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# Phospholipid Vesicle Formation and Transmembrane Protein Incorporation Using Octyl Glucoside†

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ABSTRACT: Removal of detergent from mixed micelles of egg yolk phosphatidylcholine and octyl glucoside leads to formation of unilamellar phospholipid vesicles with a diameter of about 230 nm. The same procedure applied to mixed micelles containing the transmembrane protein glycophorin A, in addition to lipid and detergent, produces vesicles of the same size with glycophorin incorporated into the bilayer. The pure lipid vesicles are highly impermeable to both anions and cations, and incorporation of up to 220 molecules of glycophorin per vesicle has little effect on permeability.

To acquire a detailed understanding of integral membrane proteins, one must be able to incorporate them in a highly purified state into unilamellar phospholipid vesicles where functional properties such as ion transport can be studied under conditions approximating the native state. Since membrane proteins are generally purified and characterized in detergent-solubilized form (Tanford & Reynolds, 1976), it is desirable to be able to form protein-containing vesicles directly from protein-containing detergent micelles. The purpose of this paper is to summarize the principles that apply quite generally to this problem and to present initial results for a simple model system, egg yolk phosphatidylcholine/octyl glucoside/human erythrocyte glycophorin. The vesicles formed in this system have been characterized with respect to size distribution, protein orientation in the membrane, and passive permeability of the membrane. The last characteristic is particularly important, for if incorporation of a transmembrane protein significantly increases the nonspecific permeability of the membrane, it would interfere with the ability to use the vesicles for specific transport studies. Glycophorin has no known transport function and was chosen for these studies because it is readily prepared in large quantity and in a pure state. It is reasonable to hope that it will be typical of transmembrane proteins, in general, in its effect on passive membrane permeability. It should be pointed out, however, that this protein has limited but finite solubility in aqueous solution in the nonliganded state (it is generally aggregated under these conditions) and that this is not a general property of transmembrane proteins.

To form complexes containing only lipid and protein from a solution of detergent-rich mixed micelles requires removal of detergent from the solution. This will decrease the solubility of the lipid and protein moieties, and the detergent-depleted micelles will aggregate to form larger particles (as one can observe visually since the solution becomes cloudy). However, these larger particles will not necessarily take the form of protein-containing lipid vesicles, and whether they do so must depend on two kinds of events that can occur during aggregation: (1) changes in local composition within a particle, both as a result of detergent removal and as a result of lipid and protein exchange between particles, and (2) molecular rearrangement in response to the compositional change.

The first process requires the movement of molecules through the aqueous solution, and the rate at which a given constituent can participate in producing a compositional change therefore depends on its solubility in an unassociated state in the aqueous medium. This solubility is very small for phospholipids and for most intrinsic membrane proteins. The attainable concentration for unassociated detergent depends on the critical micelle concentration but will always be much higher than that of lipid or protein. Detergent removal and aggregation of the remaining lipid and protein are therefore expected to occur without much change in the *local* lipid/protein ratio. It is clearly advantageous to fix this ratio at the ultimately desired value before the detergent is removed by starting with mixed micelles containing protein and lipid in the same particle.

Since purification of a membrane protein requires separation from other membrane-associated proteins originally present, it may be necessary to use such a high detergent concentration

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that delipidation accompanies the separation process. In that case, it is clearly desirable to incorporate lipid in the protein-containing micelles before the detergent is removed in a reconstitution process. The alternative procedure, which would be to start with a mixture of lipid and protein in *separate* micellar solutions, would appear to be hazardous, especially in the common situation in which the protein has negligible solubility in water. The detergent will equilibrate rapidly, but the other two components will not, leading to a kinetically stable mixture in which a relatively small number of the protein–detergent micelles contain any phospholipid. Removal of detergent from a system such as this is likely to lead in part to the formation of pure phospholipid vesicles and aggregated protein.

Little is known about the dynamics of local molecular rearrangements that occur as the local composition changes. It is probably true that multilayered structures of phospholipid represent a more stable form than single bilayers and that unilamellar vesicles will be formed as a metastable state only if the lipid is sufficiently well dispersed originally to prevent more extensive aggregation at a comparable rate. Starting with detergent-solubilized lipid is, of course, one way to achieve this. It is likely that locally high concentrations of membrane protein can likewise lead to aggregates that may be difficult to disrupt (e.g., Nozaki et al., 1978). The probability of formation of protein-containing vesicles during the rearrangement process is thus enhanced if the particles within which rearrangement is to take place already contain protein and lipid uniformly distributed in *unaggregated* form, and this requires an initially high concentration of detergent relative to protein and lipid.

The procedure adopted in response to these requirements was to deposit phospholipid as a thin film on the walls of a vessel, to dissolve the film in a solution containing protein–detergent mixed micelles, and then to remove detergent from the ternary lipid–protein–detergent micelles by dialysis or gel chromatography. The procedure is similar to that used by Helenius et al. (1977) for formation of vesicles containing membrane-incorporated viral glycoproteins, with the important difference that we used a considerably higher detergent concentration such that from statistical considerations alone the protein is expected to be distributed among the micelles in monomeric form, a condition that was not met in the experiments of Helenius et al. (1977). We also used a much higher lipid/protein ratio because the functional studies to which we eventually want to apply this technique are best performed with a relatively small number of protein molecules per vesicle.

Because experience has shown that many membrane proteins retain their native characteristics in nonionic detergent solution but not when ionic detergents or bile salts are used, it was deemed desirable to use a nonionic detergent in this study. Octyl glucoside was chosen because it has a very high critical micelle concentration, which facilitates rapid removal from mixed micellar complexes. Octyl glucoside has been used in some previous reconstitution experiments for the same reason (Helenius et al., 1977; Engelhard et al., 1978; Petri & Wagner, 1979; Racker et al., 1979), but it is likely that some intrinsic membrane proteins will require detergents with longer alkyl chains (and therefore lower critical micelle concentrations) for maintenance of structural integrity. The methods here employed should in prinicple remain applicable even though the time required for detergent removal would presumably be increased. (A note of caution is in order, however, because we do not know as yet the effect of rate of detergent removal on the structure of reconstitution products.)

