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GLYCOPHORIN-ENRICHED VESICLES OBTAINED BY A SELECTIVE EXTRACTION OF HUMAN ERYTHROCYTE MEMBRANES WITH A NON-IONIC DETERGENT

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Summary

A method is described for isolating glycophorin-enriched vesicles from human erythrocytes by extracting membranes that were incubated for 30 min at 37°C at pH 4.5 and washed at low and high ionic strength with the non-ionic detergent Triton X-100. The extracts were 11.8 ± 2.4 fold enriched in glycophorin and contained 325 ± 69 μg sialic acid/mg protein, which represented 61 ± 16% of the total sialic acid.

Upon removal of Triton X-100 one third of the total glycophorin forms glycophorin-enriched vesicles with coextracted, endogenous lipids as shown by sedimentation, dextran-density gradient centrifugation, and electron microscopy. Addition of exogenous lipids increased the fraction of glycophorin-enriched vesicles up to 87%.

The incorporation of glycophorin in the membrane was shown by hemagglutination inhibition assays using anti-M sera and by the accessibility of glycophorin to trypsin. Freeze-fractured vesicles did not reveal intramembranous particles.

The selectivity of the extraction procedure is not simply due to chemical constraints introduced by disulfide cross-linkage of protein component 3, because only 20% of this protein undergo disulfide cross-linking. The selective extraction of glycophorin implies that glycophorin is segregated from protein component 3 and thus from intramembranous particles when erythrocyte membranes have been incubated at pH 4.5. This segregation may precede aggregation of intramembranous particles.

Abbreviations: PMSF, phenylmethanesulfonyl fluoride; MalNEt, *N*-ethyl maleimide; SDS, sodium dodecyl sulfate; Me_2SO, dimethyl sulfoxide.

Introduction

The major sialoglycoprotein from human erythrocyte membranes, glycophorin A, spans the lipid bilayer (for review, see Ref. 1) and is known to carry M/N antigens [2,3] lectin and virus receptor sites [4—6]. Several investigators addressed the question whether this protein might reveal transmembrane functions in reconstituted systems. The hydrophobic segment of glycophorin was reported to induce a permeability change for potassium and water [7]. Recently, it was shown that intact glycophorin increases the conductance of black lipid films [8]. Glycophorin has further been suggested to facilitate a transmembrane flux of lysophosphatidylcholine [9]. Both the permeability change as well as the facilitated transmembrane flux of a lipid occur in liposomes containing glycophorin and show intramembranous particles in freeze-fractured preparations [10—13]. Thus, it is possible that these transmembrane changes were due to aggregates of glycophorin; they may not be detectable in reconstituted systems containing monomeric forms of glycophorin and hence most likely no intramembranous particles.

Furthermore, intramembranous particles seen in plasmatic fracture faces of erythrocyte membranes do not depend on the presence of glycophorin, because erythrocytes of the EN (a-) genotype that lack glycophorin [14,15] reveal the same number and morphology of intramembranous particles [16]. Thus, it seems unlikely that glycophorin, when present in membranes, forms intramembranous particles by itself unless it is aggregated. It appears quite probable that the procedures used to extract glycophorin [17—19] stripped off some structurally important neighboring lipids, although glycophorin obtained by lithium-diiodosalicylate retains tightly bound lipids [20,21].

By using non-ionic detergents one might preserve the supramolecular structure of a glycophorin domain, yet no reconstitutions have been performed with such preparations [22,23].

Erythrocyte membranes incubated at low pH contain a considerable fraction of spectrin and protein component 3 in disulfide-bonded polymers [24]. Thus Triton X-100, known to extract integral membrane proteins [25], is expected to become selective for those integral membrane proteins that are not polymerized with peripheral proteins and those that do not contain SH groups such as glycophorin [26]. Here, we describe a procedure to obtain such an extract enriched in glycophorin that forms glycophorin-enriched vesicles with endogenous lipids upon removal of Triton X-100. However, the selective extraction was not solely due to other membrane proteins undergoing cross-linking.

