube. The running time under this condition was about 45 min. The gels were stained for protein with Coomassie blue and scanned with a densitometer. The relative protein amounts were estimated by weighing the scanned papers. The relation between the protein amount and the stain intensity is calibrated to be approximately linear at our loading range. The total protein was measured by the method of Lowry et al. (1951).

CB Binding Assay. The freeze–thawed, reconstituted membranes were resuspended with a glass homogenizer at 4 °C. The membranes were incubated with the mixture of CB at varying concentrations and a tracer amount of ^{3}H-labeled CB (0.02 μCi) for 30 min at room temperature. The percent distribution of CB in the pellet was determined by the centrifugation method (Jung & Hampal, 1977).

Electron Microscopy. The reconstituted liposomes either before or after freeze–thawing were studied by negative staining and freeze–fracturing. For negative staining, membrane vesicles were allowed to settle on carbon-coated grids and stained with 2% ammonium molybdate. For freeze–fracturing, 0.1 μL of specimen was placed in between two thin copper foil plates. The sandwiches were rapidly plunged into liquid propane without cryoprotectant as previously described (Epand et al., 1981). The frozen specimens were fractured in a Polaron C-7500 unit and evaporated with platinum/carbon, and the replicas were floated off in 1 N nitric acid and cleaned in hypochloric acid. The negative-stained and freeze-factured specimens were observed in a Siemen 101 electron microscope.

Results

Morphology of Reconstituted Vesicles. Proteoliposomes were prepared from fixed amounts (20 mg of protein equivalents) of Triton extracts of human erythrocyte ghosts and a fixed amount (20 mg) of exogenous phospholipid containing PC and PE at varying proportions as detailed under Materials and Methods. Both natural and synthetic mixtures, namely, egg PC/soybean PE and POPC/DLPE, were used. Electron microscopy (Figure 1A) revealed that the vesicles formed after the removal of Triton X-100 consist mainly of small vesicles approximately 50 nm in diameter plus large multilamellar vesicles up to several micrometers in diameter. Intramembranous particles (IMPs) were found mostly in the very small vesicles while few IMPs are found in large multilamellar vesicles. Sucrose density gradient centrifugation studies (data not shown) have shown that the vesicles formed after removal of the detergent are separable into two populations. The lower density population has a protein/phospholipid ratio (mass) of 0.12 whereas the higher density fraction has a ratio of 0.49. Electron microscopy revealed that the lower density population mainly consists of small vesicles. In an experiment in which tritium-labeled exogenous phospholipids were used, more than 70% of the radioactivity was found to be recovered in the lower density population. Therefore, we believe that the large vesicles in the low-density population contain mostly exogenous lipids, whereas the small vesicles containing IMPs possibly consist of the Triton-extracted protein and certain endogenous lipids.

