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BARRIER PROPERTIES OF GLYCOPHORIN-PHOSPHOLIPID SYSTEMS PREPARED BY DIFFERENT METHODS

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Glycophorin was incorporated into large unilamellar dioleoylphosphatidylcholine vesicles by either a detergent dialysis method using octylglucoside or a method avoiding the use of detergents. The vesicles were characterized and the permeability properties and transbilayer movement of lipids in both vesicles were investigated as a function of the protein concentration and were compared to protein-free vesicles. An insight in the permeability properties of the vesicles was obtained by monitoring the ratio potassium (permeant): dextran (impermeant) trap immediately after separation of the vesicles from the external medium. Glycophorin incorporated without the use of detergents in 1:300 protein:lipid molar ratio induces a high potassium permeability for the majority of the vesicles as judged from the low potassium trap (K^+:dextran trap = 0.21). In contrast, the vesicles in which glycophorin is incorporated via the octylglucoside method (1:500 protein:lipid molar ratio) are much less permeable to potassium (K^+:dextran trap = 0.67 and $t_{\frac{1}{2}}$ of potassium efflux at 22°C is 7.5 h.). The relationship between protein-induced bilayer permeability and lipid transbilayer movement in both vesicle preparations is discussed. Addition of wheat-germ agglutinin to glycophorin-containing vesicles comprised of dioleoylphosphatidylcholine and total erythrocyte lipids caused no or just a small effect (less than 20% release of potassium) on the potassium permeability of these vesicles. Also, addition of lectin to dioleoylphosphatidylethanolamine-glycophorin bilayer vesicles in a 25:1 lipid:glycophorin molar ratio had no effect on the permeability characteristics of the vesicles. In contrast, addition of wheat-germ agglutinin to bilayer vesicles made of dioleoylphosphatidylethanolamine and glycophorin in a 200:1 molar ratio resulted in a release of 74% of the enclosed potassium by triggering a bilayer to hexagonal (H_{II}) phase transition. The role of protein aggregation and the formation of defects in the lipid bilayer on membrane permeability and lipid transbilayer movement is discussed.

Introduction

One approach to the acquisition of detailed information on the function of intrinsic proteins in biological membranes is to isolate the protein and reconstitute it into artificial model membranes [1]. This approach has been successful in many cases and had contributed significantly to an understanding of membrane functioning. One major drawback of this approach, in particular in the case of transport proteins, is that the presence of the protein itself can greatly increase in an aspecific manner the permeability of the membrane system. It is important to explore the nature of this aspecific permeability in lipid membranes after protein reconstitution.

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Glycophorin, a biochemically well-characterized membrane-spanning protein from the human erythrocyte which has no known transport function, has been used as a model to gain insight in the barrier properties of reconstituted systems. Glycophorin reconstituted in vesicles made of dioleoylphosphatidylcholine by the method of MacDonald and MacDonald [2] (protein-lipid molar ratio 1 : 200–1 : 500) avoiding the use of detergents, introduces an extremely high permeability to small permeants like potassium or glucose. In contrast, Mimms et al. [3] observed that incorporation of a much lower amount of glycophorin (protein:lipid molar ratio 1 : 2000 or less) by the use of a detergent dialysis method using octylglucoside resulted in only little effect on the permeability of the lipid bilayer made of egg yolk phosphatidylcholine. These data suggest that either the protein concentration or the way the protein is incorporated in the vesicle is of importance in determining the barrier properties of the vesicles. To explore these possibilities, we compared glycophorin-reconstituted dioleoylphosphatidylcholine vesicles made at different protein concentrations by the detergent dialysis method to vesicles made by the 'MacDonald' procedure. Since in the 'MacDonald' vesicles transbilayer movement of lipids is increased also [4] we also studied lipid transbilayer movement in vesicles made by octylglucoside dialysis. Another aspect of glycophorin-induced permeability is the lipid dependency of this phenomenon, as 'MacDonald' vesicles made of total erythrocyte lipids show a greatly reduced permeability [5].

It is possible that permeability is related to the aggregation state of the protein. In order to investigate the role of protein aggregation on membrane functional properties, wheat-germ agglutinin (a lectin, containing multiple binding sites for *N*-acetylneuraminic acid and *N*-acetyl-D-glucosamine [6], both present in glycophorin) was used. This lectin can be expected to influence the organization state of glycophorin in the membrane, as was suggested in previous studies [7,8]. Addition of lectin to glycophorin-containing phosphatidylethanolamine vesicles resulted in protein aggregation, membrane fusion and structural transition from a bilayer to a hexagonal (H_{II}) phase [8]. The effect of this lectin on the permeability properties of glycophorin-containing vesicles made of various lipid compositions is studied.

Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine were synthesized according to established methods [9,10], and purified by high-performance liquid chromatography. 1-Palmitoyl-*sn*-glycero-3-phosphocholine was obtained by hydrolysis of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine by means of pancreatic phospholipase A_2. The total lipids of the human erythrocyte membrane were extracted from ghosts by *n*-butanol [11] or by methanol/chloroform mixtures [12]. The solvent was evaporated under reduced pressure and the lipids were redissolved in an ethanol/hexane (3 : 7, v/v) mixture and stored at −20°C under nitrogen. 1,2-[*N-methyl*-^{14}C]Dioleoyl-*sn*-glycero-3-phosphocholine (100 mCi/mmol) was synthesized as described in Ref. 13. 1-[1-^{14}C]Palmitoyl-*sn*-glycero-3-phosphocholine (57 mCi/mmol), glycerol tri[9,10(n)-^{3}H]oleate (500 mCi/mmol) and [^{3}H]dextran (174 mCi/g) were obtained from Amersham International (Utrecht, The Netherlands). [^{14}C]Octyl-β-D-glucopyranoside (314 mCi/mmol) was obtained from New England Nuclear (Boston, U.S.A.). Dextran, M_r 60 000–90 000, was obtained from Serva Labor (Heidelberg, F.R.G.). Fatty acid-poor albumin (bovine) and octylglucoside (octyl-β-D-glucopyranoside) were obtained from Calbiochem (San Diego, U.S.A.). Wheat-germ agglutinin was purchased from Boehringer Biochemicals (Mannheim, F.R.G.). Neuraminidase from *Vibrio cholerae* (protease-free) was obtained from Koch-Light Ltd. (Colnbrook, Bucks., U.K.). Trypsin (type XI, diphenylcarbamylchloride-treated to remove chymotrypsin activity) was purchased from Sigma (St. Louis, U.S.A.). Phosphatidylcholine exchange protein purified from bovine liver according to Kamp et al. [14] was a gift from Dr. K.W.A. Wirtz. It was stored at −20°C in 50% glycerol and dialyzed against incubation buffer before use. Fresh blood in acid-citrate dextrose was obtained from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service with no

preference for special blood groups. Sephadex G-50 coarse and Sepharose 4B-CL were obtained from Pharmacia Inc. All chemicals were of analytical grade.

