Biochimica et Biophysica Acta, 600 (1980) 607—619

BBA 78892

THE TRANSBILAYER MOVEMENT OF PHOSPHATIDYLCHOLINE IN VESICLES RECONSTITUTED WITH INTRINSIC PROTEINS FROM THE HUMAN ERYTHROCYTE MEMBRANE

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(Received January 18th, 1980)

Key words: Band 3 protein; Glycophorin; Phosphatidylcholine; Transbilayer movement; Phospholipid exchange

Summary

Vesicles have been prepared from 18 : 1_c/18 : 1_c-phosphatidylcholine with or without purified glycophorin or partially purified band 3 (obtained by organomercurial gel chromatography). The vesicles have been characterized by freeze-fracture electron microscopy, binding studies to DEAE-cellulose, ^{31}P-NMR and K^+ trap measurements. Pools of phosphatidylcholine available for exchange have been investigated using phosphatidylcholine exchange protein from bovine liver. The protein-containing vesicles both exhibit exchangeable pools larger than the fraction of phosphatidylcholine in the outer monolayer, whereas in the protein-free vesicles the exchangeable pool is consistent with the outer monolayer. The results indicate that both glycophorin and the partially purified band 3 preparation enhance the transbilayer movement of phosphatidylcholine.

Introduction

Transmembrane movements of phospholipids have been studied both in biological and artificial membranes. Rapid translocation rates have been reported for bacterial [1] and microsomal [2,3] membranes. In erythrocyte membranes a much slower transbilayer movement of phosphatidylcholine has been observed [4,5], whereas virtually no transbilayer movement has been detected in the membrane of influenza virus [6,7]. In artificial phospholipid bilayers flip-flop is extremely slow [8—10] unless the equilibrium distribution is perturbed by unilateral alterations in the phospholipid composition [11,12]. Recently, it was observed that the presence of glycophorin in the bilayer of

phosphatidylcholine vesicles causes a highly increased transbilayer movement of phosphatidylcholine [13]. Another intrinsic protein, cytochrome *c* oxidase, was shown not to induce phosphatidylcholine flip-flop when incorporated in vesicles of phosphatidylcholine and phosphatidylinositol [14]. In this paper we study the effect of other intrinsic proteins of the human erythrocyte membrane on phosphatidylcholine flip-flop in order to clarify the question as to whether this protein-mediated transbilayer movement is a glycophorin-specific process.

Intrinsic proteins are extracted from the membrane using Triton X-100 [15,16]. Glycophorin, which is also solubilized by this extraction, is effectively removed by organomercurial affinity chromatography [17]. Reconstitution of proteins into phosphatidylcholine vesicles is achieved by SM-2 bead treatment of mixtures of lipid and protein in Triton X-100 [16,18]. The vesicles are fractionated by centrifugation and characterized as to their chemical composition, freeze-fracture appearance, internal volume, inside-outside distribution of phosphatidylcholine and permeability properties. The transbilayer movement of phosphatidylcholine in the vesicles is investigated by measuring the pool of phosphatidylcholine that is exchangeable with phosphatidylcholine exchange protein from bovine liver.

Materials and Methods

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine ($18:1_c/18:1_c$-phosphatidylcholine), $18:1_c/18:1_c$-[^{14}C]phosphatidylcholine and [^{3}H]cholesterol oleate were synthesized as described before [11,19]. Cardiolipin was obtained from Sigma, Triton X-100 from Rohm and Haas, Bio-Beads SM-2 and Affigel 501 from Bio-Rad, Sephadex G-50 coarse from Pharmacia and DEAE-cellulose (DE 52) from Whatman. Phosphatidylcholine exchange protein, purified from beef liver according to the method of Kamp et al. [20], 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. Glycophorin was purified as described by Verpoorte [21].

Ghosts were prepared from fresh human blood according to the method of Dodge et al. [22] and stored at −20°C. Extraction of ghosts with Triton X-100 was performed as outlined previously [16]. Organomercurial gel chromatography [17] was carried out in 0.5% Triton X-100, 150 mM NaCl, 0.2 mM EDTA, 10 mM Tris-acetic acid (pH 7.0), at 4°C, with a flow rate of 30 ml/h. The protein bound to the gel was eluted with 100 mM L-cysteine in the same buffer. Cysteine was removed by dialysis against 200 vols. of buffer for 5 h at 4°C, with one change of buffer after 2 h. For reconstitutions, only freshly prepared protein preparations were used. The organomercurial gel was regenerated according to the instructions of the manufacturer.