An unexpected benefit from the use of octyl glucoside was the finding that vesicles formed by our procedure, with or without incorporated protein, are fairly uniform in size and much larger than vesicles obtained in most preparations described heretofore. The large size has a great advantage for transport studies; small vesicles such as are formed by sonication (Huang, 1969) or from cholate–lipid mixed micelles (Brunner et al., 1976) have an external diameter of the order of 25 nm, with an internal volume of $2 \times 10^{-18}$ mL/vesicle, which corresponds to inclusion of only a single molecule of solute per vesicle when the solute concentration is 1 mM. The vesicles prepared by the use of octyl glucoside, as described in this paper, have a 10-fold larger diameter and therefore a 1000-fold larger internal volume.

## Experimental Procedures

*Materials.* Hen egg lecithin was purchased from Lipid Products, South Nutfield, U.K. It was tested by high-pressure liquid chromatography and found to contain no component other than diacylphosphatidylcholine (detection limit 0.1% by weight). Octyl glucoside (octyl $\beta$-D-glucopyranoside) was purchased from Calibiochem and [$^{14}$C]octyl glucoside was obtained from New England Nuclear. $^{22}$NaCl, Na$^{36}$Cl, and $^{86}$RbCl were purchased from Amersham Corp. TPCK-trypsin was a Worthington Biochemical Corp. product, and neuraminidase was a gift from Dr. L. Glasgow. Sodium dodecyl sulfate (chain length purity 99.8%) was purchased from BDH Chemicals, Ltd., Poole, U.K. Resins used for gel chromatography (Sephadex G-25 and G-50 and Sepharose 4B-CL) were obtained from Pharmacia, Inc. Water was deionized and filtered to remove particulate matter. All other chemicals were reagent grade.

*Preparation of Glycophorin A.* Human red blood cell ghosts were prepared from freshly drawn blood by the method of Dodge et al. (1963). Glycophorin A was isolated from ghosts as described by Grefrath & Reynolds (1974) and Grefrath (1975) with the exception that the Sepharose 4B column was equilibrated in 0.1 M sodium phosphate buffer, pH 7.1, 10 mM sodium dodecyl sulfate, and 1 mM $NaN_3$. Fractions from the front half of the sialic acid containing peak were pooled and dialyzed exhaustively against 10 mM Tris-HCl, pH 7.5, and 1 mM $NaN_3$, containing ion-exchange resin, until all sodium dodecyl sulfate had been removed (3–4 days). Glycophorin A solutions were concentrated by lyophilization and stored frozen up to 6 months. These preparations are greater than 95% glycophorin A as judged by polyacrylamide gel electrophoresis and quantitative amino acid analysis. Glycophorin A was radioiodinated to high specific activity by the chloramine T method of Cuatrecasas (1971).

To obtain solutions for reconstitution a small amount of [$^{125}$I]glycophorin (in 1–2 $\mu$L) was added to 20–300-$\mu$L unlabeled glycophorin at a concentration of 1 mg/mL. A volume of 0.2–0.5 mL of 20 mg/mL (68.5 mM) octyl glucoside solution was added to the protein solution. Since octyl glucoside has a critical micelle concentration near 20 mM (deGrip & Bovee-Geurts, 1979) and a micelle aggregation number near 100 (D.McCaslin, unpublished data), these conditions correspond to a range of about 1–130 detergent micelles/protein molecule.

*Formation of Vesicles.* Egg lecithin was dissolved in 2:1 (v/v) chloroform–methanol in a 30-mL Corex tube, and the mixture was dried to a thin film under a stream of argon and then put under vacuum for several hours to remove the residual traces of organic solvent. (Sometimes a portion of the desired final detergent solution was deposited with the lipid, but no advantage in doing so was observed.) The lipid film was

redissolved in 0.4–0.6 mL of an appropriate detergent (or detergent-protein) solution and dialyzed against two 1-L changes of buffer for 12 h each. The composition of the buffer in this step included whichever salt it was desired to trap in the vesicles together with 0.01 M Tris-HCl, pH 7.5 or 8.5. Most of the detergent is removed at this point in the procedure, and the solution becomes turbid. In several cases, gel filtration on Sephadex G-25 or G-50 was used instead of dialysis (total column volume = 44.0 mL, sample size = 0.4–0.6 mL). Vesicles obtained from the dialysis step or the gel-filtraiton procedure were then passed down a Sepharose 4B-CL column (total column volume = 45.2 mL) equilibrated with the same salt and buffer to minimize the amount of residual detergent. This last procedure was always done at 4 °C.

For many of the permeability measurements, the external solution needed to contain a different salt from that of the trapped solution, and this was accomplished by use of a Sephadex G-50 column equilibrated with the desired salt and buffer (e.g., Tris–nitrate in place of Tris-HCl). The column dimensions were 2 × 8 cm, and the exchange was completed in 10–16 min.

The amount of initially deposied lipid was varied between 2 and 10 mg, and the molar ratio of detergent/lipid in the final solution was varied from 4 to 15. Dissolution was carried out with and without bath sonication (2–10 min) with no detectable effect on the final vesicle preparation.

*Electron Microscopy.* For negative staining, vesicle suspensions were applied to carbon-coated grids, washed with stain solution (2% uranyl acetate in water), and blotted with filter paper. Freeze–fracture experiments employed the technique of Costello & Corless (1978). Vesicle suspensions were sandwiched between two copper strips and plunged into liquid propane (−190 °C). Specimens were fractured in a Balzers 360 M freeze–fracture apparatus at −160 °C and were shadowed with platinum and carbon, without etching.

A Philips EM-301 electron microscope was used, with an objective aperture of semiangle 0.005 rad. Grids were scanned at low magnificiation to select regions having a good distribution of vesicles and an even deposit of stain. These areas were imaged randomly.