Methods

Purification of glycophorin

Glycophorin was isolated and purified from ghosts according to a modification of the method of Verpoorte [15] as described previously [16]. The purified protein showed two bands (PAS I and PAS II) upon sodium dodecyl sulfate polyacrylamide gels stained with Coomassie blue and with periodate-Schiff reagent [17], and was more than 95% glycophorin A [18]. The glycophorin contained 2.1 μmol sialic acid/mg protein and less than 0.4 mol phosphorus per mol glycophorin.

Preparation of vesicles

The reconstitution of glycophorin into large unilamellar vesicles made of dioleoylphosphatidylcholine was performed in two ways. Firstly according to MacDonald and MacDonald avoiding the use of detergents [2] and secondly via a detergent dialysis method using octylglucoside [3]. In both methods, a mixture of glycophorin and lipid was dissolved in chloroform/methanol/water (150 : 75 : 1, v/v) and dried by evaporation. The dry film was stored under vacuum for several hours to remove traces of organic solvent. In the case of the MacDonald procedure [2], the film was hydrated in the appropriate buffer leading to the formation of a mixture of large unilamellar glycophorin-containing vesicles (diameter 1000–5000 Å) and lipid structures containing no or a small amount of glycophorin. These latter structures were removed by centrifugation at $10\,000 \times g$ for 10 min at 4°C [19]. In order to pellet the glycophorin-containing vesicles, the supernatant was centrifuged at $37\,000 \times g$ for 30 min at 4°C. Incorporation of glycophorin by the use of the detergent octylglucoside was performed by redissolving the dry lipid-protein film (10 μmol dioleoylphosphatidylcholine) in 0.5 ml of the appropriate buffer containing octylglucoside in a final detergent : lipid molar ratio of 15 : 1 [3]. The mixture was sonicated briefly (1–2 min) under nitrogen in a bath-type sonicator (Bransonic 12) and the suspension was dialyzed overnight against 1 litre of buffer at room temperature. The vesicles formed after the dialysis procedure were run over a Sepharose 4B-CL column (1.5 × 30 cm) at 4°C, equilibrated with the dialysis buffer in order to remove free glycophorin and non-trapped material (see section on potassium permeability). The fractions containing the vesicles (void volume) were centrifuged at $100\,000 \times g$ for 1 h at 4°C. The vesicle pellet was redissolved in 1 ml buffer and passed over a Sephadex G-50 coarse column (1.5 × 30 cm) equilibrated in the same buffer at 4°C, in order to minimize the amount of residual detergent.

The residual detergent content of the vesicles was determined using ^{14}C-labeled octylglycoside and was found to be about 0.5 mol% in both protein-free and protein-containing vesicles.

Unilamellar glycophorin-dioleoylphosphatidylethanolamine vesicles were prepared via the MacDonald procedure [2], as described in detail before (diameter 300–1500 Å) [16], or by cosonication of a dry film of dioleoylphosphatidylethanolamine and a buffer solution containing the appropriate amount of glycophorin at 0°C (diameter of the vesicles, 250–1000 Å) [8].

The reconstitution of glycophorin in vesicles made from the total lipids of the human erythrocyte membrane was performed by the method according to MacDonald and MacDonald [2] as described for dioleoylphosphatidylcholine (see above).

Large unilamellar protein-free dioleoylphosphatidylcholine vesicles were prepared via the octylglucoside method as described above. Protein-free liposomes made of the total lipids from the human erythrocyte membrane were prepared by hydration of a dry lipid film in the appropriate buffer followed by vortex mixing. Small unilamellar vesicles (for use as acceptor vesicles in the phospholipid exchange experiments) were prepared by sonication of a dispersion of lipids in buffer under nitrogen for 15 × 30 s intervals at 0°C using a Bransonic tip sonicator at 80 W. Metal particles from the probe were removed by centrifugation at $37\,000 \times g$ for 30 min at 4°C. Prior to use the vesicles were recentrifuged at $160\,000 \times g$ for 45 min at 4°C and the supernatant was used in the subsequent incubations.

Potassium permeability

In order to correct for possible differences in vesicle sizes between different preparations and in order to detect extremely high permeability rates of potassium, a method was used in which the trap of a small permeant (potassium) and a large non-permeable molecule (dextran) were simultaneously determined [5]. Vesicles with and without glycophorin and of various lipid compositions were prepared in 0.5–1.0 ml of buffer containing 150 mM KCl, 10 mM Tris-HCl pH 7.4, 0.20% (w/v) dextran, 0.02% NaN_3 and 10^6 dpm [^{3}H]dextran/0.5 ml. Non-trapped dextran was removed on a Sepharose 4B-CL column (1.5 × 30 cm) equilibrated in 150 mM KCl/10 mM Tris-HCl (pH 7.4)/0.02% NaN_3 at 4°C. Large vesicles recovered in the void volume were pelleted by centrifugation at 100000 × *g* for 1 h at 4°C and resuspended in 1 ml buffer (150 mM KCl/10 mM Tris-HCl (pH 7.4)/0.02% NaN_3). The vesicles were run over a Sephadex G-50 coarse column (1.5 × 15 cm) equilibrated with 150 mM choline chloride/10 mM Tris-HCl (pH 7.4) in order to remove non-trapped potassium at 4°C. The [^{3}H]dextran content of the vesicles was measured by radioactivity counting and phospholipid phosphorus determination [20]. The internal volumes of vesicles were derived from the dextran-entrapment measurements and could be used to calculate the outer radii, as was described before [5]. Efflux of K^+ was measured at room temperature within 5 min after removal of non-trapped K^+ using a potassium-selective electrode as was described previously [21]. K^+ efflux obeyed first-order kinetics. Therefore, a semilogarithmic plot of the relative K^+ trap (K^+ trap at *t* min/dextran trap at zero time) versus time will yield a straight line. Statistical analyses were by the least-squares method. From the slope of this curve, the half-time ($t_{1/2}$) of the K^+ permeation could be obtained.