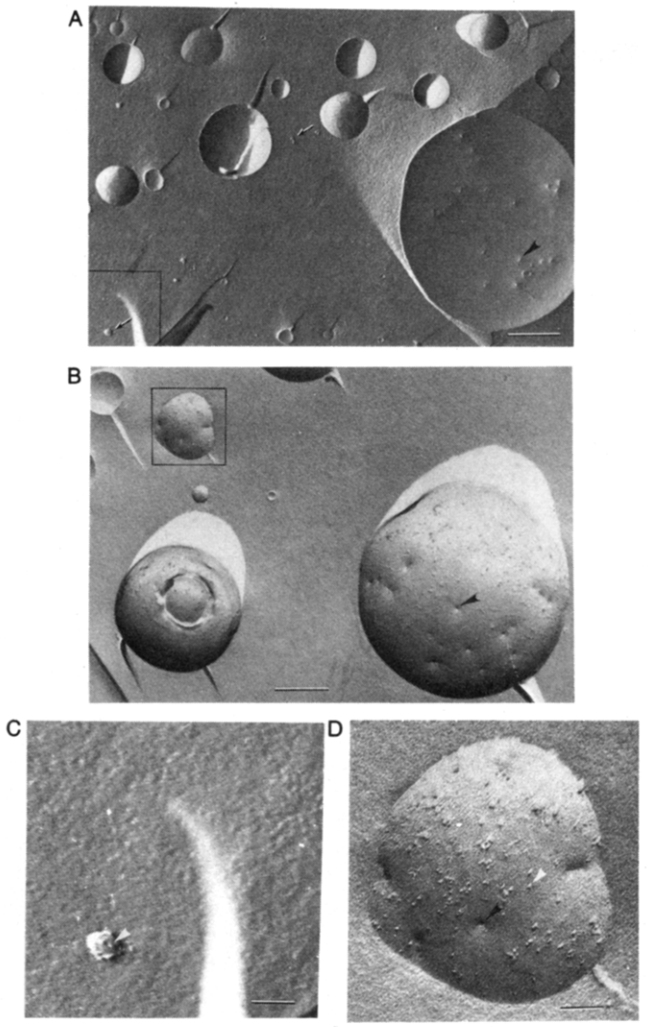

FIGURE 1: Freeze–fracture electron micrograph of reconstituted products after detergent removal and before (A) and after (B) the freeze–thaw procedure. The exogenous lipids used are 25% egg PC and 75% soybean PE. Larger, lipidic particles (black arrowheads) are seen in large vesicles. Smaller, protein-containing particles (white arrowheads) are seen only in small vesicles (arrowed) in (A) but appear frequently in large vesicles in (B). (C) and (D) are enlarged from portions of (A) and (B). Bar = 500 nm in (A) and (B) and 100 nm in (C) and (D).

The large vesicles also contain lipidic particles (LIPs) that are usually found in lipids of such compositions (Hui et al., 1981a,b). Thus, the reconsitution is incomplete without the freeze–thaw process.

After freeze–thawing, the population of small vesicles drastically decreased, and the large multilamellar vesicles were seen with a much higher density of IMPs. A typical view of the samples after freeze–thawing is shown in Figure 1B. It seems that the proteins associated with IMPs in small vesicles are now incorporated into the large vesicles. These IMPs are easily distinguishable from LIPs by their smaller size and their hemispherical shape, in contrast to the conical shape of the LIPs (Hui et al., 1981a,b). The distributions of both LIPs and IMPs seem to be random and are unrelated to each other. The frequency of LIPs varies with the composition in approximately the same manner as in lipid mixtures alone.

Composition of the Reconstituted Proteoliposomes. The post freeze–thaw yield of reconstituted proteoliposomes in terms of lipid is almost independent of the ratio of exogenous PC to PE, as shown in Table I. The relative ratio of PC to PE on these reconstituted proteoliposomes shows almost a linear relationship with that of exogenously added PC and PE

Table I: Total Phospholipid Recovered from Reconstituted Proteoliposomes

PC/PE ratio of exogenous lipid	total phospholipid (mg/mL)	
	egg PC + soy PE	POPC + DLPE
no exogenous lipid	0.96 ± 0.62	0.50 ± 0.03
0/100	1.43 ± 0.75	1.67 ± 0.03
15/85	1.40 ± 0.71	1.58 ± 0.18
25/75	1.48 ± 0.79	1.50 ± 0.01
50/50	1.69 ± 0.95	1.38 ± 0.11
75/25	1.66 ± 0.83	1.81 ± 0.38

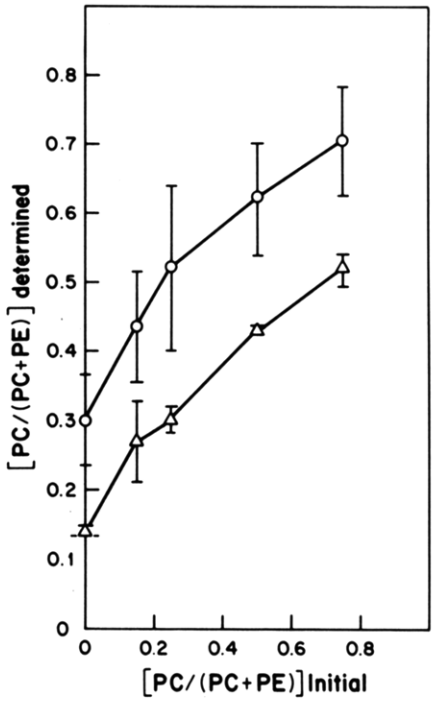

FIGURE 2: PC/(PC + PE) ratio in reconstituted proteoliposomes in relation to that in exogenously added lipid. The exogenous lipids used are egg PC/soybean PE (○) and POPC/DLPE (△). Each point represents an average of the results of four (○) or two (△) independent reconstitution experiments with bars representing standard errors (○) or the range (△).

but indicates that the incorporation of PC into proteoliposomes is less than that of PE (Figure 2). This relationship applies to both natural and synthetic mixtures. The Triton extract contains almost equal amounts of endogenous PC and PE (data not shown).