*Internal Volume.* Total trapped volume in a vesicle suspension was obtained by passing vesicles prepared in 0.25 M NaCl or KCl down a Sephadex G-50 column to exchange the $Cl^-$ in the external medium for $NO_3^-$. External $Cl^-$ concentrations after this exchange ($C_0$) were measured by using chloride electrodes (see below) and were generally between $10^{-5}$ and $10^{-4}$ M. The concentrations were measured again after lysis of the vesicles by addition of excess detergent ($C_\infty$). The ratio of internal to total volume is obtained as $(C_\infty - C_0)/0.25$. The phospholipid concentration was measured in terms of organic phosphate by the method of Bartlett (1959) to allow calculation of the internal volume per mole of phospholipid.

*Ion-Flux Measurements.* For measurement of $Cl^-$ efflux, an external solution of 0.25 M $NaNO_3$ was used. External $Cl^-$ was monitored continuously for 2–3 h by using a solid-state ($Ag_2S$–AgCl) electrode obtained from Orion Research, Inc., together with a homemade reference calomel electrode (Nozaki & Tanford, 1967), which was modified to provide a second bridge solution (1 M $NaNO_3$) between the KCl salt bridge and the sample solution. The electrode assembly was housed in an aluminum chamber for shielding and attached to a Radiometer Model 4 pH meter for emf measurements. The instrument was calibrated daily with KCl solutions in 0.25 M $NaNO_3$ and readings were found to be stable for several hours and unaffected by the presence of vesicles or detergent. Measurements were made at room temperature.

For influx measuremens using $^{36}Cl$, $^{22}Na$, or $^{86}Rb$, internal and external solutions of the same composition were used. A solution (10–20 μL) containing the labeled ion of interest was added to 2–3 mL of isotope-free vesicle suspensions, and 0.1–0.3-mL aliquots were removed at appropriate intervals and assayed as described by Toyoshima & Thompson (1975). The ratio of internal vesicle volume to total volume is required for interpretation of the results and was obtained as described above on one aliquot of each preparation.

*Other Methods.* Radioactivity of [$^{14}C$]octyl glucoside, $^{36}Cl$, and $^{86}Rb$ were measured in a Beckman LS-100 scintillation counter, using a toluene–Triton X-100 (2:1) based cocktail. $^{22}Na$ and $^{125}I$ were measured directly in a Beckman Model 3000 γ counter.

## Results

*Optimal Detergent/Lipid Ratio.* Because relatively high lipid/protein ratios were used in this investigation, the properties of the vesicle preparation even in the presence of protein are determined primarily by the detergent/*lipid* ratio in the solution from which detergent is to be removed rather than by the detergent/*protein* ratio. How high this ratio has to be to assure good lipid dispersal and formation of reasonably uniform vesicles was thus determined by using lipid–detergent systems without added protein. Two procedures were used for detergent solubilization: (1) an appropriate concentration of detergent solution was added to the lipid film directly, and (2) and a portion of the desired final detergent concentration was deposited with the lipid and the remainder added in solution, as described by Helenius et al. (1977). As expected, since the final detergent/lipid ratio is the determining factor, the two procedures gave identical results. One possible advantage of codeposition is that it might increase the *rate* of solubilization of the lipid film, but we did not test this.

(No difference between the two procedures was observed also in the subsequent experiments in which protein was incorporated into the vesicle. In these experiments, there is, in any case, a limit to how much of the detergent may be deposited with the lipid film because sufficient detergent must be present in the protein–detergent solution to disperse all protein aggregates.)

The amount of lipid deposited on the walls of the vessel used for solubilization was designed to give a final concentration of about 20 mM. It was found that octyl glucoside had to be present in the final mixture at minimally a 4-fold higher concentration to obtain a clear solution. Since the critical micelle concentration is about 20 mM, this means that the molar ratio of detergent to lipid in the mixed micelles has to be at least 3:1 to avoid the presence of particles large enough to cause visible turbidity.

When detergent was removed from the mixed micellar solutions, the clear solutions became turbid again, and examination by electron microscopy (Figure 1) showed that unilamellar vesicles were now present. However, when the amount of detergent was close to the minimal required for obtaining a clear solution (molar ratio in the solution usually 5:1), only about 50% of the lipid was found to be in vesicular form, the remainder consisting of other kinds of aggregates, as seen in Figure 1A. The principles outlined in the introduction imply that these aggregates must have been present before detergent was removed from the sample, and the reason for this is easy to understand. When the detergent/lipid ratio in the solution is 5:1, the ratio in the micelles is 4:1, but this is an average value, and standard statistical considerations (Boas, 1966)