Determination of the transbilayer movement of phosphatidylcholine

The phosphatidylcholine exchange protein was used to determine the exchangeable fraction of dioleoylphosphatidylcholine in protein-free and glycophorin-containing vesicles using a procedure similar to that described previously [22]. Large unilamellar donor vesicles (1.0 μmol dioleoylphosphatidylcholine, $2.5 \cdot 10^5$ dpm ^{14}C-labeled dioleoylphosphatidylcholine) were incubated with small unilamellar acceptor vesicles (10 μmol dioleoylphosphatidylcholine, $5 \cdot 10^6$ dpm glycerol tri[^{3}H]oleate and 500 μl (approx. 60 μg) exchange protein in a total volume of 1.5 ml buffer (150 mM KCl/10 mM Tris-HCl (pH 7.4)/0.02% NaN_3). The mixtures were rotated slowly at 37°C. After each hour of incubation, a 200 μl sample was withdrawn and diluted 20-fold with buffer, whereafter the donor vesicles were pelleted at 100000 × *g* for 45 min at 4°C. The vesicle pellet was resuspended in buffer and assayed for ^{14}C- and ^{3}H-radioactivity and phospholipid phosphorus [20]. The percentage [^{14}C]phosphatidylcholine remaining in the vesicles was calculated from ^{14}C-radioactivity and phospholipid phosphorus after correction for amounts of contaminating sonicated vesicles (2.5–20%) as determined from ^{3}H-radioactivity.

Determination of the transbilayer movement of lysophosphatidylcholine

Transbilayer movement of lysophosphatidylcholine was determined in large unilamellar vesicles by extraction of lysophosphatidylcholine from the outer monolayer of the vesicles with fatty acid-poor albumin (bovine) in a way similar to that described previously [5]. Large unilamellar protein-free and glycophorin-containing vesicles (10 μmol dioleoylphosphatidylcholine) containing 1 mol% 16:0-lysophosphatidylcholine, $4 \cdot 10^5$ dpm [1-^{14}C]16:0-lysophosphatidylcholine and $4.5 \cdot 10^6$ dpm glycerol tri[9,10(n)-^{3}H]oleate were prepared by either the MacDonald or the octylglucoside dialysis method. In case of this latter method, detergent was removed by dialysis of 0.5 ml suspension against twice 1 litre buffer (100 mM NaCl/10 mM Tris-HCl (pH 7.4)/0.02% NaN_3) at room temperature. After dialysis overnight, which caused no detectable loss of radioactivity, a sample was withdrawn to determine the ratio of non-extractable marker [^{3}H]glycerol trioleate and [^{14}C]lysophosphatidylcholine (100% value). The vesicles were passed over the Sepharose 4B-CL and Sephadex G-50 columns equilibrated in dialysis buffer. Then 0.5 ml vesicle suspension (1 μmol lipid) was mixed with 0.5 ml of freshly prepared 2% (w/v) albumin solution in the same buffer

solution, at room temperature. At different times, a sample of 150 μl was withdrawn, diluted with buffer to a final volume of 3 ml, whereafter the vesicles were pelleted by centrifugation at 100000 × *g* for 45 min at 4°C. The supernatant was discarded and the amount of lysophosphatidylcholine in the pellet was determined by radioactivity counting.

Determination of the effect of wheat-germ agglutinin on the potassium permeability

Wheat-germ agglutinin was added to a vesicle suspension of 0.06–1.0 μM lipid, after removal of non-trapped dextran and potassium in a total volume of 1.0 ml. After addition of lectin to a final wheat-germ agglutinin/glycophorin molar ratio of 2.0, the vesicles were incubated for 5 min at room temperature. Within this time, agglutination of the vesicles was completed as judged from the stabilization of the increase in turbidity of the vesicles as measured at 450 nm. Aggregation of glycophorin-containing vesicles could also be visualized by freeze-fracture electron microscopy as had been shown previously for glycophorin-containing sonicated vesicles [7]. After 5 min of incubation, the amount of trapped potassium was measured as described above.

NMR

Proton noise decoupled ^{31}P-NMR spectra were obtained on a Bruker WP 200 NMR spectrometer operating at 81 MHz. Approx. 1.0 ml of 100 mM NaCl/10 mM Tris-HCl (pH 7.4)/0.2 mM EDTA containing the dioleoylphosphatidylethanolamine-glycophorin recombinants and 0.2 ml of the ^2H_2O analogue of this buffer were transferred to a 10 mm NMR tube and examined at 30°C. Spectra were accumulated from up to 3000 transients by employing a 90° pulse (18 μs), a 25 kHz sweep width and a 1 s interpulse time using gated proton noise decoupling (4 W input power during the 0.0819 s acquisition time).

General methods

The glycophorin content of the vesicles was measured by sialic acid determination according to Warren [23]. Phospholipid phosphorus was determined according to Rouser et al. [20]. The orientation of the protein and the permeability characteristics of glycophorin-containing vesicles made by the detergent dialysis method was established by adding neuraminidase (50 units/mg protein) or trypsin (5%, w/w, with respect to glycophorin) to the vesicles as described before [19]). After 1 h incubation at 37°C, the vesicles were separated from sialic acid-containing fragments by centrifugation at 100000 × *g* for 60 min at 4°C. Comparison of the amount of sialic acid present in the vesicles before and after enzymatic treatment gives insight into the protein orientation in the membrane.

Freeze-fracture electron microscopy was performed as described previously [24]. 25% (v/v) glycerol was added to the samples to prevent freeze damage.

Results

Characterization of large unilamellar glycophorin-dioleoylphosphatidylcholine vesicles prepared by octylglucoside dialysis

The internal volume of vesicles can be determined by entrapment of ^{3}H-labeled dextran, a large non-permeable molecule. From the trapped volume of unilamellar vesicles, it is possible to calculate the vesicle size. An essential step is that the vesicles are effectively separated from non-enclosed dextran. To test this for vesicles prepared via the octylglucoside dialysis method, ^{3}H-labeled dextran was added to the outside of the vesicles, which had been prepared in the absence of [^{3}H] dextran. The results presented in Fig. 1 demonstrate that non-trapped dextran is completely and efficiently separated from large unilamellar glycophorin-containing vesicles by passing the vesicles over a Sepharose 4B-CL column. Examination of Fig. 1 also reveals that in this particular experiment only 20% of the glycophorin co-eluted with the vesicles in the void volume of the column, resulting in a protein : lipid molar ratio of 1 : 600, both before and after pelleting the vesicles (100000 × *g*, 45 min, 4°C). The glycophorin fraction co-eluting with the lipid was dependent on the initial protein-lipid molar ratio, in good agreement with earlier results reported by Mimms et al. [3].