Vesicles were prepared by SM-2 bead treatment of mixtures of lipid and protein in Triton X-100, as described before [16]. Large structures were removed by two or three centrifugations at $25\,000 \times g$ for 30 min. Where indicated, vesicles were sedimented at $150\,000 \times g$ for 90 min. Sonicated vesicles with or without glycophorin were prepared as described previously [23], starting with 0.2 mg glycophorin (dry weight) per μmol lipid, and centri-

fuged at 150 000 × *g* for 45 min before use. The binding of vesicles to DEAE-cellulose was studied using columns prepared from 1 ml DEAE-cellulose slurry (20%, w/v) in 10 mM Tris-HCl, 10 mM sodium acetate, pH 7.0. After washing with 10 mM Tris-acetic acid (pH 7.0), 150 mM KCl, the columns were loaded with 50 μl of vesicles in the same buffer, and subsequently eluted with 0.5 ml buffer. The total eluent was assayed for lipid phosphorus. The internal volume of vesicles was determined from the amount of trapped K^+. The vesicles were prepared in 10 mM Tris-acetic acid (pH 7.0), 150 mM KCl, and the non-trapped K^+ removed by column-centrifugation through Sephadex G-50 [24]. The columns were prepared from 6 ml of Sephadex G-50 coarse, pre-swollen in 10 mM Tris-acetic acid (pH 7.0), 150 mM choline chloride. They were loaded with 0.4 ml vesicle suspension and centrifuged at 300 × *g* for 2 min. The K^+ content of the vesicles in the effluent was released by the addition of Triton X-100 and measured with a K^+-specific glass electrode [25]. To measure K^+ leakage from the vesicles, effluents from G-50 columns were incubated at 23°C for 1 h, after which the K^+ content of the vesicles was determined.

^{31}P-NMR was used to measure the inside-outside distribution of phosphatidylcholine in vesicles using Nd^{3+} (2 mM) as a paramagnetic reagent to shift the outside resonances [26]. Vesicles were concentrated by vacuum dialysis using Sartorius Collodion Bags, and thereafter again centrifuged twice at 25 000 × *g* for 30 min. It was checked that the internal volume, as determined from the K^+ trap, did not increase by this concentration step. The ^{31}P-NMR measurements were performed on a Bruker WH 90 spectrometer, operating at 36.4 MHz [26]. Accumulated free induction decays were obtained from 500 transients, using 90° pulses with interpulse time of 1.7 s. Peak intensities were determined via computer integration with respect to an external triphenylphosphine reference.

The internal volumes of vesicles were determined from K^+ trap measurements and used to calculate outer radii, assuming unilamellar vesicles, a bilayer thickness of 35 Å and an area per molecule of phosphatidylcholine of 72 Å^2 [27]. Vesicle outer radii were also calculated from ^{31}P-NMR linewidths, using the formula, $\Delta\nu = ca^3 + d$, where $\Delta\nu$ is the full peak width at half-height, a is the vesicle radius and c and d are constants, for which values of $c = 2.9 \cdot 10^{-6}$ s^{-1} · Å^{-3} and $d = 2$ Hz were used [28]. Inner monolayer pools of phosphatidylcholine were calculated from vesicle outer radii and bilayer thickness, assuming equal packing densities in the inner and the outer monolayer.

Phosphatidylcholine exchange reactions using the exchange protein from bovine liver were studied by a modification of the procedure described previously [13]. Small donor vesicles of 18 : 1_c/18 : 1_c-phosphatidylcholine, labelled with 18 : 1_c/18 : 1_c-[^{14}C]phosphatidylcholine and a trace of [^{3}H]-cholesterol oleate, were prepared by sonication or by the SM-2 bead procedure. Vesicles prepared by SM-2 bead treatment were centrifuged twice at 25 000 × *g* for 30 min before use. The supernatants contained 20—40% of the total phospholipids. It was checked that the ^{14}C/^{3}H ratio in the supernatant was the same as that in the material sedimented in the first centrifigations. Acceptor vesicles were prepared from a mixture of 18 : 1_c/18 : 1_c-phosphatidylcholine and 5 mol% cardiolipin [29] by SM-2 bead treatment of a 10 mM lipid disper-

sion in 0.5% Triton X-100 containing buffer. After removal of the beads the vesicles were sedimented once at 30 000 × *g* for 15 min, and subsequently three times at 20 000 × *g* for 15 min. About 50% of the starting material was recovered this way. These vesicles were a sufficiently good substrate for the exchange protein, and could effectively be separated from the donor vesicles by centrifigation. For the exchange reactions 0.5 ml of donor vesicles (0.25—0.75 μmol 18 : 1_c/18 : 1_c-phosphatidylcholine, 3—9 · 10^4 dpm 18 : 1_c/18 : 1_c-[^{14}C]-phosphatidylcholine, 3—9 · 10^4 dpm [^{3}H]-cholesterol oleate) was incubated with 0.25 ml of acceptor vesicles (18 : 1_c/18 : 1_c-phosphatidylcholine with 5 mol% cardiolipin, 25 μmol P_i/ml) and 0.5 ml exchange protein (30 μg). The ratio of the donor-to-acceptor phosphatidylcholine varied from 1 : 7 to 1 : 25. The mixtures were slowly rotated at 37° C for 45 min. At the end of an incubation the acceptor vesicles were sedimented at 25 000 × *g* for 30 min, and aliquots were taken from the supernatant for counting and phosphorus assay. The recovery of donor vesicles in the supernatant was 90—100%, as determined from ^{3}H radioactivity, and only 0—0.5% of the acceptor vesicles did not sediment, as calculated from lipid phosphorus, ^{3}H radioactivity and specific ^{3}H radioactivity of the donor vesicles. The remaining part of the supernatant was reincubated with a new amount of acceptor vesicles in such a way that the ratio of donor-to-acceptor phosphatidylcholine was kept constant, and this procedure was repeated five times. At the start of the fourth incubation also 0.2 ml (12 μg) of new exchange protein was added to the incubation mixture to compensate for any loss or inactivation of exchange protein. The percentage of 18 : 1_c/18 : 1_c[^{14}C]-phosphatidylcholine remaining in the donor vesicles was calculated from the ^{14}C/^{3}H ratio in the supernatants and the ^{14}C/^{3}H ratio of the starting vesicles. In control experiments it was established that in the absence of exchange protein the decrease in the ^{14}C/^{3}H ratio was negligible.