Protein incorporation in these reconstituted proteoliposomes, however, shows an interesting pattern according to the PC content in the exogenously added lipid mixture. For egg PC/soybean PE mixtures, the protein/lipid ratio increases as the PC content increases, showing about 50% more incorporation at 25% PC, and then decreases gradually as the PC content further increases (Figure 3). The peaking at 25% PC is significantly above experimental error. For POPC/DLPE, on the other hand, the peaking of the protein/lipid ratio is less apparent: The protein incorporation showed a gradual increase as the PC in the mixture increased up to 50% and then leveled off when the PC content further increased (Figure 3).

The Triton extract represents a mixture of many intrinsic membrane polypeptides and includes band 4.5, a presumed glucose carrier protein. Figure 4 shows that the relative compositions of the protein incorporated in reconstituted vesicles are approximately the same as those of the extract used for most lipid compositions. No preferential incorporation is noted for any individual peptide bands except band 3. Band

Table II: Protein/Phospholipid Ratios and Cytochalasin B Binding Activities of Proteoliposomes Reconstituted with Various Exogenous Lipids

variable[a] exogenous lipid (60%)	protein concn (mg/mL)	protein/lipid ratio (mg/mg)	B_T/protein (nmol/mg)	no. of experiments
soybean PE	0.45 ± 0.13	0.57 ± 0.06	0.30 ± 0.10	4
TPE	0.28 ± 0.04	0.27 ± 0.05	0.62 ± 0.10	3
egg PC	0.56 ± 0.04	0.50 ± 0.03	0.44 ± 0.08	5
DMPC	0.71 ± 0.13	0.33 ± 0.04	0.74 ± 0.06	3
cholesterol (40%) + egg PC (20%)	0.45 ± 0.01	0.72 ± 0.01[b]	0.84 ± 0.22	2

[a] Other exogenous lipids are 25% soy PE and 15% egg PC. [b] Cholesterol is not included in this ratio.

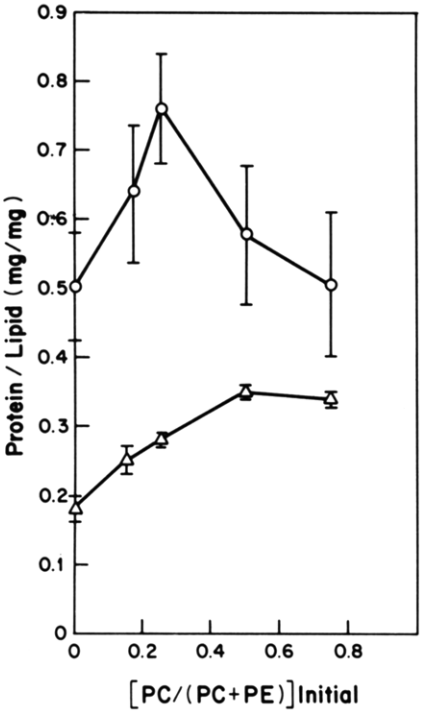

FIGURE 3: Ratio of protein to phospholipid in reconstituted proteoliposomes as a function of the PC/(PC + PE) ratio of exogenous lipids. Symbols are the same as those in Figure 2.