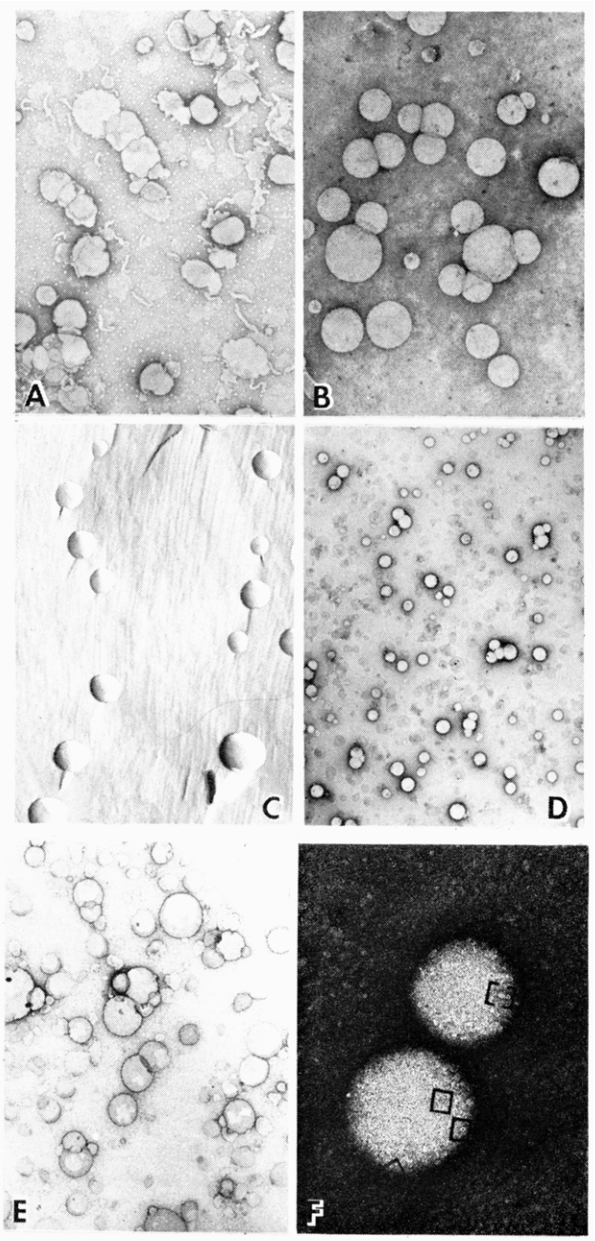


FIGURE 1: Electron microscopy of egg phosphatidylcholine vesicles. (A) Vesicles were prepared by using a molar detergent/lipid ratio of 5:1, examined by negative staining. The electron micrograph shows one of the worst portions of the total field. The origin of the small round particles in the background is not known (×27 500). (B) Vesicles were prepared by using a molar detergent/lipid ratio of 15:1, examined by negative staining. The electron micrograph shows an *average* area of the total field (×27 500). (C) Representative freeze–fracture electron micrograph of vesicles prepared at a 15:1 molar detergent/lipid ratio (×22 000). (D) The same preparation as in (B), examined at lower magnification to show the homogeneity of the preparation. Dark round shapes in the background probably represent vesicles that collapsed during the staining procedure (×8250). (E) Glycophorin-containing vesicles (50 protein molecules/vesicle) prepared at a 15:1 molar detergent/lipid ratio. Freeze–fracture studies were not made (×16 500). (F) Visualization of membrane-bound glycophorin, using ferritin-conjugated wheat germ agglutinin, which was added to the vesicle preparation before the final chromatography step. This preparation contained an average of 20 glycophorin molecules/vesicle. The most prominent ferritin molecules seen on negative staining with uranyl acetate are indicated by brackets (×49 500).

indicate that the actual ratio in individual particles will be distributed over a broad range: about one-fourth of the mixed micelles at a 4:1 average ratio are likely to have an actual detergent/lipid ratio <3:1. No studies of phase separation within mixed micelles have been reported, but it is not unreasonable to expect that patches of aggregated lipid will exist in mixed micelles in which more than one-third of the constituent molecules are phospholipids. (Since each lipid molecule contributes two hydrocarbon chains, the molar ratio of lipid hydrocarbon chains to the much shorter octyl glucoside chains becomes 1:1 when the molar detergent/lipid ratio is 2:1).

Table I: Calculated Properties of Vesicles[a]

| external diameter (nm) | phospholipid molecules per vesicle | internal volume | |
|---|---|---|---|
| | | per vesicle (mL × $10^{15}$) | per mole of lipid (mL) |
| 100 | 83 000 | 0.408 | 2960 |
| 150 | 192 000 | 1.50 | 4710 |
| 200 | 345 000 | 3.71 | 6470 |
| 220 | 419 000 | 4.99 | 7170 |
| 240 | 500 000 | 6.54 | 7870 |
| 260 | 588 000 | 8.38 | 8580 |

[a] Based on a bilayer thickness of 4.0 nm and an average surface area of 0.70 $nm^2$/phospholipid molecule.

Much better vesicle preparations were obtained when the detergent/lipid ratio was increased to 10:1 or above: the electron micrographs of Figure 1B–D were obtained from samples prepared at a 15:1 detergent/lipid ratio, corresponding to a 14:1 ratio in the mixed micelles. We estimate from the electron micrographs that about 90% of the lipid is in vesicular form in these preparations.

These results are confirmed by comparing measured internal volumes with internal volumes calculated on the basis of vesicle dimensions. The calculated volumes, shown in Table I, are based on a bilayer thickness of 4.0 nm and an average surface area per phospholipid molecule of 0.7 $nm^2$, but the results would not have been significantly different if we had used a bilayer thickness of 4.6 nm, as was done in similar calculations by Hauser et al. (1973). A membrane thickness of 3.7 nm has been measured on the basis of trapping volumes for egg phosphatidylcholine vesicles much smaller than the vesicles used here (Johnson, 1973; Huang & Mason, 1978). On the basis of the data in Table I, the internal volume per mole of phospholipid is expected to be about 7500 mL for vesicles with an external diameter of about 230 nm (see below). When the procedure described under Experimental Procedures is used, values of 6000–7400 mL were measured for vesicles prepared at a molar detergent/lipid ratio of 10:1 or 15:1 whereas values of 3600 to 5000 mL were measured when the detergent/lipid ratio was close to its minimal value for obtaining a clear solution, indicating that a much larger fraction of the lipid was incapable of trapping solute under the latter conditions.