Protein was measured by using the method of Lowry et al. [30] with 3% sodium dodecyl sulfate in the alkaline copper reagent. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to the method of Steck and Yu [31], using 7.5% acrylamide gels and bromophenol blue as tracking dye. Phospholipids were determined as phosphorus according to the method of Rouser et al. [32] and sialic acid was measured according to the method of Warren [33].

Results

Intrinsic proteins can be rather selectively extracted from the human erythrocyte membrane with Triton X-100 [15]. The major protein components in such extracts are band 3 and glycophorin (Fig. 1A and B). Also phospholipids are extracted [16], resulting in 2.6—2.9 μmol P_i/mg protein. Organomercurial gel chromatography [17] was found to be very effective in separating both glycophorin and most of the phospholipids from the other protein components. In the procedure described by Shami et al. [17] the sialoglycoproteins are eluted from the gel with 34 mM phosphate, pH 3.5, and the other proteins with a cysteine-containing buffer. In our hands this procedure was not very satisfactory with respect to two points. Firstly, often the sialoglycoproteins were not completely eluted and 10—15% of the sialic acid was recovered in the

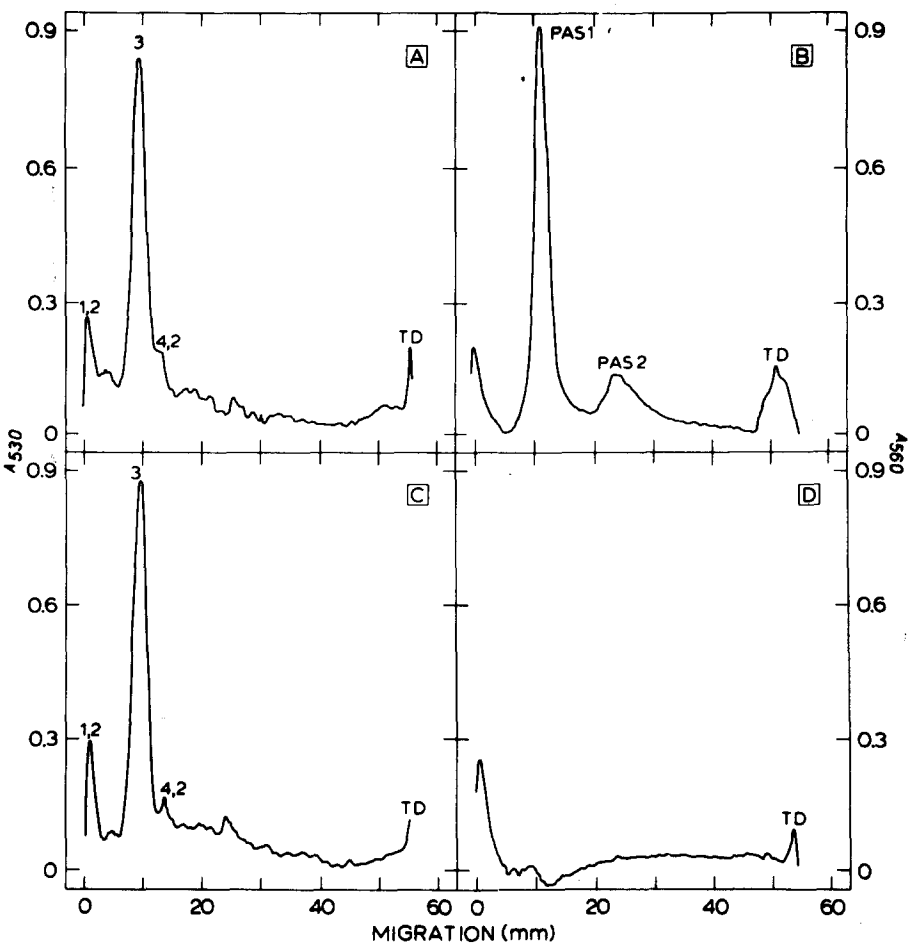

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of a crude Triton X-100 extract of ghosts (A,B) and the protein fraction eluted with cysteine from organomercurial gel (C,D). The gels A and C were loaded with 10—15 μg of protein and stained with Coomassie brilliant blue. The gels B and D were loaded with 40 μg of protein and stained with periodic acid Schiff reagent.