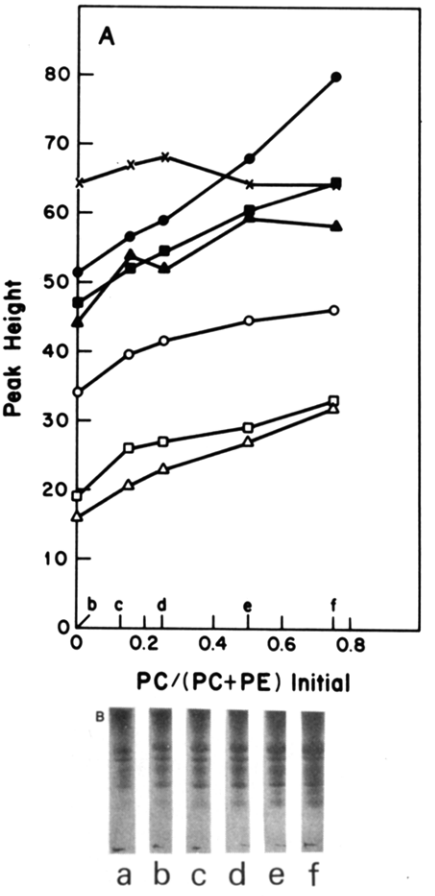

FIGURE 4: Protein incorporation in reconstitution as a function of the exogenous POPC/DLPE ratio. (A) Relative distribution of different membrane peptides in reconstituted proteoliposomes corresponds to bands 3 (●), 4.1 (■), 4.5 (○), 5 (▲), 6 (△), and 7 (□) and aggregates of molecular weight greater than 200 000 (×). Relative distribution was estimated by measuring the peak height of each major peptide band on SDS gel scanning. (B) SDS–polyacrylamide gel electrophoreses of proteoliposomes reconstituted from the Triton extract and lipids of varying PC/(PC + PE) ratio. The PC/(PC + PE) ratios are 0 (b), 0.15 (c), 0.25 (d), 0.50 (e), and 0.75 (f). (a) shows the Triton extract only without exogenous lipids.

3 showed somewhat higher incorporability to liposomes of higher PC contents.

Binding of CB to Proteoliposomes. Equilibrium binding of CB to the proteoliposome prepared from egg PC/soybean PE mixtures shows both saturable and linear components (Figure 5A). The linear component appears to increase as the PC content of the proteoliposomes increases, the significance of which is not immediately clear. Theoretically, the linear binding component should include ligands partitioned into lipid, trapped in the aqueous phase (intravesicular as well as extravesicular), and bound nonspecifically to proteins. The percentage of entrapped tritiated water accounts for only 3.7%, 3.9%, and 1.1% out of 6.8%, 9.8%, and 6.9% linear binding for proteoliposomes reconstituted from 15%, 75%, and 0% egg PC in soybean PE, respectively. The ligand trapped in the aqueous phase thus accounts only partially for this linear binding. The partitioning of CB into liposomes prepared from the lipids without protein accounts for only a small portion (1.1% out of 6.8% for 15% PC) of the observed linear binding.

Regardless of the exact nature of this linear component, the binding was analyzed on the basis of a simple two-component model, one saturable and one linear, and the saturable component alone was plotted according to Scatchard (Figure 5A, inset). It is clear in this analysis that the saturable binding is essentially a single component. It shows an apparent dissociation constant (K_D) of (1.2–1.6) × 10^{-7} M, which increases slightly as the PC content of proteoliposomes increases (Figure 6B). The total binding capacity (B_T), however, is a distinct function of the PC content. It increases greatly and almost linearly as the PC content of the exogenous lipid mixture increases (Figure 6A). The total binding capacity of 75% egg PC is 2.6-fold as large as that of 0% egg PC, or more than 3-fold as large as that (B_T = 0.09 ± 0.01 nmol/mg) of the vesicle with no exogenously added lipids.

A series of experiments similar to those described above were performed with POPC and DLPE instead of egg PC and soybean PE. Equilibrium binding of CB to POPC/DLPE proteoliposomes (Figure 5B) shows a pattern not significantly different from those of egg PC/soybean PE proteoliposomes (Figure 5A). A Scatchard plot of these data with the linear