*Characterization of Pure Lipid Vesicles.* Lipid vesicles eluted at the void volume when chromatographed on Sepharose 4B-CL (Figure 3 provides an example), which indicates that they are very much larger than egg phosphatidylcholine vesicles prepared by sonication (Huang, 1969; Huang & Mason, 1978) or by detergent removal from cholate–lipid mixed micelles (Brunner et al., 1976). Numerous preparations were examined by electron microscopy and invariably yielded mean diameters in excess of 200 nm. Typical electron micrographs of vesicles prepared at a 15:1 molar detergent/lipid ratio are shown in Figure 1. The vesicles are seen to be smooth and round. The smooth fracture faces shown in Figure 1C are representative of the entire field, and the absence of multistep surfaces unambiguously demonstrates the unilamellar nature of the preparation. The vesicles are seen to be fairly homogenous in size. Figure 2A shows a size distribution histogram of one such preparation, which yields a mean diameter of 240 ± 60

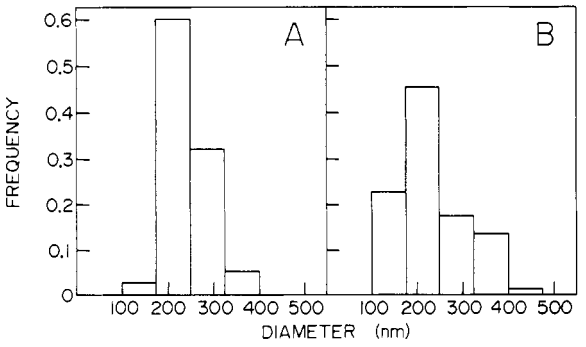


FIGURE 2: Size distribution histograms of vesicles prepared at a 15:1 molar detergent/lipid ratio. (A) Pure lipid vesicles and (B) Vesicles containing about 50 glycophorin molecules/vesicle. Diameters of 75 randomly chosen vesicles were measured in each case from representative fields of the electron micrographs. Average values were 240 ± 60 nm in (A) and 225 ± 85 nm in (B). Particles with a diameter of <100 nm (mostly nonvesicular) were not included in this analysis.

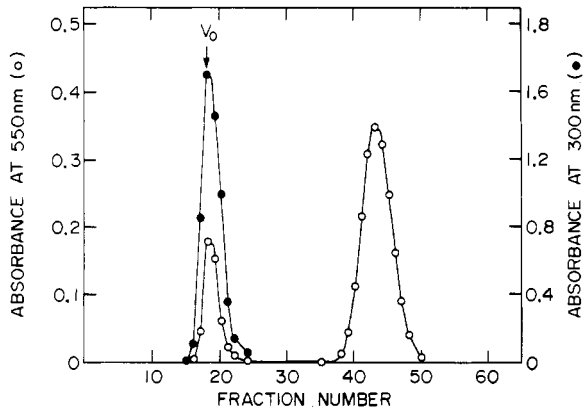


FIGURE 3: Elution profile of cytochrome *c* (measured at 550 nm) and phospholipid (measured by optical density at 330 nm arising from light scattering). Cytochrome *c* was added to the lipid–detergent mixture before vesicles were formed, and the portion coeluting with lipid (20%) represents the protein trapped in the internal vesicular volume (see text). Sepharose 4B-CL was used for chromatography, and the column was eluted (at 4 °C) with the same buffer in whch the vesicles were prepared, but without detergent. The elution profile of the lipid in this experiment is typical of all vesicle preparations used in the course of this investigation.

nm. Fewer than 20% of the vesicles had diameters outside the range 175–325 nm.

When vesicles were prepared by using the minimal molar detergent/lipid ratio of 5:1, about half the lipid is seen to be present in nonvesicular forms (Figure 1A), but the vesicles formed by the remainder of the lipid have about the same size as vesicles formed at higher detergent/lipid ratios.

The vesicular nature of one preparation was demonstrated by trapping of cytochrome *c*, as shown in Figure 3. Vesicles were prepared as before, but a lipid concentration of 30 mM was used, and cytochrome *c* was added to the mixture before the detergent was removed. With an internal volume of about 7500 mL/mol of lipid, vesicles formed under these conditions are expected to trap 22% of the cytochrome *c*, and this corresponds within experimental error with the fraction of cytochrome *c* eluting in the void volume in Figure 3. The trapped fraction of cytochrome *c* could not be reduced with sodium ascorbate (to which lipid vesicles are impermeant), but it was reducible after the vesicles were lysed with detergent. In a control experiment, cytochrome *c* added to preformed vesicles did not coelute with vesicles at the void volume.

$^{14}$C-Labeled octyl glucoside was used in one experiment to determine the amount of detergent remaining in the vesicle preparation, in terms of how much was found to coelute with

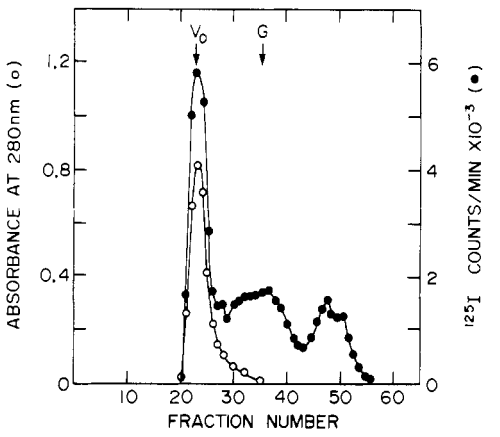


FIGURE 4: Elution profile of glycophorin-containing vesicles on Speharose 4B-CL. The protein was $^{125}$I labeled and was monitored by radioactivity; the lipid was monitored on the basis of optical density at 280 nm arising from light scattering. Half of the glycophorin was observed to coelute with the lipid at the void volume ($V_0$). Much of the remainder elutes at the same position (*G*) as glycophorin aggregates that normally form in aqueous solution when the protein is dissolved in the absence of detergent or lipid. Initial protein solution contained 3.5 μM glycophorin and 68.5 mM octyl glucoside, corresponding to about 130 detergent micelles/protein molecule. Final concentrations prior to detergent removal were 3.5 μM glycophorin, 16 mM phospholipid, and 240 mM octyl glucoside, corresponding to 7 lipid molecules/average micelle and 600 micelles/protein molecule.

the lipid on chromatography. After the first chromatographic step (Sephadex G-25), the residual detergent was less than 0.01% of what was added originally, corresponding to about 1 octyl glucoside molecule/200 lipid molecules. After chromatography on Sepharose 4B-CL, the radioactivity coeluting with the lipid was barely detectable, about 1 ± 1 molecule of detergent/400 lipid molecules. Even this small amount of residual detergent might partly represent molecules that were trapped in the internal vesicular space.