cysteine-eluted fraction. Secondly, after exposure to pH 3.5, new bands of low molecular weight appeared on the gels, presumably due to proteolytic degradation [34]. However, in a Tris-saline buffer, pH 7.0 (10 mM Tris-acetic acid (pH 7.0), 150 mM NaCl, 0.2 mM EDTA, 0.5% Triton X-100), there was no binding of the sialoglycoproteins to the gel, and in the protein fraction subsequently eluted with 100 mM cysteine virtually no sialic acid could be detected. Periodic acid Schiff reagent (PAS)-stained gels of the cysteine-eluted proteins showed the complete absence of PAS 1 and PAS 2 (Fig. 1D), whereas there was no change in the Coomassie blue pattern (Fig. 1C). Most of the phospholipids were also removed this way: the cysteine-eluted fraction contained only 0.05—0.07 μmol P_i/mg protein. Elution with 100 mM cysteine resulted in a broad, asymmetrical peak in which 55—65% of the total protein was recovered. Attempts to separate the individual components of this mixture by elution with a cysteine gradient were unsuccessful. For reconstitution

experiments, peak fractions were pooled to give a final protein concentration of approx. 0.3 mg/ml.

It has been shown previously [16] that band 3 can be incorporated into phosphatidylcholine vesicles by SM-2 bead treatment of a mixture of lipid and crude Triton X-100 extract. After sedimentation at 150 000 × *g* these vesicles have a rather uniform size, with diameters ranging from 500 to 1500 Å, and 80-Å particles on the fracture faces [16]. When a band 3 fraction, prepared by organomercurial gel chromatography, was used instead of a crude Triton X-100 extract, the vesicles obtained by high-speed centrifugation were more heterogeneous in size, most of them being either smaller than 500 Å or larger than 2000 Å (Fig. 2A). The large structures have smooth fracture faces, whereas many of the small vesicles (250—500 Å) show one or two 80 Å particles on the fracture faces. The large structures could be removed by centrifugation at 25 000 × *g* prior to high-speed centrifugation. In this way a rather homogeneous fraction of small vesicles was obtained,

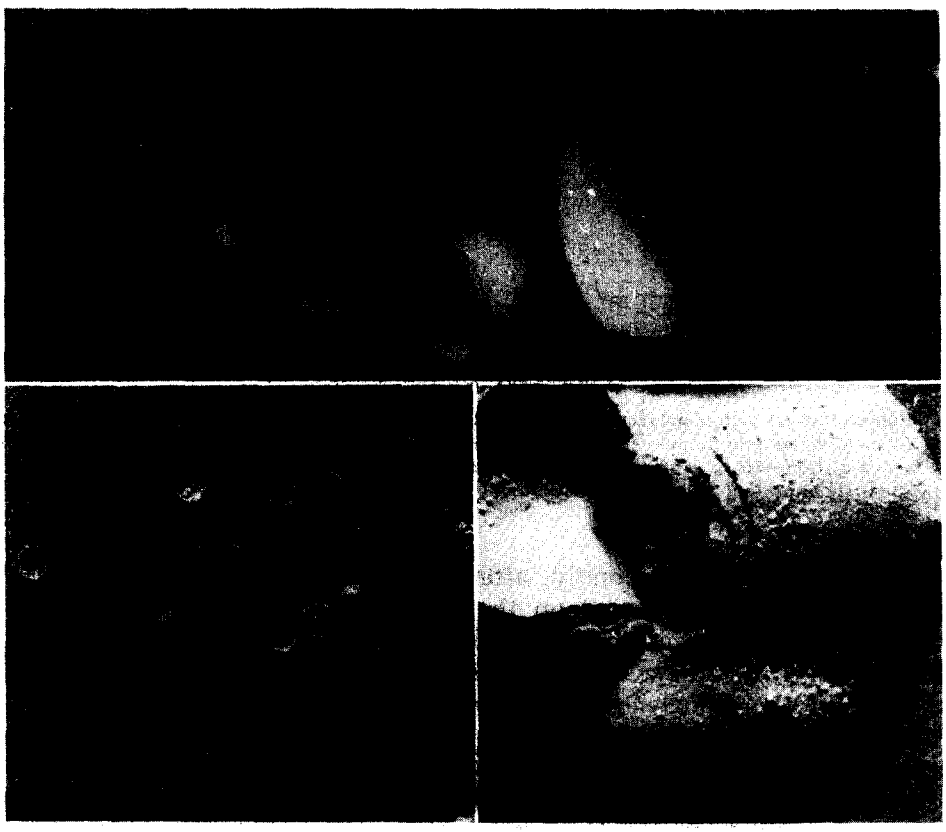

Fig. 2. Freeze-fracture electron micrographs of 18 : 1_c/18 : 1_c-phosphatidylcholine vesicles, prepared with the band 3-containing protein fraction, obtained by organomercurial gel chromatography (5 μmol lipid and 0.3 mg protein per ml 0.5% Triton X-100, 10 mM Tris-acetic acid (pH 7.0), 150 mM NaCl, 0.2 mM EDTA). After SM-2 bead treatment the vesicles were sedimented at 150 000 × *g* for 90 min (A) or fractionated by centrifugation at 25 000 × *g* for 30 min, followed by sedimentation at 150 000 × *g* for 90 min (B) and fused by repeated freeze-thawing (C). The lipid/protein weight ratio in samples B and C is 7 : 1. Magnification, × 100 000.

containing 30—40% of the total lipid and 60—70% of the total protein (Fig. 2B). On repeated freeze-thawing, these vesicles fused and formed large structures which show many 80 Å particles on the fracture faces (Fig. 2C).