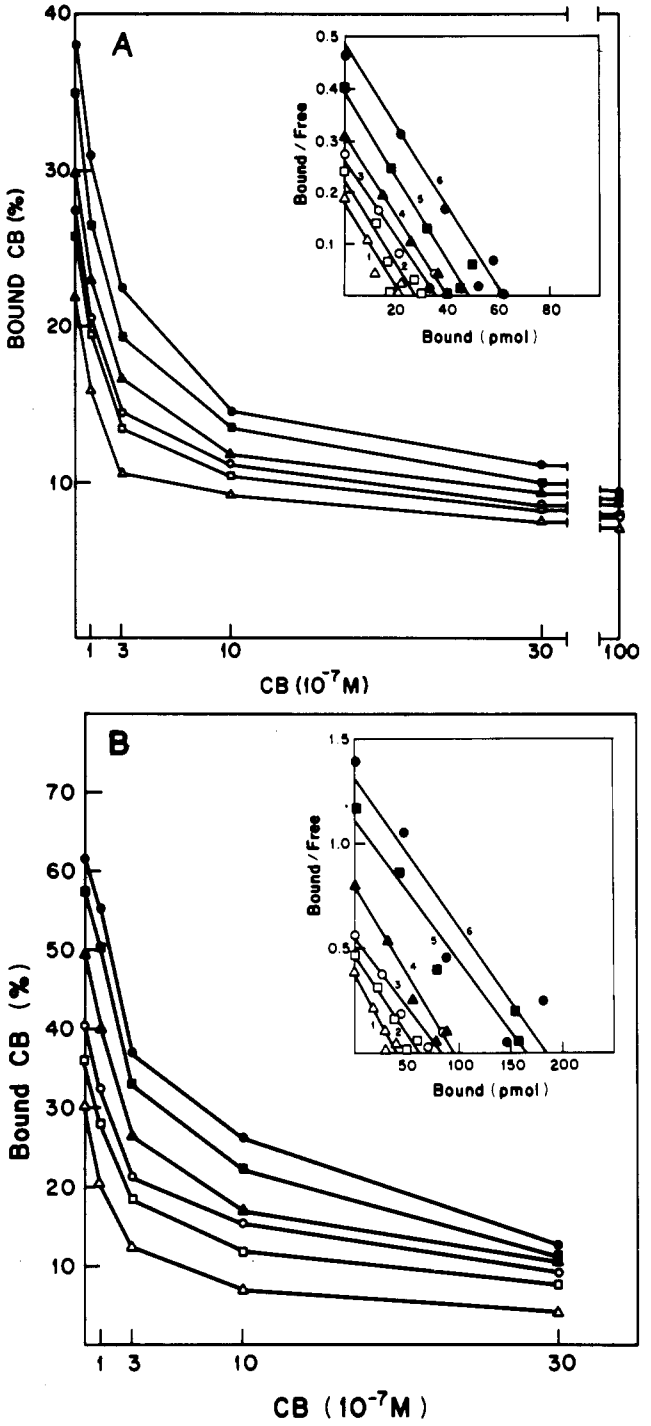

FIGURE 5: Equilibrium binding of cytochalasin B to proteoliposomes reconstituted from fixed amounts of erythrocyte Triton extracts and lipid mixtures of varying PC/PE ratios. Data are before normalization against protein or lipids incorporated. (A) Egg PC and soybean PE. The PC/PE ratios in liposomes used are 0/100 (□), 15/85 (○), 25/75 (▲), 50/50 (■), and 75/25 (●). (△) represents the Triton extract only without exogenous lipids. Scatchard analysis of the data is shown in the inset. Straight lines are drawn as the best fit to the data by least-squares analyses. The regression coefficients are 0.94 (△), 0.92 (□), 0.95 (○), 0.99 (▲), 0.96 (■), and 0.98 (●). (B) POPC with DLPE. Symbols represent the same PC/PE ratios as those used in panel A. Regression coefficients in the Scatchard plots are 0.96 (△), 0.96 (□), 0.94 (○), 0.98 (▲), 0.97 (■), and 0.94 (●).

component corrected for (Figure 5B, inset) revealed that neither B_T (when normalized against protein) nor K_D is affected significantly as the PC/PE ratio is raised (Figure 6).

Equilibrium binding of CB to proteoliposomes also varied when other lipids were substituted for soybean PE in reconstitution. Table II lists the B_T values and the protein/lipid ratios of proteoliposomes using various lipids. All substituted lipids, including a less unsaturated TPE, resulted in significant increases in B_T. TPE is a transphosphatidylated product from egg PC. The major hydrocarbon compositions of egg PC and TPE are palmitic acid (16:0, 33%) and oleic acid (18:1, 32%).

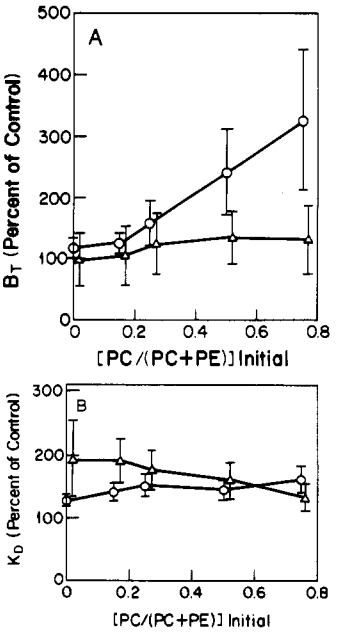

FIGURE 6: Total binding capacities (B_T) and dissociation constants (K_D) of cytochalasin B binding to proteoliposomes reconstituted from the Triton extract and exogenous lipids of different PC/PE ratios. Egg PC with soybean PE (○); each point represents an average of the results of four independent reconstitution experiments with bars indicating standard errors. POPC with DLPE (△); each point represents an average of the results of two reconstitution experiments with bars showing ranges. The binding parameters are expressed as a percent of those of the vesicles of the Triton extract without exogenous lipids (control).