*Characterization of Glycophorin-Containing Vesicles.* Vesicles prepared from mixed micelles containing glycophorin were similar in their morphological characteristics to protein-free vesicles, but they had a broader size distribution. Figure 1E shows a typical electron micrograph and Figure 2B a typical size distribution histogram. The average diameter is 225 ± 85 nm, compared with 240 ± 60 nm obtained in the absence of protein. Although the statistical size measurements shown in this figure were made on only one preparation, all preparations were superficially indistinguishable by electron microscopy. When a minimal detergent/lipid ratio was used, the amount of vesicular material decreased, as was observed in the absence of protein, but the appearance of the vesicles was not altered. Residual detergent was determined for one preparation and was found to be of the same order of magnitude as for protein-free vesicles.

Figure 4 shows a typical elution profile of glycophorin-containing vesicles on Sepharose 4B-CL. In this particular experiment, the added amount of glycophorin corresponded to about 100 molecules/vesicle, but only about half that amount coeluted with the lipid at the void volume. As the figure shows, most of the remainder eluted at the position where aggregated glycophorin elutes in aqueous solution in the absence of lipid and detergent, but some radioactive counts were also found in a peak corresponding to a smaller particle size. A similar fraction of the protein migrated with lipid vesicles on sucrose gradient centrifugation (results not shown). Since the separation is, in this case, on the basis of density rather than particle size, this experiment demonstrates that the protein coeluting with the lipid in Figure 4 must be in-

corporated with the lipid in the same particle. Similar results were obtained with four different preparations at molar ratios of protein to lipid ranging from 1:500 to 1:4400. From the data of Table I, this corresponds to 100–900 glycophorin molecules added per vesicle formed. The fraction of protein molecules incorporated, on the basis of coelution with the lipid, ranged from 25% to 50%. It is possible that the inability to incorporate all of the protein into vesicles is a result of the aqueous solubility of glycophorin, representing a thermodynamic distribution between soluble and membrane-bound states at the time that vesicles are formed, in which case better yields of incorporated protein can be expected for more typical intrinsic membrane proteins of lower aqueous solubility.

The initial phospholipid concentration used to prepare vesicles for these experiments was lower than that used for the cytochrome *c* trapping experiment shown in Figure 3, and the calculated internal volume was correspondingly smaller. In the case of Figure 4, for example, the lipid concentration was 15.6 mM, and the calculated fraction of internal volume was 11%. The amount of glycophorin eluting with the lipid (50%) is much higher than this, and a similar result was obtained in all experiments of this kind. It is therefore concluded that most of the protein coeluting with the lipid is membrane bound. This conclusion was confirmed by showing that the glycophorin coeluting with the lipid is susceptible to the action of neuraminidase and trypsin. (Trapped cytochrome *c* in Figure 3 was found to be inaccessible to chemical reagents.) A total of 50–75% of the protein's sialic acid was found to be removed by neuraminidase in several experiments whereas 100% was removed when the vesicles were first lysed by use of detergents. Similar results were obtained for trypsin cleavage. These data are consistent with the view that the membrane-bound glycophorin does not have a unique orientation in the membrane.

Membrane incorporation of glycophorin was also demonstrated by use of ferritin-conjugated wheat germ agglutinin, as shown in the electron micrograph of Figure 1F.

*Ion Permeability.* Ion-permeation measurements obeyed first-order kinetics within experimental error, i.e., where $C_{in}$ and $C_{ex}$ represent internal and external concentrations, respectively

$$-dC_{in}/dt = k(C_{in} - C_{ex}) \quad (1)$$

Where $\rho$ is the ratio of internal to external volume, conservation of mass requires that $\rho C_{in} + C_{ex} = (1 + \rho)C_\infty$, $C_\infty$ being the concentration inside and outside at equilibrium, i.e., $t = \infty$. With this condition, the integrated form of eq 1 is the same for efflux measurements, in which $C_{ex}$ is determined as a function of time, and for influx measurements, in which $C_{in}$ is determined, i.e., where $C$ is the measured concentration at time $t$ and $C_0$ the initial value:

$$\ln (C_\infty - C) = \ln (C_\infty - C_0) - (1 + \rho)kt \quad (2)$$

Experimental plots showing that this equation is obeyed within experimental error are shown in Figure 5. Since the vesicles become diluted during the final chromatography, the ratio of internal to total volume is considerably smaller under the conditions of these measuremenmts than when the vesicles are first formed, so that, with $\rho \ll 1$, the slopes of the plots of Figure 5 give the rate constant $k$ within experimental error. Rate constants obtained in this way are tabulated in Table II. The table also shows permeability coefficients ($P$) obtained from the rate constants by use of the relation, $P$(membrane area/vesicle) = $k$(internal volume/vesicle), which follows from the normal definition of $P$ as the ion flux per unit membrane area at unit concentration gradient. An external vesicle diameter of 230 nm was used for the calculation.