Reconstitutions by the Triton X-100/SM-2 bead procedure were also carried out with glycophorin, the other major component present in a crude Triton X-100 extract. Purified glycophorin [21] was dissolved in 0.5% Triton X-100 containing buffer up to a concentration of 1 mg dry material per ml (17 μM), and this was mixed with 18 : 1_c/18 : 1_c-phosphatidylcholine in a concentration of 5 mM. After incubation with SM-2 beads [16], the material was sedimented at 150 000 × g for 90 min. Freeze-fracture electron microscopy showed the presence of small vesicles (diameters of 250—400 Å) and large structures (diameters more than 2000 Å) (Fig. 3A). The large structures have smooth fracture faces. Centrifugation at 25 000 × g for 30 min sedimented 60—70% of the lipid, but virtually no sialic acid. When the supernatant was centrifuged at 150 000 × g nearly all lipid and sialic acid were sedimented, but no firm pellet was formed. Extended fracture faces were obtained by concentrating at 25 000 × g supernatant by vacuum dialysis and fusing the vesicles by repeated freeze-thawing. On these fracture faces, small ill-defined particles are observed (Fig. 3B) similar to those observed on vesicles reconstituted with glycophorin by other procedures [35—37].

To study the effect of intrinsic erythrocyte membrane proteins on the transbilayer movement of phosphatidylcholine, we used vesicles prepared with purified glycophorin and with the band 3-containing fraction, obtained by organomercurial gel chromatography, and also protein-free vesicles prepared by the same procedure. The vesicle preparations were repeatedly centrifuged at 25 000 × g to remove large structures, but not sedimented by high-speed centrifugation as this could induce the formation of multilamellar structures

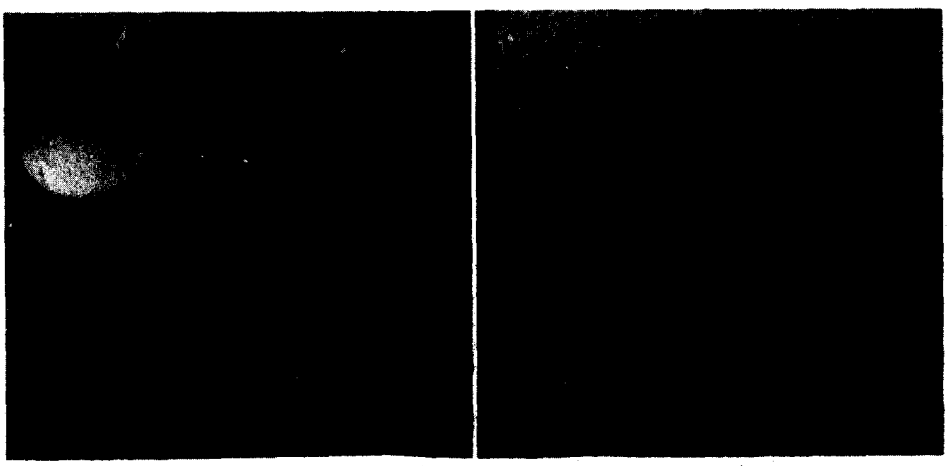

Fig. 3. Freeze-fracture electron micrographs of 18 : 1_c/18 : 1_c-phosphatidylcholine vesicles prepared with purified glycophorin (5 μmol lipid and 1 mg glycophorin per ml 0.5% Triton X-100, 10 mM Tris-acetic acid (pH 7.0), 150 mM KCl). After SM-2 bead treatment, the vesicles were sedimented at 150 000 × g for 90 min (A) or fractionated by centrifugation at 25 000 × g for 30 min, concentrated by vacuum dialysis and fused by repeated freeze-thawing (B). The lipid/protein molar ratio in sample (B) is 90 : 1. Magnification, × 100 000.

[16]. Vesicles were characterized by binding studies to DEAE-cellulose, K^+ trap measurements and ^{31}P-NMR studies (Table I). The vesicles prepared with protein were retained to a large extent (83—93%) by the ion exchange resin, whereas protein-free vesicles showed little binding (14%). This indicates that in the protein-containing vesicle preparations, most of the phospholipids are indeed present in protein-containing structures. For the internal volumes, determined from the amounts of trapped K^+, values of 0.6—0.7 μl/μmol P_i were found (Table I), in the same range as the 0.8 ± 0.3 μl/μmol P_i reported for sonicated vesicles of egg phosphatidylcholine [38]. The K^+ leak from protein-free vesicles was 11% per h, and the same value was found for vesicles prepared with glycophorin. Vesicles prepared with the band 3 fraction from an organomercurial gel showed a K^+ leak of 31% per h. From the internal volume of a unilamellar vesicle its size can be estimated. Outer radii calculated