Discussion

Freeze–thawing has been widely used as a key step in membrane reconstitution studies (Gerritsen et al., 1979; Wolosin, 1980; Wheeler & Hinkle, 1981; Yu & Branton, 1976). Most authors found that the proteins associated mainly with the smallest liposomes after detergent removal if the freeze–thaw procedure was not instituted (Gerritsen et al., 1979; Froman et al., 1981). Freeze–thawing apparently facilitates the incorporation of protein into larger lipid vesicles. This is especially effective if the protein-containing small vesicles are pelleted with the exogenous lipid before freezing. Since the exogenous lipids and membrane proteins still reside in separate vesicles after detergent removal, the freeze–thaw process is the crucial reconstitution step. It is very likely that during the slow freezing, ice crystals grow and force the membrane vesicles into areas between the ice crystals. The pressure exerted by the ice crystals on the membrane vesicles can be substantial. This strong force destroys the bilayer integrity and thereby facilitates the penetration of proteins. Because water is removed to form ice crystals during the freezing process, the membranes are increasingly dehydrated. This situation is not unlike that created by many chemical fusogens, such as poly(ethylene glycol), which dehydrate and disrupt the lipid bilayer at the same time (Boni et al., 1981). Existing defects or structural disruption in the bilayer may render the incorporation of proteins or any other foreign molecules into the bilayer easier. The linear relation between the compositions of the exogenous lipids added and the final

lipids in proteoliposomes (Figure 2) indicates that the combination is a purely mechanical process without any chemical preference. Reconstitution by the freeze–thaw process does not favor any particular lipid species. The same argument applies to the proteins. The ratio of the amounts of different membrane proteins incorporated into lipids of different compositions remained approximately constant (Figure 4), indicating that this is also a simple mixing procedure.

The reconstitution efficiency of the freeze–thaw process, i.e., the amount of protein in small vesicles incorporated in a given amount of lipid in large vesicles, is, however, a function of the lipid composition. As shown in Figure 3, the maximum incorporation occurred at a region between 15% and 35% of egg PC in soybean PE. If we compare the efficiency of the protein incorporations with the known partial phase diagram of these lipid mixtures (Hui et al., 1981a), we find that the maximal incorporation efficiency region coincides with the highest occurrence of LIP or interbilayer connections in the egg PC/soybean PE phase diagram. This is the so-called "bilayer instability range". According to the partial phase diagram of DLPE and POPC (Boni & Hui, 1983), this mixture shows only a few scattered LIPs existing at room temperature and at a POPC percentage higher than 15%. As the PE content increases, the mixture transforms from a lamellar to a hexagonal structure via a cubic phase, instead of via an instability range as in the case of egg PC and soybean PE (Hui et al., 1981a). When DLPE and POPC are used for reconstitution, the efficiency of protein incorporation is lower, and no peaking is apparent as the PC/PE ratio changes (Figure 3). Therefore, it seems that the reconstitution efficiency is intimately related to the presence of the disruption of the exogenous bilayer in the form of isolated LIPs. The coincidence of the unstable range with the maximal incorporation is an example of the importance of lipid bilayer instability on the reconstitution process. The importance of the lipid environment on protein incorporation has previously been suggested (Froman et al., 1980).

Although many experiments on the reconstitution of the human erythrocyte membrane proteins into liposomes have been reported, very few have investigated the effect of the lipid composition. Gerritsen et al. (1979) have incorporated a protein extract similar to that used in our experiment to a mixture of phospholipids prepared from human erythrocyte membranes. They found that the distribution of IMPs is apparently not affected by the composition of the lipid used, although more IMPs were seen in mixtures containing PE, PS, and PC (2:1:1) than in mixtures containing PE and PS (3:1). Gerritsen et al. also reported that liposomes containing distearoylphosphatidylcholine and certain erythrocyte membrane proteins were highly permeable to certain cations and that the addition of distearoylphosphatidylethanolamine or lysolecithin will restore the permeability barrier. They interpreted these observations as a better geometric fitting introduced by the wedged-shape molecules of PE or lysolecithin in the protein/bilayer interface, thus reducing the defect or mismatching at the lipid/protein boundary. Wolosin (1980) also measured anion transport as a function of the PC/cholesterol mixture composition. High cholesterol mixtures favored a high rate of anion transport.