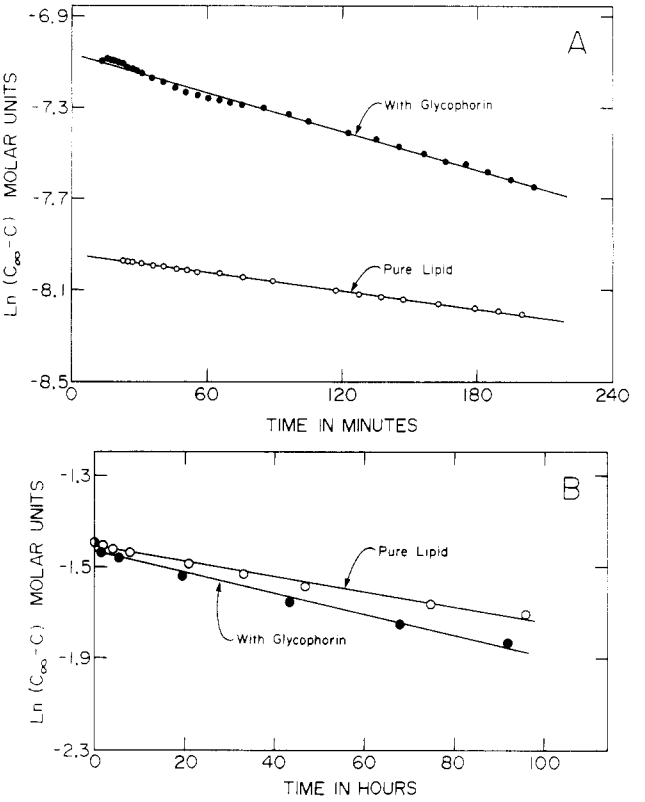


FIGURE 5: Typical ion-flux data, plotted according to eq 2. (A) Chloride efflux, monitored continuously with a $Cl^-$ electrode. Protein-containing vesicles contained 50 glycophorin molecules/vesicle. (B) Rubidium influx, measured by withdrawing aliquots of the solution at appropriate intervals and determining radioactivity trapped in chromatographically removed vesicles. Protein-containing vesicles contained 70 glycophorin molecules/vesicle. The concentration scales are different in the two experiments because the external solution volume is much larger than the internal vesicular volume, so that $C_\infty$ is close to 0.25 M in an influx experiment but less than 1 mM in an efflux experiment.

Table II: Ion-Flux Measurements[a]

| | method | glycophorin (molecules/ vesicles) | $k$ ($s^{-1}$) | $P$ (cm/s) |
|---|---|---|---|---|
| $Cl^-$ | *b* | none | 2.1 (± 0.6) × $10^{-5}$ | 7.6 × $10^{-11}$ |
| | *c* | 50 | 4.7 × $10^{-5}$ | 1.7 × $10^{-10}$ |
| | *c* | 70 | 8.3 × $10^{-5}$ | 3.0 × $10^{-10}$ |
| | *c* | 220 | 9.3 × $10^{-5}$ | 3.4 × $10^{-10}$ |
| $Na^+$ | *d* | none | 2.6 × $10^{-7}$ | 9.5 × $10^{-13}$ |
| | *d* | 70 | 4.4 × $10^{-7}$ | 1.6 × $10^{-12}$ |
| $Rb^+$ | *e* | none | 9.0 × $10^{-7}$ | 3.3 × $10^{-12}$ |
| | *e* | 70 | 1.3 × $10^{-6}$ | 4.7 × $10^{-12}$ |

[a] At 24 °C, pH 7.3–8.5, 0.25 M salt concentration. Glycophorin molecules per vesicle were obtained from measured protein/lipid ratios by assuming a vesicle diameter of 230 nm, which corresponds by Table I to 460 000 lipid molecules/vesicle. [b] Average of six measurements: four efflux measurements using chloride electrodes, with NaCl inside and $NaNO_3$ outside; one similar measurement, with KCl inside and $NaNO_3$ outside; one influx measurement using $^{36}Cl^-$, with KCl inside and outside. [c] Efflux measurements using chloride electrodes, with NaCl inside and $NaNO_3$ outside. [d] $^{22}Na$ influx, with 0.25 M NaCl on both sides of the membrane. [e] $^{86}Rb$ influx, with 0.25 M KCl on both sides of the membrane.

The results given in Table II for protein-free vesicles are comparable with permeability data for sonicated egg phosphatidylcholine vesicles obtained by Hauser et al. (1973) and by Toyoshima & Thompson (1975), in spite of the fact that the sonicated vesicles are much smaller (external diameter

20–30 nm) and were prepared in the absence of detergent. In making the comparison with these earlier studies, it should be noted that the measurements of Hauser et al. (1973) were made at 4 °C, where permeability is about 7-fold lower than at room temperature, and that our $Cl^-$ vs. $NO_3^-$ measurements were made in buffered solutions, where one does not expect to see the large difference between $Cl^-/NO_3^-$ and $Cl^-/Cl^-$ exchange that has been observed in unbuffered solutions (Toyoshima & Thompson, 1975).

The data in Table II for vesicles containing glycophorin show that protein incorporation has only a small effect on permeability properties, and this is perhaps the most significant aspect of the results. Our data in this regard differ from previous reports showing strong enhancement of lipid bilayer permeability when glycophorin is incorporated (Tosteson et al., 1973; Tosteson, 1978; van Zoelen et al., 1978 a,b).

It should be noted that some of the data of Table II (including the experiment in which the vesicles contained 220 copies of the protein) were obtained with vesicles prepared from solutions at the minimal detergent/lipid ratio, i.e., when only about 50% of the lipid forms vesicles during detergent removal. The permeability properties of these vesicles did not differ from those of vesicles prepared from solutions at a higher detergent/lipid ratio.

## Discussion

The best characterized unilamellar phospholipid vesicles previous to the present study were obtained by sonication of aqueous suspensions of phospholipid. The vesicles obtained by this procedure are mostly very small (external diameter <30 nm), and the bilayer envelope is for that reason extremely asymmetric (Chrzeszczyk et al., 1977; Huang & Mason, 1978). The permeability of the walls of such vesicles to ionic solutes has been studied and was found to be extremely low (Hauser et al., 1973; Toyoshima & Thompson, 1975). Less extensive studies of vesicles formed by removal of cholate from cholate–lipid mixed micelles show that vesicles obtained in this way have similar dimensions to those made by sonication and that they are equally resistant to ion permeation (Brunner et al., 1976). There are no reports on the effects of incorporation of transmembrane proteins on the size or permeability of vesicles made by these procedures. It has been shown, however, that an enzyme complex that probably enters the membrane only partially from one side can be incorporated without effect on the vesicle size (Brunner et al., 1978). The effect on $Na^+$ permeability was also found to be small, but not as small as we have observed for glycophorin.