The dextran trap of vesicles made by octylglucoside dialysis at different protein : lipid molar ratios is summarized in Table I. The vesicles were

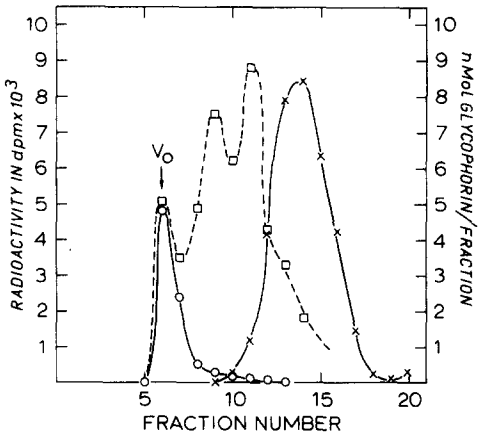

Fig. 1. Elution profile obtained for glycophorin-containing dioleoylphosphatidylcholine vesicles prepared by the octylglucoside dialysis method (initial protein : lipid ratio approx. 1 : 100) in the presence of externally added dextran on a Sepharose 4B-CL column (1.5 × 30 cm). The ^{14}C-labeled dioleoylphosphatidylcholine (○) and [^{3}H]dextran (×) profiles were monitored by radioactive counting. Glycophorin (□) is detected by sialic acid determination.

prepared by extensively dialyzing a clear mixed micellar solution of octylglucoside, dioleoylphosphatidylcholine and different amounts of glycophorin in buffer containing 0.2% [^{3}H]dextran. No change in concentration of dextran was observed in the dialysis bag during dialysis. The lipid : protein ratios present in the mixed micellar solution prior to dialysis (initial ratio) and in the vesicles after passage over the Sepharose 4B-CL column (incorporated) are also summarized in Table I.

It is observed that the vesicles which are devoid of glycophorin have a similar dextran trap of 14 μl/μmol lipid as glycophorin-dioleoylphosphatidylcholine (1 : 1300 molar ratio) vesicles. At higher protein concentrations the dextran trap decreases, indicative of a vesicle population with a decreased mean diameter. Freeze-fracture electron microscopy analyses of the vesicles confirm such a decrease in vesicle size. In the various vesicle populations prepared by the octylglucoside procedure, no multilamellar structures were seen by the electron microscopy technique. All vesicles showed smooth fracture faces, whereas vesicles made by the method of MacDonald revealed small particles, probably representing small aggregates of glycophorin [19,25]. To obtain insight in the orientation of glycophorin, the vesicles were treated with trypsin. When glycophorin is present in a 1 : 2000 protein : lipid molar ratio, 56% of the sialic acid could be removed by tryptic digestion, which is in agreement with results obtained by Mimms et al. [3], suggesting a slight preferential orientation of the protein toward the outside of large unilamellar vesicles (Table II). However, at higher glycophorin content (1 : 300–1 : 570 protein/lipid molar ratios) only 30–36% of the sialic acid could be removed. This was an unexpected result, as one would expect, a random orientation of glycophorin or possibly a slight preference for an outward orientation as has been previously found to be the case for vesicles prepared by sonication or via the MacDonald procedure [19]. It might be possible that at a high initial glycophorin concentration the free (water soluble) glycophorin is trapped within the vesicles during their formation and therefore cannot be degraded by the trypsin. Assuming a 50/50

TABLE I

THE DEXTRAN ENTRAPMENT IN GLYCOPHORIN-DIOLEOYLPHOSPHATIDYLCHOLINE VESICLES MADE BY OCTYLGLUCOSIDE DIALYSIS AT DIFFERENT PROTEIN CONCENTRATIONS

The internal volumes of the vesicles (trapped volume) were used to calculate the outer radii as described before [5].

Glycophorin : dioleoylphosphatidylcholine molar ratio		Dextran trap (μl/μmol lipid)	Calculated vesicle size (diameter in Å)
initial ratio	incorporated		
1 : 40	1 : 340	9.3	2500
1 : 100	1 : 530	11	3000
1 : 500	1 : 1300	14	3800
no glycophorin	no glycophorin	14	3800

TABLE II

PERCENTAGE OF SIALIC ACID REMOVABLE BY TRYPSIN FROM GLYCOPHORIN-CONTAINING VESICLES

The results are for glycophorin-containing dioleoylphosphatidylcholine vesicles made by the use of octylglucoside.

Glycophorin : lipid molar ratio	Removable sialic acid (%)	glycophorin content in the membrane – molar ratio
1 : 300	30	1 : 500
1 : 340	34	1 : 500
1 : 570	36	1 : 790
1 : 2000	56	1 : 2000

inside/outside distribution of the sialic acid residues for the 1 : 570 vesicle preparation, in which 36% of the sialic acid is removed by trypsin, it can be calculated that 72% of the sialic acid would be membrane-bound and 28% trapped within the vesicles. That this is indeed the case is indicated by the finding that 32% of the glycophorin was recovered in the supernatant after lyzing the vesicles by adding a 20-fold volume of H_2O to the vesicles. This experiment demonstrates that at high initial glycophorin concentrations a significant portion of the glycophorin can be trapped within the vesicles and is not incorporated into the membrane. The molar ratio of the membrane incorporated glycophorin and dioleoylphosphatidylcholine based upon the amount of removable sialic acid and an assumed random orientation of the protein, is summarized in Table II. The lipid : protein ratios referred to beyond this point in the manuscript have been corrected for the amount of free protein trapped in the vesicles, estimated from the trypsin-treatment experiments.

Bilayer permeability of large unilamellar dioleoylphosphatidylcholine vesicles as a function of the glycophorin content

Glycophorin was incorporated in large unilamellar dioleoylphosphatidylcholine vesicles by using both the octylglucoside and MacDonald procedure. The influence of the protein on the permeability of the lipid bilayer was determined by a method in which the trap of a small molecule like potassium is related to that of a large non-permeable molecule such as dextran. By this method, extremely high potassium permeability can be detected, resulting in a low ratio of the potassium/dextran trap determined directly after separation of the vesicles from the external medium. The efflux of potassium from the vesicles prepared by the detergent dialysis method containing increasing amounts of glycophorin is depicted in Fig. 2. The relative potassium trap and the half-time ($t_{1/2}$) of the potassium efflux derived from these data are summarized in Table III. Protein-free dioleoylphosphatidylcholine vesicles prepared by the use of octylglucoside are highly impermeable to potassium and show a half-time of potassium efflux of 27 h. The relative potassium trap determined within 5 min after removal of non-trapped potassium is 0.65. The difference between an expected K^+/dextran trap of 1.0 and the determined value in the vesicles made by the octylglucoside dialysis may be the result of the way the vesicles are formed. During removal of octylglucoside closed bilayer vesicles are formed which trap both potassium and dextran. At this stage, a significant amount of detergent will still be present. Upon further dialysis, this amount is reduced by that decreasing the membrane surface area, resulting in shrinking of the vesicles, leading to concentration of the impermeable dextran. Incorporation of increasing amounts of glycophorin in the vesicles does not affect the relative potassium