TABLE I

VESICLES OF 18 : 1_c/18 : 1_c-PHOSPHATIDYLCHOLINE (DOPC) PREPARED BY THE TRITON X-100/SM-2 BEAD PROCEDURE, WITH OR WITHOUT INTRINSIC PROTEINS OF THE HUMAN ERYTHROCYTE MEMBRANE, AND FRACTIONATED BY REPEATED CENTRIFUGATION AT 25 000 × *g*

Parameter	Vesicles		
	Without protein	With glycophorin (0.2 mg protein/ml)	With band 3 [a] (0.3 mg protein/ml)
Lipid protein ratio (w/v)	—	5.3 : 1 [b]	5.2 : 1 [c]
Binding to DEAE-cellulose (% P_i)	14	83	90
K^+ trap			
internal volume (μl/μmol P_i)	0.6	0.7	0.7
leak (% in 1 hr)	11	11	31
^{31}P-NMR			
linewidth $\Delta\sqrt{}$(Hz)	9	12	22
Nd^{3+}-unshifted resonance (%)	31	29	<10
Vesicle outer radius (Å)			
determined from K^+ trap	150	160	155
determined from ^{31}P-NMR linewidth	134	151 [d]	190 [e]
Inner monolayer DOPC (%)			
determined from K^+ trap	35	36	36
determined from ^{31}P-NMR linewidth	33	35	38
determined from Nd^{3+}-unshifted resonance	31	29	— [f]

[a] The band 3 preparation was obtained by organomercurial gel chromatography; it contained about 60% band 3 protein, as determined from densitometric tracings of Coomassie blue-stained gels, but no glycophorin.

[b] The molar ratio of glycophorin and phosphatidylcholine is about 1 : 100, using a molecular weight for the protein part of glycophorin of 14 000 [58].

[c] The molar ratio of band 3 and phosphatidylcholine is about 1 : 1100, using a molecular weight for band 3 of 90 000 [59] and a band 3 content of 60% of the total protein (see footnote a).

[d] Assuming that all glycophorin is incorporated into vesicle bilayers, from the molar ratio 1 : 100 (see footnote b) and a vesicle outer radius of 150 Å, it is calculated that each vesicle contains an average of about 60 glycophorin molecules.

[e] Assuming that all band 3 is incorporated into vesicle bilayers, from the molar ratio 1 : 1100 (see footnote c) and a vesicle outer radius of 190 Å, it is calculated that each vesicle contains an average of eight band 3 molecules.

[f] It is not possible to differentiate between inner and outer surfaces as the vesicles are permeable to Nd^{3+}.

from the K^+ trap measurements have been indicated in Table I. Also, from the measured ^{31}P-NMR linewidths (Table I), vesicle sizes can be calculated (Table I). The vesicle sizes calculated in the two ways correlate fairly well with, as the only exception, the relatively low figure from the K^+ trap measurements for vesicles prepared with band 3 obtained from organomercurial gel chromatography. This, however, may be caused by the relatively high permeability of these vesicles to K^+. The presence of Nd^{3+} causes a downfield shift of the ^{31}P-NMR resonances from the phospholipids accessible to the paramagnetic ions [26,39]. For the various vesicles the percentage of the total signal intensity which was not shifted by Nd^{3+} is shown in Table I. If there is no inward leak of Nd^{3+} during the data accumulation (15 min), this percentage indicates the fraction of phospholipid molecules in the inner monolayer. From the values in Table I it is obvious, however, that the band 3-containing vesicles are permeable to Nd^{3+}, as more than 90% of the signal was shifted. With the protein-free vesicles, 31% of the signal was not shifted with Nd^{3+} after 15 min, and this value remained constant upon further incubation up to 30 min. As shown in Table I, the value of 31% for the inner monolayer pool of phosphatidylcholine agrees fairly well with values calculated from the amount of trapped K^+ (35%) and the ^{31}P-NMR linewidth (33%). For the vesicles prepared with glycophorin, the Nd^{3+}-unshifted ^{31}P-NMR resonance would indicate a value of 29% for the inner monolayer pool of phosphatidylcholine, whereas values of 36 and 35% were calculated from K^+ trap and ^{31}P-NMR linewidth measurements, respectively. This discrepancy might be caused by a small fraction of the vesicles being permeable to Nd^{3+}. For the band 3-containing vesicles, K^+ trap and ^{31}P-NMR linewidth measurements suggest a value of about 38% for the inner monolayer pool of phosphatidylcholine.