Materials and Methods

The blood was obtained from the Swiss Red Cross, Zürich, and had been collected in citrate/phosphate/dextrose. In all experiments erythrocytes (O, Rh^+) were used from donors that were homozygous for M. Rabbit anti-M sera were obtained from Ortho (Cilag, Schaffhausen). Phenylmethane sulfonyl fluoride (PMSF), dansylhydrazine, dithiothreitol, *N*-ethyl maleimide (MalNEt), trypsin inhibitor from soybean, and sialic acid were purchased from Sigma (St.

Louis, MO, U.S.A.). Bio Beads SM 2 is a product of Bio Rads (München, F.R.G.). Triton X-100 was obtained from Serva (Brunschweig, Basel). All other chemicals except for staining purposes were of analytical grade.

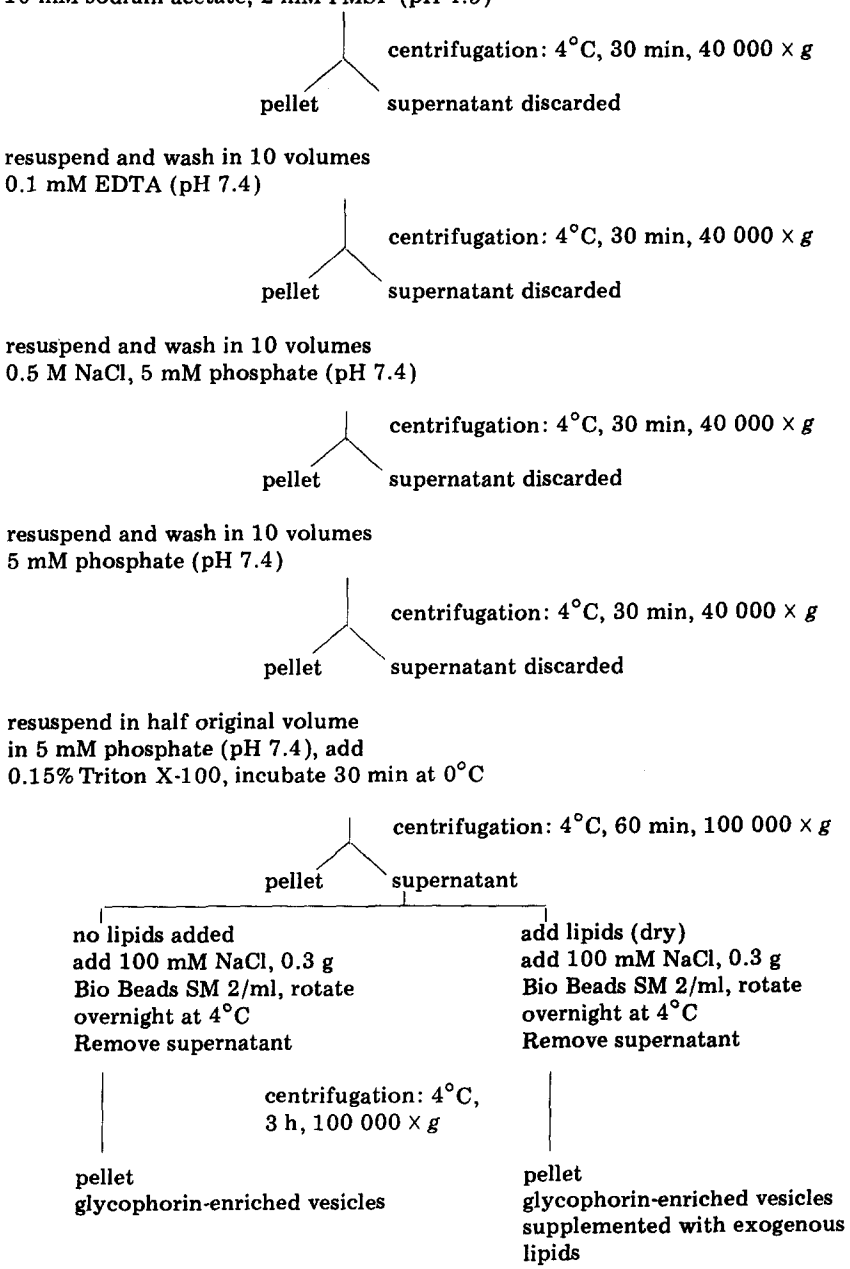
Isolation of erythrocyte membranes. One unit of fresh blood was spun the day it had been collected for 7 min at 4000 rev./min in a Sorvall SS 34 rotor. Plasma and buffy coat were removed and the cells resuspended in 150 mM NaCl, 5 mM $NaKHPO_4$ (pH 7.4) at 50% hematocrit. In order to minimize white cell and platelet contamination this suspension was passed through a cotton column (30—40 × 3 cm) that was equilibrated in the same buffer and kept at room temperature. From the eluate (kept in ice) 1.2—1.5 times the original volume was collected and spun for 10 min at 4000 rev./min. The pelleted cells were further washed 4 times with buffered saline. Following this procedure the packed cells were lysed at 0—4°C in 30 volumes 5 mM phosphate buffer (pH 7.4). The pelleted membranes were washed two more times in 30 volumes of 5 mM phosphate buffer yielding white membranes. The final supernatant was completely removed to get packed erythrocyte membranes.