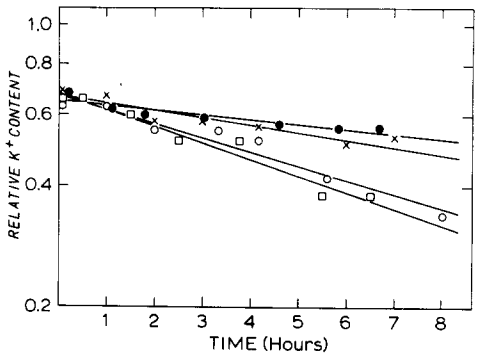

Fig. 2. The relative potassium content of dioleoylphosphatidylcholine vesicles made by the use of octylglucoside in absence (●) and presence of glycophorin at different protein : lipid ratios in the membrane: 1 : 1300 (×), 1 : 740 (○) and 1 : 500 (□). The relative K^+ content is defined as K^+ trap at t = t/dextran trap at $t = 0$.

trap at $t = 0$, but causes an increase in potassium permeability such that in the 1 : 500 (protein : lipid molar ratio) vesicles the potassium efflux shows a half-time of 7.5 h. Treatment of these glycophorin-containing vesicles (1 : 500 molar ratio) with neuraminidase (which removes the negatively charged sialic acid residues on the protein) or with trypsin (which removes the bulky hydrophilic carbohydrate headgroup of glycophorin at the outside of the vesicles) results in both cases in a further increase in potassium efflux (half-time of 4.5 h). In glycophorin-dioleoylphosphatidylcholine vesicles prepared by the method of MacDonald, the protein : lipid molar ratio can be varied only within a limited range between 1 : 500 and 1 : 100. Vesicles prepared by this method and therefore containing more protein (1 : 300), show a relative potassium trap of 0.21, indicating an extremely fast release of 79% of the trapped potassium during removal of non-trapped material from the glycophorin-containing vesicles, in agreement with previous data [5]. The half-time of the efflux of residual potassium from these 1 : 300 vesicles is about 12 h, suggesting the presence of a fraction of vesicles in the preparation which shows a slow potassium efflux. These vesicles might contain only a limited amount of glycophorin [5].

TABLE III

POTASSIUM PERMEABILITY OF LARGE UNILAMELLAR DIOLEOYLPHOSPHATIDYLCHOLINE (DOPC)-GLYCOPHORIN VESICLES

The relative K^+ trap is the K^+ trap/dextran trap determined within 5 min after removal of non-trapped K^+.

	Glycophorin : DOPC calculated molar ratio in membrane	Relative K^+ trap	$t_{1/2}$ of the K^+ efflux (h)
DOPC vesicles made by the use of octylglucoside	no glycophorin	0.65 ± 0.06	27 ± 5
	1 : 3000	0.67 ± 0.06	17 ± 3
	1 : 740	0.67 ± 0.06	8.6 ± 0.9
	1 : 500	0.68 ± 0.06	7.5 ± 0.7
DOPC vesicles made in the absence of detergent	1 : 300	0.21 ± 0.06	12 ± 3

Transbilayer movement of lipids in large glycophorin-containing unilamellar dioleoylphosphatidylcholine vesicles made by different methods

In order to explore the possible relationship between permeability and transbilayer movement of lipids and to determine the origin of these phenomena, studies concerning transbilayer movement of phosphatidylcholine and lysophosphatidylcholine were undertaken.

Transbilayer movement of dioleoylphosphatidylcholine in large glycophorin-containing vesicles was studied by determining the size of the dioleoylphosphatidylcholine pool which can be exchanged by the phosphatidylcholine exchange protein. In large unilamellar protein-free dioleoylphosphatidylcholine vesicles made by the use of octylglucoside, 50% of the ^{14}C-labeled dioleoylphosphatidylcholine could be exchanged (Fig. 3A). Upon the incorporation of glycophorin either by the method of MacDonald or by detergent dialysis, 60–70% of the ^{14}C-labeled dioleoylphosphatidylcholine could be exchanged. It should be realized that prolonged incubation of large donor vesicles with an excess of small acceptor vesicles in the presence of exchange protein will lead to an underestimation of the exchangeable pool due to back exchange of labeled dioleoylphosphatidylcholine molecules. The results depicted in Fig. 3A suggest that there is no large difference in transbilayer movement of dioleoylphosphatidylcholine in glycophorin-containing vesicles prepared by the two different methods. In both cases, a fraction of the vesicles appears to show a transbilayer movement (more than 50% exchange), whereas in a larger fraction this process appears to be too slow to be detected.

Transbilayer movement of 16:0-lysophosphatidylcholine in large unilamellar vesicles comprised of dioleoylphosphatidylcholine, containing 1 mol% 16:0-lysophosphatidylcholine, was assessed by determining the fraction of ^{14}C-labeled 16:0-lysophosphatidylcholine which is extracted by externally added albumin as a function of time [5]. In large protein-free vesicles made by the use of octylglucoside, about 54% of the original lysophosphatidylcholine content remains in the vesicles after addition of albumin, suggesting no transbilayer movement of lysophosphatidylcholine in the time of the incubation period (Fig. 3B). In