The exchangeability of phosphatidylcholine in the reconstituted vesicles was studied with phosphatidylcholine exchange protein from bovine liver. The results obtained with the sonicated vesicles (Fig. 4A) are very similar to those described before [13], despite the experimental differences. They confirm that in sonicated vesicles glycophorin enhances the transbilayer movement of phosphatidylcholine. In the protein-free vesicles prepared from a Triton X-100 buffer, a non-exchangeable pool of 31—34% phosphatidylcholine was detected (Fig. 4B). As this agrees very well with the values obtained for the inner monolayer pool of phosphatidylcholine (Table I), it can be concluded that these vesicles do not show any rapid transbilayer movement of phosphatidylcholine. The vesicles prepared with glycophorin from a Triton X-100-containing buffer showed a clearly increased exchangeability of phosphatidylcholine: after the incubations approx. 83% of the phosphatidylcholine had been transferred (Fig. 4B). These vesicles have a good permeability barrier for ions like K^+ and Nd^{3+}, and at least 29% of the phosphatidylcholine is present in the inner monolayer (Table I). This indicates that, like in the sonicated vesicles, the transbilayer movement of phosphatidylcholine is enhanced by the incorporation of glycophorin. In the vesicles prepared with the band 3-containing protein fraction, obtained by organomercurial gel chromatography, about 90% of the phosphatidylcholine is available for exchange (Fig. 4B). According to their size, 36—38% of the phosphatidylcholine of the vesicles is present in the inner monolayer, and although the permeability is enhanced, there is still a barrier

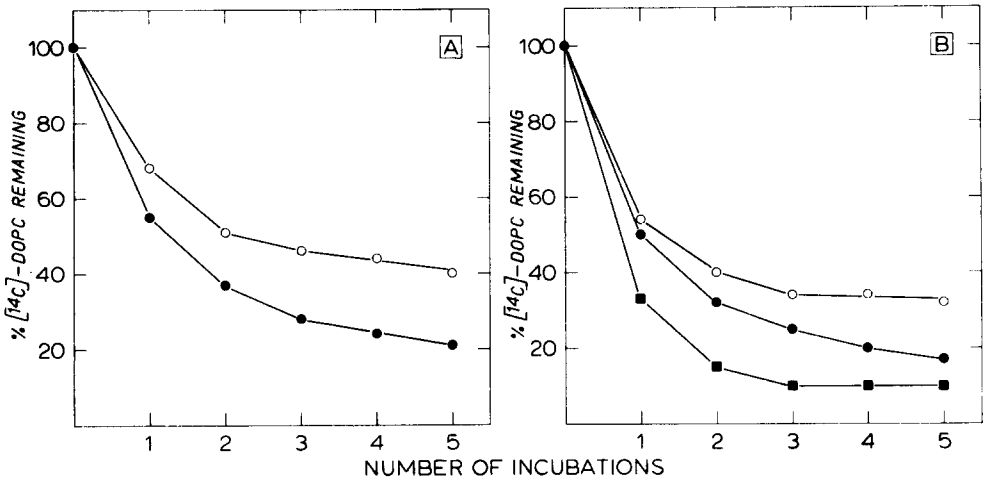

Fig. 4. Percentage of 18 : 1_c/18 : 1_c-[^{14}C]phosphatidylcholine (DOPC) exchangeable with phosphatidylcholine exchange protein from bovine liver in (A) sonicated vesicles and (B) vesicles prepared by the Triton X-100/SM-2 bead procedure, without protein (○), with glycophorin (●) or with a partially purified band 3 fraction (■). The incubations were carried out at 37°C for 45 min as outlined in Materials and Methods. The initial amounts of 18 : 1_c/18 : 1_c-phosphatidylcholine in the donor vesicles were as follows: protein-free sonicated vesicles 0.75 μmol, glycophorin-containing sonicated vesicles 0.45 μmol, protein-free SM-2 bead vesicles 0.32 μmol, glycophorin-containing SM-2 bead vesicles 0.30 μmol and band 3-containing SM-2 bead vesicles 0.23 μmol. The initial amount of 18 : 1_c/18 : 1_c-phosphatidylcholine in acceptor vesicles was 5.33 μmol in the case of the incubations with protein-free vesicles and 5.75 μmol in the incubations with protein-containing vesicles. The initial amount of exchange protein was 30 μg.

for K^+ and it seems unlikely that the exchange protein would again access to the inside of the vesicles. This indicates that also a protein other than glycophorin, and presumably band 3, enhances the transbilayer movement of phosphatidylcholine. The non-exchangeable pool of 10% might reflect a fraction of vesicles which contains no protein.

Discussion

Methods described for isolation and purification of band 3 include selective extraction with Triton X-100 [15,18,40], DEAE-cellulose chromatography [41] and concanavalin A affinity chromatography [42—44]. These methods, however, either do not completely separate the sialoglycoproteins (glycophorin) from band 3 [18,43] or give low yields [41]. A separation between these major proteins in a Triton X-100 extract can be achieved by sulfhydryl affinity chromatography [17,45], as the sialoglycoproteins are free of sulfhydryl groups [45]. In this study we show that an organomercurial gel (Affigel 50, Bio-Rad) can give a fast and complete separation of the sialoglycoproteins from band 3, even without the pH 3.5 treatment described by Shami et al. [17]. Simultaneously, about 95% of the phospholipids of the Triton X-100 extract are removed. The band 3-containing protein fraction obtained in this way still contains contaminants (band 4.2, band 4.5 and traces of bands 1 and 2) and can be further purified by ion exchange chromatography [41], However, to study protein-mediated transbilayer movement of phos-

phatidylcholine in reconstituted vesicles, at this state the removal of glycophorin and most of the phospholipids was considered to be sufficient.