The vesicles obtained in the present investigation by removal of detergent from mixed micelles of phospholipid and octyl glucoside are much larger in size (average diameter >200 nm). Vesicles of similar size were formed regardless of whether detergent was removed by dialysis or by gel chromatography and even when only part of the lipid formed vesicles at all because a low detergent/lipid ratio was used initially. Incorporation of glycophorin into the vesicle walls was readily achieved by starting with mixed micelles containing this protein, and this, too, had no significant effect on vesicle dimensions. From a theoretical point of view, our results are consistent with the view that unilamellar vesicles are metastable species, a "kinetic trap" rather than an equilibrium state. Kinetic events during removal of octyl glucoside from mixed micelles evidently dictate formation of a bilayer with less curvature than when cholate is used or when vesicles are formed by sonication.

As was noted in the introduction, reconstitution studies using octyl glucoside have been carried out in other laboratories. Electron microscope pictures of the preparations obtained in these studies show that the vesicles obtained were more heterogeneous and generally smaller than ours. No permeability measurements were made.

Because vesicles of the size obtained in this study are better suited for transport measurements than the smaller vesicles obtained by sonication or from cholate-containing mixed micelles, the observation that the larger size does not significantly affect passive permeability of the vesicle wall is an important one. The small effect of glycophorin incorporation on passive permeability (if it proves to be representative of inserted membrane proteins in general) is especially encouraging from this point of view, because it suggests that transport studies can be carried out in reconstituted vesicles that contain only a very small number of copies of a purifed transport protein, which is desirable if one wants to avoid the presence of contaminant proteins in the same vesicle as the protein of interest. For example, the highest permeability for $Cl^-$ given in Table II (for 220 glycophorin molecules/vesicle) corresponds to a leak rate of fewer than 50 $Cl^-$ ions $s^{-1}$ per vesicle at a transmembrane concentration gradient of 0.15 M. This may be contrasted with the turnover number of the erythrocyte anion exchanger, which corresponds to the transport of about $5 \times 10^4$ $Cl^-$ ions $s^{-1}$ per binding site (Knauf, 1979). Thus even a *single* exchanger molecule per vesicle would lead to a transfer rate 3 orders of magnitude higher than the passive permeability we have measured. A similar comparison applies between passive transport of univalent cations and the turnover rate of the ATP-driven Na,K pump.

It should be pointed out in conclusion that this paper is a preliminary survey of several aspects of the reconstitution process. Variations in the conditions used for initial solubilization prior to detergent removal and in the rate at which detergent is removed were not studied systematically. The effect of such variations (particularly on the yield of incorporated protein) remains a subject for future investigation.

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# Lipid–Protein Interactions in Bacteriorhodopsin–Dimyristoylphosphatidylcholine Vesicles†

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ABSTRACT: Bacteriorhodopsin (BR) was incorporated into large unilamellar dimyristoyl- and dipalmitoylphosphatidylcholine vesicles (100–300-nm radius). The effect of this intrinsic membrane protein on the order and dynamics of the lipids and on the cooperativity and transition temperature ($T_c$) of the gel to liquid-crystalline phase transition was investigated as a function of the lipid:protein ratio (L/BR). The lipid phase transition induces protein segregation. Above $T_c$, bacteriorhodopsin is in the monomeric state. Below $T_c$, BR is aggregated in the same hexagonal lattice as in the purple membrane (PM). In this reconstituted system, BR has a photochemical cycle similar to that in the PM and is active as a light-driven proton pump. The lipid phase transition which was monitored by using the steady-state anisotropy of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) broadens with decreasing L/BR but occurs at approximately the same $T_c$. Below $T_c$, the fluorescence anisotropy of DPH is quite high (0.35) and independent of the L/BR. Above $T_c$, however, the anisotropy increases markedly with decreasing L/BR. It was recently pointed out that the fluorescence anisotropy of probes like DPH contains information not only on the dynamics (correlation times) but also on the order parameters of the lipids [Heyn, M. P. (1979) *FEBS Lett. 108*, 359–364]. The most likely explanation of the observed increase in anisotropy above $T_c$ is that the perturbation of the lipid bilayer caused by the incorporation of BR leads both to an increase in order and to a slowing of the rotational diffusion of the lipids (increased viscosity). In agreement with this latter dynamical effect, the rotational diffusion constant of BR itself decreases above $T_c$ with decreasing L/BR. Above $T_c$, the membrane viscosity as determined from the rotational diffusion constant of BR is at least 1.5 times larger than that obtained from the fluorescence depolarization of DPH. The formation of the BR lattice as a function of temperature was followed by using the circular dichroism (CD) exciton effect together with measurements of the rotational diffusion of BR. Both methods show similar transition curves for the protein crystallization whose midpoints, however, occur several degrees below $T_c$.

The study of lipid–protein interactions is of considerable importance in membrane biology. Such interactions affect the fluidity and order of the bilayer, modulate the enzymatic activity of membrane proteins, and are responsible for changes in the melting temperature and cooperativity of the gel to liquid-crystalline phase transition. Deuterium nuclear magnetic resonance (Seelig & Seelig, 1978; Oldfield et al., 1978), spin-labeling (Watts et al., 1979; Davoust et al., 1980), and Raman spectroscopy (Curatolo et al., 1978) have provided a detailed but as yet not consistent picture of the effect of intrinsic membrane proteins on lipid order. Much less is known about the perturbation caused by the incorporation of proteins on the dynamics of the lipid motion and on the rotation of the proteins themselves. Little is also known about the change in the thermodynamic parameters of the lipid-phase transition as a function of the lipid to protein ratio.

Reconstituted bacteriorhodopsin–phosphatidylcholine vesicles provide a well-characterized system for the study of lipid–protein interactions (Cherry et al., 1978; Dencher & Heyn, 1979). Bacteriorhodopsin (BR)[1] is an intrinsic membrane protein which spans the bilayer. Mixing of a suspension of solubilized monomeric BR with synthetic lipids above $T_c$

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[1] Abbreviations used: CD, circular dichroism; L/BR, molar phospholipid to bacteriorhodopsin ratio; BR, bacteriorhodopsin; PM, purple membrane; DPH, 1,6-diphenyl-1,3,5-hexatriene; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; $T_c$, midpoint temperature of the lipid gel to liquid-crystalline phase transition.

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