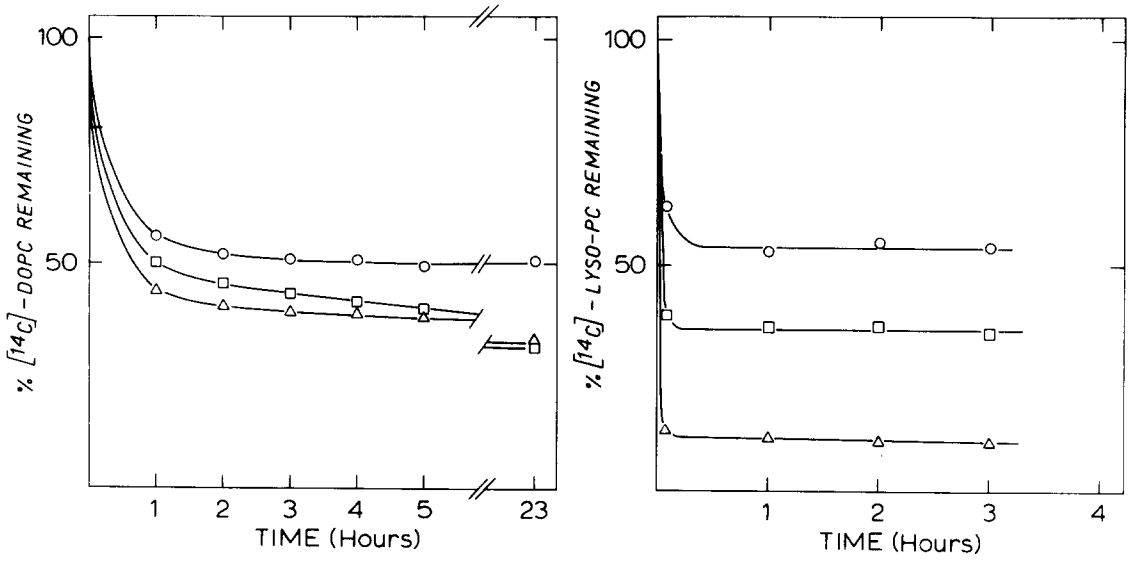

Fig. 3. A. Percentage of ^{14}C-labeled dioleoylphosphatidylcholine exchangeable at 37°C with phosphatidylcholine exchange protein in protein-free vesicles (○) and glycophorin-containing vesicles in a 1 : 700 protein : lipid molar ratio made by the use of octylglucoside (□) and in glycophorin-containing vesicles (△) (1 : 300) made by the method of MacDonald and MacDonald [2] as a function of time. B. Percentage of ^{14}C-labeled lysophosphatidylcholine extractable by albumin from vesicles made of 99 mol% dioleoylphosphatidylcholine and 1 mol% [^{14}C]16:0-lysophosphatidylcholine at 22°C in protein-free vesicles (○), glycophorin-containing vesicles (□) prepared by detergent dialysis in a 1 : 700 protein : lipid molar ratio and in glycophorin-containing vesicles (△) (1 : 300) made by the method of MacDonald.

large unilamellar glycophorin-containing vesicles made by the MacDonald method, 10% of the lysophosphatidylcholine remains in the vesicles after extraction of lysophosphatidylcholine from the outer monolayer, suggesting a fast transbilayer movement of lysophosphatidylcholine in the majority (80%) of the protein-containing vesicles, which is in good agreement with previous results [4,5]. Transbilayer movement of lysophosphatidylcholine is also apparent in the recombined vesicles prepared by the octylglucoside dialysis method, but to a lesser extent (a smaller fraction of vesicles) than was encountered for the MacDonald vesicles (compare in Fig. 3B, □ and △).

Effect of wheat-germ agglutinin on the permeability properties of glycophorin-containing lipid bilayers

It is generally accepted that lectin binding to carbohydrate receptors present in membrane-embedded glycoproteins and glycolipids results in receptor rearrangements [7,8,26]. Following the binding event, redistribution of intrinsic membrane glycoproteins (e.g., changes in aggregation state) may occur resulting in structural alteration at localized areas within the membrane. In order to investigate the possibility that lectin-glycophorin interaction could affect barrier properties, the permeability to potassium was assessed following addition of lectin to the vesicles. The glycophorin-containing systems that were utilized for this study were selected on their property of being only slightly permeable or impermeable to small ions such as potassium. These systems include dioleoylphosphatidylcholine-glycophorin (500 : 1 molar ratio) vesicles prepared by the octylglucoside method and MacDonald vesicles prepared from total erythrocyte lipids [5]. Also, the effect of lectin on pure total erythrocyte lipid extracts was investigated. The permeability characteristics of the vesicles in the absence or presence of wheat-germ agglutinin are summarized in Table IV. Agglutination of vesicles after lectin addition was detected as described in Methods.

Dioleoylphosphatidylcholine-glycophorin vesicles prepared in a 1 : 500 protein-to-lipid molar ratio by detergent dialysis were determined to

TABLE IV

INFLUENCE OF WHEAT-GERM AGGLUTININ (WGA) ON PERMEABILITY PROTERTIES OF MODEL MEMBRANES

The glycophorin-DOPC vesicles in this table were made by the detergent dialysis method. (A) Erythrocyte lipids purified by extraction with chloroform and methanol [12] and containing 1.9 μmol sialic acid/mol lipid. (B) Erythrocyte lipids purified by extraction with butanol [11] and containing 22 μmol sialic acid/mol lipid.

Lipid	Glycophorin : lipid calculated molar ratio in membrane	Dextran trap (μl/μmol lipid)	K^+ : dextran trap before addn. of WGA	% K^+ trap after addn. of WGA
DOPC	1 : 500 *	9.2	0.63	82
Erythrocyte	no protein	5.0	1.05	94
lipids (A)	1 : 490	5.7	0.82	89
Erythrocyte	no protein	9.8	0.97	55
lipids (B)	1 : 450	8.2	0.75	97

have a K^+/dextran trap of 0.63 prior to lectin addition. Following addition and a 5 min incubation period causing extensive vesicle aggregation [8], 82% of the original amount of trapped potassium is still present in the vesicles (Table IV). No further change in the half-time of the potassium efflux was observed, suggesting a rapid release of potassium (18%) during the first 5 min of incubation with lectin. Addition of wheat-germ agglutinin to glycophorin-containing vesicles made of total lipids from the human erythrocyte (containing different amounts of glycolipids as is shown in the legend of Table IV) by the MacDonald method had a negligible effect on the permeability properties of these vesicles. 89–97% of the original amount of trapped potassium remained in the vesicles following lectin addition. In contrast to these results, addition of wheat-germ agglutinin to protein-free multilamellar liposomes of human erythrocyte lipids which had been extracted with butanol and contained a significant amount of glycolipid (22 μmol sialic acid/mol lipid) caused a marked release (45%) of the trapped potassium. Liposomes made of the same lipids which had been extracted by the method of Reed et al. [12] and therefore contained less glycolipid (1.9 μmol sialic acid/mol lipid) showed little release of potassium following wheat-germ agglutinin addition. These data suggest that lectin-glycophorin interaction does not significantly affect barrier functions in these systems, but that lectin-glycolipid interactions can result in a large increase in membrane permeability.

Glycophorin-containing dioleoylphosphatidylethanolamine vesicles produced by the MacDonald method which have been extensively characterized before [16] show almost no permeability to potassium (K^+/dextran trap = 0.83), despite an extremely high glycophorin content (1 : 28 protein/lipid molar ratio) as shown in Fig. 4. In contrast, as discussed above, glycophorin-dioleoylphosphatidylcholine 'MacDonald' vesicles,

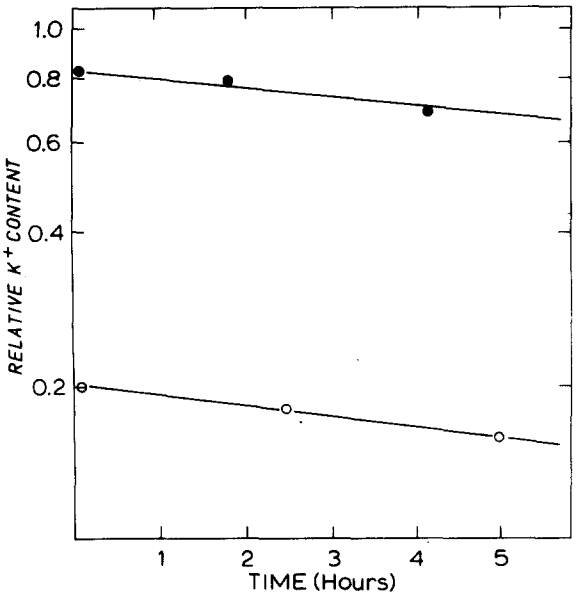

Fig. 4. The relative K^+ content of glycophorin-containing dioleoylphosphatidylcholine vesicles (1 : 300) (○) and glycophorin-containing dioleoylphosphatidylethanolamine vesicles (1 : 28) (●) both made by the method of MacDonald and MacDonald [2] as a function of time. The relative K^+ trap is defined as K^+ trap at $t = t$/dextran trap at $t = 0$.

containing a much lower amount of protein, are much more permeable.