Extraction of glycophorin. Packed erythrocyte membranes were incubated for 30 min at 37°C in sodium acetate (10 mM acetic acid adjusted to pH 4.5 with NaOH) containing 1—2 mM PMSF. PMSF was dissolved in absolute ethanol and added to the buffer at 37°C to give a final concentration of 1—2% ethanol. Following the addition of PMSF the pH was readjusted *. After incubation the suspended membranes were chilled, pelleted, and washed as outlined in Scheme 1. Prior addition of Triton X-100 the pellets were resuspended in exactly half the original volume and samples withdrawn for protein measurements and sodium dodecyl sulfate (SDS) gel electrophoresis.

If membranes pretreated at pH 4.5 were directly resuspended in 5 mM phosphate buffer (pH 7.4) and extracted with Triton X-100 (0.5 or 0.15%), only a small fraction of glycophorin could be extracted that contained yet a considerable amount of protein component 6 (see Table V for enrichment in glycophorin using 0.5% Triton). The membranes pretreated at pH 4.5 had to be washed at least once at pH 7.4 in a low ionic strength buffer to yield a high enrichment in glycophorin upon extraction with Triton (not shown). The first wash with 0.1 mM EDTA was necessary to reduce a small contamination by spectrin and actin when 0.5% Trition X-100 was used, and could be dropped when 0.15% was used. The high salt wash reduced a contamination by protein component 6 under both conditions. However, this wash had to be followed by a 5 mM phosphate or bicarbonate wash to keep the ionic strength low during the extraction with Triton X-100; otherwise a residual amount of protein component 6 was coextracted (not shown). In order to compare experiments with 0.15 and 0.5% Triton X-100 the complete washing procedure was used throughout this paper. The recommended protocol to extract glycophorin with 0.15% Triton X-100 requires only the high and low salt washing at pH 7.4.

In some experiments the resuspended membranes were stored overnight

* When PMSF was omitted no significant change in the specific content of sialic acid/mg protein was found in the glycophorin extract. However, the amount of protein component 3 was slightly higher than with PMSF and glycophorin was partially degraded, especially when erythrocytes were not well washed (data not shown).