Vesicles reconstituted with a crude Triton X-100 extract from ghosts or (partially) purified band 3 preparations have been used for electron microscopy [18,44,46] and transport studies [43,47—50]. Reconstitution of band 3 results in the appearance of 80-Å intramembrane particles, observed by freeze-fracture electron microscopy [18,44,46]. Reconstitution of the band 3-containing protein fraction, obtained by organomercurial gel chromatography, with $18:1_c/18:1_c$-phosphatidylcholine by SM-2 bead treatment [16,18], produces small vesicles (diameters less than 500 Å) showing the intramembrane particles. Large structures containing little or no protein are also formed, and these can be removed by centrifugation. Also reconstitution of purified glycophorin by the Triton X-100/SM-2 bead procedure results in the formation of small and large structures, with glycophorin preferentially associated with the small vesicles. This heterogeneity in size and protein content was not recognized at first, and it was assumed that glycophorin, reconstituted from Triton X-100, did not form intramembrane particles [37]. However, after isolation of the small vesicles, followed by fusion, the fracture faces show intramembrane particles similar to those observed in vesicles reconstituted with glycophorin by other methods [36,37].

Small vesicles, containing either band 3 or glycophorin, were obtained by SM-2 bead treatment, followed by repeated low-speed centrifugation, to remove large structures which contain little or no protein. Also, small protein-free vesicles were obtained in this way. The protein-free and glycophorin-containing vesicles have a size comparable to that of sonicated vesicles (about 150 Å outer radius), are impermeable to Nd^{3+} and not leaky to K^+. The band 3-containing vesicles are somewhat larger (about 200 Å outer radius), permeable to Nd^{3+} and more leaky to K^+. After concentration by vacuum dialysis, which takes several hours to complete, the size of these vesicles is still comparable to that of the protein-free vesicles. This indicates that they are stable structures, not undergoing fusion to a significant extent, and therefore the observed ion leakage should really be due to increased transmembrane permeation and not to disruption of the vesicles. Increased membrane permeability, not related to transport activity of a protein, has been observed in a number of reconstituted systems containing band 3 [43,48] or other membrane proteins [16,51, 52]. In two cases the permeability properties were shown to depend strongly on the lipid composition [16,43]. The enhanced permeability may be caused by irregularities in the packing of lipid molecules around the protein [16,53] and this packing may be influenced by the shape of the lipid molecules [16].

The transbilayer movement of phosphatidylcholine in the reconstituted vesicles has been investigated by measuring the pool of phosphatidylcholine exchangeable with phosphatidylcholine exchange protein from bovine liver. The observation that glycophorin induces flip-flop of phosphatidylcholine in sonicated vesicles [13] has been confirmed. The glycophorin used in this study had been purified without the use of lithium diiodosalicylate [21,54] and the vesicles had not been treated with neuraminidase before the exchange assay. These changes did not substantially affect the result. In protein-free vesicles, prepared by the Triton X-100/SM-2 bead procedure, no transbilayer

movement of phosphatidylcholine has been detected. Incorporation of glycophorin into these vesicles induces flip-flop of phosphatidylcholine, like in the sonicated vesicles. Also, with the band 3-containing vesicles the exchangeability of phosphatidylcholine strongly suggests a rapid transbilayer movement. This indicates that the protein-mediated translocation of phosphatidylcholine is not a glycophorin-specific process. More generally, it may be a consequence of the incorporation of an intrinsic protein in the lipid bilayer caused by discontinuities in the packing of lipid molecules around the protein [13,23,55]. Yet, in cytochrome *c* oxidase-containing vesicles no enhanced transbilayer movement of phosphatidylcholine has been observed [14]. However, with phosphatidylcholine as the only lipid no high respiratory control cytochrome *c* oxidase vesicles can be obtained [56]. The vesicles used for the phosphatidylcholine exchange experiments [14] had high respiratory control ratios, and so were impermeable to H^+, due to the choice of the lipid mixture which contained, apart from phosphatidylcholine, also phosphatidylinositol (cholate dialysis vesicles) and phosphatidylethanolamine (direct incorporation vesicles) [56,57]. Obviously, these lipid mixtures provide a good packing of lipids around the protein, and therefore the absence of flip-flop in these vesicles is fully consistent with the proposed mechanism for protein-mediated transbilayer movement of lipids. The effect of the lipid composition on flip-flop in protein-containing vesicles will be a topic of future studies.

Acknowledgements

The investigations were carried out under the auspices of the Netherlands Foundation for Chemical Research (SON) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO). We would like to thank Dr. A.T.M. van der Steen for the gift of the glycophorin, Mr. J. Westerman and Dr. K.W.A. Wirtz for the gift of the exchange protein, Dr. A.J. Verkleij and Mrs. J. Leunissen-Bijvelt for performing the freeze-fracture experiments, Mr. H. Munzert for printing the photographs and Dr. C.J.A. van Echteld for carrying out the ^{31}P-NMR measurements.

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