Addition of wheat-germ agglutinin to dioleoylphosphatidylethanolamine-glycophorin vesicles (25 : 1) produced by the MacDonald method failed to significantly cause potassium to be released from the vesicles (data not shown). In order to gain insight into the factors responsible for maintaining the permeability barrier in these vesicles, it is desirable to vary the lipid : protein ratio and to determine the structural organization of the lipids. It has been previously reported [16] that reconstitution of dioleoylphosphatidylethanolamine with glycophorin by the MacDonald method always results in vesicles which have a 25 : 1 lipid : protein molar ratio, regardless of the intended lipid : protein ratio. However, using a cosonication procedure which has recently been described [8], it is possible to vary the glycophorin : dioleoylphosphatidylethanolamine ratio. The internal volumes determined from the amount of trapped potassium of sonicated glycophorin-containing dioleoylphosphatidylethanolamine vesicles (1 : 25 and 1 : 200 protein : lipid molar ratio) in the absence and presence of wheat germ agglutinin are presented in Table V. In vesicles with a 1 : 25 protein : lipid molar ratio, there is no loss of trapped potassium after lectin addition, in agreement with the results obtained from the vesicles prepared via the MacDonald procedure. However, 74% of the amount of originally trapped potassium is released after lectin addition in sonicated vesicles with a 1 : 200 glycophorin : dioleoylphosphatidylethanolamine molar ratio.

TABLE V

THE POTASSIUM ENTRAPMENT IN GLYCOPHORIN COSONICATED DIOLEOYLPHOSPHATIDYLETHANOLAMINE VESICLES BEFORE AND AFTER ADDITION OF WHEAT-GERM AGGLUTININ (WGA)

Glycophorin-dioleoylphosphatidylethanolamine (molar ratio)	K^+ trap (μl/μmol lipid)	
	before addition of WGA	after addition of WGA
1 : 25	0.26	0.26
1 : 200	0.27	0.07

Recently it was demonstrated that glycophorin stabilizes the bilayer organization in mixtures with dioleoylphosphatidylethanolamine which in the absence of glycophorin would prefer an hexagonal (H_{II}) phase above 0°C. The protein was found to stabilize dioleoylphosphatidylethanolamine into unilamellar bilayer vesicles up to a lipid : protein molar ratio of 200 : 1 [8]. Addition of wheat-germ agglutinin to cosonicated glycophorin-dioleoylphosphatidylethanolamine vesicles (1 : 200) triggered a transition from a bilayer to hexagonal (H_{II}) phase in these vesicles [8]. Reconstitution of glycophorin and dioleoylphosphatidylethanolamine in a 1 : 25 molar ratio by cosinication results in the formation of small, unilamellar vesicles (approx. 300 Å diameter) which gives rise to a narrow symmetric ($\nu_{1/2}$ = 10 Hz) ^{31}P-NMR spectrum (Fig.

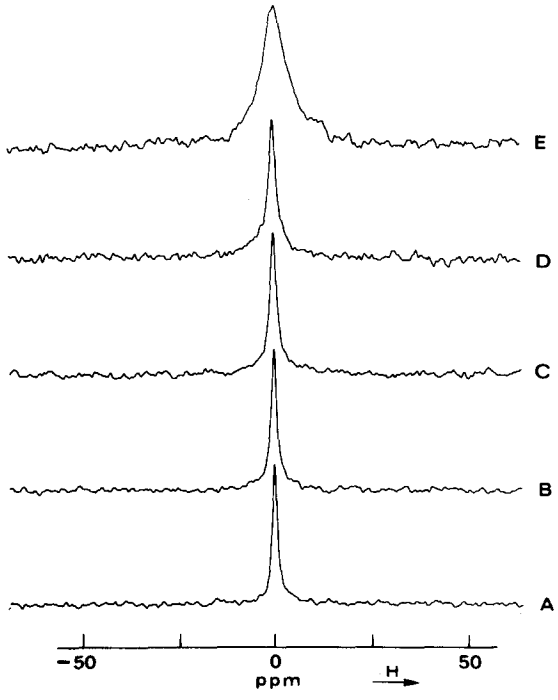

Fig. 5. Proton-decoupled ^{31}P-NMR spectra (81.0 MHz) of dioleoylphosphatidylethanolamine-glycophorin vesicles (15 μmol) dioleoylphosphatidylethanolamine, 30 mg glycophorin, protein : lipid molar ratio 1 : 25) in the absence and presence of wheat-germ agglutinin at 30°C. A, No WGA; and wheat-germ agglutinin : glycophorin ratios; B, 0.35;C 0.70; D, 1.0; E, 1.4. Wheat-germ agglutinin was added to the NMR tube in aliquots of dry lectin, vortexed and allowed to incubate at 30°C for 15 min prior to acquisition.

5A). Addition of lectin leads to a substantial broadening ($\nu_{1/2}$ = 500 Hz) of the NMR spectrum (Fig. 5E); however, no hexagonal (H_{II}) phase component, which would appear 6.7 ppm downfield from the isotropic component [16], is detected. Freeze-fracture electron microscopy showed aggregation and some fusion into larger bilayer types of vesicle, responsible for the broadening of the isotropic NMR spectrum, but demonstrated the absence of any hexagonal (H_{II}) phase lipid (data not shown). Wheat-germ agglutinin is unable to trigger a bilayer → hexagonal (H_{II}) phase transition in vesicles having a high protein content (25 : 1). Thus wheat-germ agglutinin causes agglutination and non-leaky fusion of sonicated glycophorin-dioleoylphosphatidylethanolamine (1 : 25) vesicles. An increase in potassium permeability is only observed in vesicles having the lower protein content (1 : 200) and which contain non-bilayer lipid structures after the addition of lectin.