D-Glucose-sensitive CB binding activity of human erythrocyte ghosts is approximately 0.4 nmol/mg of protein (Jung & Rampal, 1977). Since Triton extracts 40–50% of the membrane proteins, specific CB binding activity of the Triton extract is expected to be 0.8 nmol/mg or greater (Sogin & Hinkle, 1980). The CB binding activity (0.09 ± 0.01 nmol/mg of protein) of our Triton extract before addition of exogenous lipid mixtures is much lower than this expected value, the reason not immediately clear. This low specific activity was drastically improved by addition of appropriate lipid mixtures (Figure 6). The addition of 100% soybean PE did not affect the activity significantly. However, a lipid mixture of 75% egg PC/25% soybean PE increased the specific activity more than 3-fold. The presence of cholesterol in the lipid mixture further improved the activity to give approximately 50% the maximal specific activity expected (Table II). It appears that an appropriate lipid environment is required to reactivate the CB binding of the glucose carrier protein.

There seems to be no positive correlation of the CB binding with the instability regions of the phase diagrams of the exogenous PC/PE mixture used. The Triton extract of erythrocyte ghosts includes both proteins and endogenous lipids, and the addition of the extract to the initial PC/PE mixtures is expected to alter the physical states of the exogenous lipids (Bosterling et al., 1981). Thus, any correlation made between the phase diagrams of the exogenous lipid mixture alone and the properties of the reconstituted proteoliposomes would have no direct meaning. The phase diagram of reconstituted proteoliposomes is not available at this time. Recently, we have incorporated band 4.5 protein, the putative glucose transport protein (Kasahara & Hinkle, 1977; Phutrakul & Jones, 1979; Froman et al., 1981), into our lipid system. Our preliminary data on CB binding to the purified band 4.5 protein in these proteoliposomes seem to be similar to the results of the Triton extract. We also observed that the IMP distribution was independent of the distribution of the LIP. If the LIPs are truly the bilayer instability or attachment sites, they also have very little topographical effect on the distribution of the IMP. This finding agrees with that of Gerritsen et al. (1979).

The observed increase of CB binding with increasing PC content, on the other hand, suggests that CB binding activity has either a head-group preference for PC or a preference for more saturated lipids. In order to clarify this ambiguity, we substituted PC with other bilayer-forming lipids in reconstitution experiments. Indeed, all lipids we used, including the more saturated DMPC and TPE, resulted in an increase in CB binding (Table II). Although both have the same acyl chains, TPE causes a slightly higher binding capacity than does egg PC, and the values of both lipids are significantly higher than that of soybean PE. It seems that the reduction of the highly unsaturated acyl chain is more important than the increase of PC content in affecting an increase in CB binding in the reconstituted vesicles. Since the additional cholesterol also resulted in increased CB binding, it strongly suggests that the CB binding is affected more by the general reduction of lipid fluidity rather than by the head-group specificity of the phospholipids. However, the relation between CB binding activity and bilayer fluidity may not be simple (Yuli et al., 1981). Nevertheless, based on our findings that (1) the B_T of CB binding as expressed per milligram of protein is significantly lowered by unsaturated PE, (2) the proportion of the putative glucose carrier protein (band 4.5) to the total protein in proteoliposomes is approximately constant, and (3) the K_D of CB binding for all proteoliposomes does not vary beyond experimental error, we suspect that a subpopulation of the glucose carriers may have been inactivated by the bilayer disruptor (soybean PE). The mechanism of this inactivation is not clear at this time.

In conclusion, we found that the composition and the physical states of the lipids have definite effects on both the protein incorporation and the reconstitution of CB binding

activity of human erythrocyte membrane protein extract. The findings suggest that protein incorporation is facilitated by the preexistence of bilayer disruptions (defect) in the lipid mixture, whereas the activity of the CB binding protein incorporated increases with reduction of lipid fluidity. Both effects are apparently based on the physical properties rather than the chemical preference of the phospholipids.

Registry No. CB, 14930-96-2; POPC, 6753-55-5; DLPE, 55252-82-9; DMPC, 13699-48-4; cholesterol, 57-88-5.

References

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Boni, L. T., & Hui, S. W. (1983) *Biochim. Biophys. Acta 731*, 177–185.

Boni, L. T., Stewart, T. P., Alderfer, J. L., & Hui, S. W. (1981) *J. Membr. Biol. 62*, 65–70.

Bosterling, B., Trudell, J. R., & Galla, H. J. (1981) *Biochim. Biophys. Acta 643*, 547–556.

Carter-Su, C., Pessin, J. E., Mora, R., Gitomer, W., & Czech, M. P. (1982) *J. Biol. Chem. 257*, 5419–5425.

Dodge, J. T., Mitchell, C., & Hanahan, D. J. (1963) *Arch. Biochem. Biophys. 100*, 119–130.