Discussion

Previous investigations from this laboratory have demonstrated that incorporation of glycophorin into dioleoylphosphatidylcholine vesicles by the procedure of MacDonald and MacDonald [2] results in a high permeability to potassium and enhanced phospholipid transbilayer movement [5]. These processes have been shown to be lipid-dependent, as vesicles comprised of dioleoylphosphatidylcholine and glycophorin are highly permeable to ions and show significant lipid transbilayer movement, whereas those comprised of total erythrocyte lipids and glycophorin are only slightly permeable to ions and demonstrate no enhanced lipid transbilayer movement. Based on these results, it was suggested that packing defects caused by a mismatch in dynamic shapes of the protein and lipids might be responsible for the decreased barrier function. In addition, it could be that the protein aggregation state in the membrane, which might also be lipid-dependent, was responsible for the observed differences. Extension of these studies was limited to some degree by the MacDonald procedure as the lipid : protein ratio could not be extensively varied (for dioleoylphosphatidylcholine) and the vesicles which result are quite heterogeneous in size and protein content [5], rendering quantitation of the result difficult. In an attempt to overcome these difficulties, the octylglucoside dialysis procedure described by Mimms et al. [3] was chosen, since the preliminary results obtained in that study suggested that the procedure yielded more homogeneous vesicle preparations in which the lipid : protein ratio could be more extensively varied.

In the current study, dioleoylphosphatidylcholine-glycophorin vesicles were prepared at a variety of lipid : protein ratios (1300 : 1–500 : 1) by the octylglucoside dialysis method. At high lipid : protein ratios, the vesicles were found to be only slightly permeable to potassium, in good agreement with that reported by Mimms et al. [3] for egg phosphatidylcholine-glycophorin vesicles (up to 2000 : 1 lipid : protein). At higher protein incorporation (500 : 1), a slight increase in potassium permeability was observed ($t_{1/2}$ of potassium efflux about 7.5 h); however, it was much less than was measured for dioleoylphosphatidylcholine-glycophorin (300 : 1) MacDonald vesicles (K^+ : dextran trap ratio 0.21). Freeze-etch electron microscopy revealed that the octylglucoside vesicles were devoid of protein particles which are thought to result from aggregates of protein. These results suggest that the aggregation state of the protein plays a significant role in determining the barrier properties of the membrane as was also demonstrated for the hydrophobic peptide of glycophorin [29]. Incorporation of monomers of the hydrophobic peptide resulted in a slow ion efflux, probably due to perturbations of the bilayer, and a fast ion efflux was found on the presence of peptide aggregates (resulting in large particles as seen by freeze-fracture electron microscopy). In glycophorin-containing vesicles prepared by detergent dialysis only a small fraction (about 30%) of the vesicle population showed transbilayer movement of phosphatidylcholine and lysophosphatidylcholine (Fig. 3). In the MacDonald vesicles also, transbilayer movement of phosphatidylcholine is found in a limited fraction of the vesicles. In contrast, the majority of the MacDonald vesicles show a fast transbilayer movement of lysophosphatidylcholine. We propose a model in which irregularities in the structural organization of the membrane at the lipid/protein interfaces cause transbilayer movement of lipids accompa-

nied by a slightly increased potassium permeability. Extremely fast potassium permeability as observed in the MacDonald vesicles is probably due to the formation of pores formed by protein aggregates. The observed fast transbilayer movement of lysophosphatidylcholine in the majority of the MacDonald vesicles might be the result of transport at lipid/protein interfaces next to permeation of lysophosphatidylcholine monomers through aqueous protein channels in the membrane. This latter possibility is supported by a determined pore size of 15–18 Å diameter (Van Hoogevest, P., unpublished data). In order to ascertain further the origin of protein-induced permeability, the permeability properties of dioleoylphosphatidylethanolamine-gycophorin vesicles were investigated. This system was chosen, since a previous study [16] had revealed that these vesicles had a high content of glycophorin (25 : 1) and exhibited very large protein particles when examined by freeze-fracture electron microscopy, suggestive of the presence of large aggregates of glycophorin in the membrane. It was quite remarkable, therefore, to find that these vesicles had a much higher potassium trap and were significantly less permeable to potassium than dioleoylphosphatidylcholine-glycophorin vesicles that had a much lower protein content (300 : 1) (Fig. 4). On the basis of these results, it can be speculated that the size of the pores formed by protein aggregates is lipid-dependent. Related to this point, it has been found [27] that the addition of 10% dioleoyl phosphatidylethanolamine to dioleoylphosphatidylcholine-glycophorin MacDonald vesicles reduced their permeability to Dy^{3+}. Furthermore, vesicles prepared from total erythrocyte lipids and glycophorin, which contain a full array of lipid species, were only slightly permeable to ions [5].

Binding of effector molecules (e.g., wheat-germ agglutin) to carbohydrate receptors present on glycoproteins and glycolipids, which are embedded in membranes, is known to lead to changes in receptor mobility and distribution [7,8]. Thus, we attempted to manipulate the distribution (aggregation state) of glycophorin in various reconstituted systems and relate these changes to observed differences in permeability which might occur as a result of this event. Dioleoylphosphatidylcholine-glycophorin vesicles prepared by the octylglucoside procedure and cosonicated dioleoylphosphatidylethanolamine-glycophorin vesicles prepared in a 25 : 1 ratio did not show a significant increase in permeability following binding with lectin (Tables IV and V). Freeze-fracture electron microscopy established that the vesicles were agglutinated by the lectin; however, no change in appearance of protein particles was observed. On the contrary, addition of wheat-germ agglutinin to 200 : 1 dioleoylphosphatidylethanolamine-glycophorin vesicles resulted in an outflow of about 74% of the entrapped potassium. A previous ^{31}P-NMR and freeze-fracture electron microscopic study of these vesicles [8] revealed that addition of lectin caused a transition from a bilayer to a hexagonal (H_{II}) phase, which is most probably the event responsible for the observed potassium release.

The absence of permeability changes in the other vesicles can be due to the fact that the lectin binding may result in cross-linking of the receptor headgroup regions of the protein without causing extensive aggregation of the hydrophobic membrane-spanning segment of the proteins. A similar suggestion has recently been presented concerning the binding of concanavalin A to concanavalin A receptor glycoprotein in lipid bilayers [28].

The results obtained from this study infer that protein concentration and aggregation state might play key roles in determining membrane structure (at the lipid/protein interface) and function. It is possible that the aggregation state of the protein and, related to this, the size of the pores are lipid-dependent as evidenced from the differences in dioleoylphosphatidylethanolamine-glycophorin and dioleoylphosphatidylcholine-glycophorin systems leading to the observed differences in the permeability characteristics of the two systems. Unfortunately, this is rather speculative as data concerning the aggregation state of glycophorin in lipid bilayers are lacking. Therefore, further investigations will be undertaken to establish the aggregation state of the protein in various lipid systems in a further attempt to correlate membrane structure and function.

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