Epand, R. M., Epand, R. F., Stewart, T. P., & Hui, S. W. (1981) *Biochim. Biophys. Acta 649*, 608–615.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Froman, G., Acevedo, F., Lundahl, P., & Hjerten, S. (1980) *Biochim. Biophys. Acta 600*, 489–501.

Froman, G., Lundahl, P., & Acevedo, F. (1981) *FEBS Lett. 129*, 100–104.

Gerritsen, W. J., Verkleij, A. J., & Van Deenen, L. L. M. (1979) *Biochim. Biophys. Acta 555*, 26–41.

Hui, S. W., Stewart, T. P., Yagle, P. L., & Albert, A. D. (1981a) *Arch. Biochem. Biophys. 207*, 227–240.

Hui, S. W., Stewart, T. P., Boni, L. T., & Yeagle, P. L. (1981b) *Science (Washington, D.C.) 212*, 921–923.

Jung, C. Y., & Rampal, A. L. (1977) *J. Biol. Chem. 252*, 5456–5463.

Kagawa, Y., Kandrach, A., & Racker, E. (1973) *J. Biol. Chem. 248*, 676–684.

Kasahara, M., & Hinkle, P. C. (1977) *J. Biol. Chem. 252*, 7384–7390.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lukacovic, M. F., Feinstein, M. B., Sha'afi, R. I., & Perrie, S. (1981) *Biochemistry 20*, 3145–3151.

Marsh, D., Watts, A., & Knowles, P. F. (1976) *Biochemistry 15*, 3570–3578.

Phutrakul, S., & Jones, M. N. (1979) *Biochim. Biophys. Acta 550*, 188–200.

Pownall, H. J., Massey, J. B., Kusserow, S. K., & Gotto, A. M. (1979) *Biochemistry 18*, 574–579.

Sogin, D. C., & Hinkle, P. C. (1980) *Biochemistry 19*, 5417–5420.

Wheeler, T. J., & Hinkle, P. C. (1981) *J. Biol. Chem. 256*, 8907–8914.

Wickner, W. T. (1977) *Biochemistry 16*, 254–258.

Woldegiorgis, G., Shrago, E., Gipp, J., & Yatvin, M. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 746.

Wolosin, J. M. (1980) *Biochem. J. 189*, 35–44.

Yu, J., & Branton, D. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3891–3895.

Yuli, I., Wilbrandt, W., & Shinitzky, M. (1981) *Biochemistry 20*, 4250–4256.

Chemical Nature of the Porphyrin π Cation Radical in Horseradish Peroxidase Compound I†

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ABSTRACT: The electron paramagnetic resonance (EPR) and Mössbauer properties of native horseradish peroxidase have been compared with those of a synthetic derivative of the enzyme in which a mesohemin residue replaces the natural iron protoporphyrin IX heme prosthetic group. The oxyferryl π cation radical intermediate, compound I, has been formed from both the native and synthetic enzyme, and the magnetic properties of both intermediates have been examined. The optical absorption characteristics of compound I prepared from mesoheme-substituted horseradish peroxidase are different from those of the compound I prepared from native enzyme [DiNello, R. K., & Dolphin, D. (1981) *J. Biol. Chem. 256*, 6903–6912]. By analogy to model-compound studies, it has been suggested that these optical absorption differences are due to the formation of an A_{2u} and an A_{1u} π cation radical species, respectively. However, the EPR and Mössbauer properties of the native and synthetic enzyme and of their oxidized intermediates are quite similar, if not identical, and the data favor an A_{2u} radical for both compounds I.

Peroxidases and catalases are ferric protoporphyrin IX containing proteins that react with hydroperoxides to produce an oxidized enzyme intermediate referred to as compound I (Keilin & Hartree, 1951; George, 1952, 1953; Chance, 1952). Titration of compound I with ferrocyanide has shown it be 2-equiv oxidized above the native resting enzyme state. The compounds I formed by horseradish peroxidase (HRP)[1] and chloroperoxidase (CPO) have been subjected to a great deal of study in recent years (LaMar & de Ropp, 1980; Schulz et al., 1979; Roberts et al., 1981a,b; Rutter & Hager, 1982). These studies indicate that one of the two oxidation equivalents

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[1] Abbreviations: HRP, horseradish peroxidase; M-HRP, mesoheme-substituted horseradish peroxidase; CPO, chloroperoxidase; Cat, catalase; EPR, electron paramagnetic resonance; ENDOR, external nuclear double resonance; EDTA, ethylenediaminetetraacetic acid.

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