Scheme 1. Isolation of glycophorin-enriched vesicles. Protocol.

following the complete washing procedure before adding Triton X-100 with no effect on the final result. Triton was then added and the suspensions incubated for 30 min at 0°C with occasional mixing. The tubes were centrifuged immediately after incubation (Scheme 1) and the clear supernatant was

removed carefully to avoid contamination from the turbid pellet overlying the densely packed residual membranes.

Formation of glycophorin-enriched vesicles. With coextracted, endogenous lipids only. The extracts were added to washed Bio Beads SM 2 [27] and were supplemented with NaCl up to 100 mM (above 30—100 mM composition and structure of glycophorin-enriched vesicles were the same). These suspensions were rotated overnight at 4° C yielding slightly turbid supernatants that contained 200—300 μg protein/ml.

With additional erythrocyte lipids. Human erythrocyte membranes were isolated as outlined before and lipids were extracted following the protocol of Broekhuyse [28]. The lipid extracts were kept in chloroform/methanol under argon at —20° C for up to 2 months. Although we added the whole lipid extract we only referenced its content of phosphorus. An appropriate sample of the extract was dried under nitrogen on the wall of a glass tube. The Triton X-100 extract was added to the tube and this was rotated at 4° C until all the material had dissolved. This mixture was then supplemented with NaCl and added to Bio Beads SM 2 and treated as the samples without additional lipids.

Characterization of glycophorin-enriched vesicles. Triton-free extracts were (i) layered on dextran-density gradients containing 15 mM $NaHCO_3$ (pH 7.4) as outlined in the text or (ii) were spun for 3 h at 100 000 × *g* to collect glycophorin-enriched vesicles in the pellet. The vesicles were resuspended in 5 mM phosphate, 100 mM NaCl (pH 7.4) to roughly the same protein concentration as found in the Triton-free extract.

For hemagglutination inhibition assays the titer of anti-M serum was twice that to agglutinate $5 \cdot 10^7$ cells/ml. From both Triton-free extract and glycophorin-enriched vesicles dilutions were made and 25 μl were incubated for 60 min at room temperature with 25 μl anti-M in phosphate-buffered saline. 25 μl from each mixture were added to 25 μl red cell suspension (O Rh^+M/M) containing $1 \cdot 10^8$ cells/ml. After 30 min at room temperature the agglutination titer was determined.

The orientation of glycophorin in glycophorin-enriched vesicles was analyzed by incubating 1 ml of vesicles (200—300 μg protein/ml) in 10 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$ and 100 μg trypsin/ml for 20 min at 37° C. The reaction was stopped by adding trypsin inhibitor (200 μg/ml). The chilled suspension was centrifuged for 3 h at 100 000 × *g* and the supernatant removed. Following digestion with sulfuric acid, sialic acid was determined in the supernatants. Controls contained no vesicles.

For negative staining the Triton-free extract was directly added to carbon-coated formvar grids and stained with 2% uranyl acetate. Samples processed for freeze-fracturing were suspended in 5 mM phosphate (pH 7.4) containing 35% glycerol and frozen in liquified Freon 22. The frozen samples were freeze-fractured according to standard techniques with a Balzers (Liechtenstein) apparatus. Pictures were taken with a Philips 300 electron microscope.

Chemical characterization. Protein was measured in triplicates from previously frozen samples by standard techniques according to Lowry. For sialic acid determinations 0.5—1.0 ml were digested for 1 h at 80° C in 0.1 N H_2SO_4 in screw cap tubes. Triplicates from these digests were analyzed according to Warren [29]. Phosphorus was measured following the protocol of Chen et al.

[30] from perchloric acid digests of glycophorin-enriched vesicles. In those experiments from which samples were withdrawn for lipid phosphorus determination the protocol was changed, instead of 5 mM phosphate 15 mM $NaHCO_3$ (pH 7.4) was used to wash and resuspend the erythrocyte membranes prior extraction with Triton X-100.

SDS-polyacrylamide gel electrophoresis. All samples withdrawn for electrophoresis contained 1% SDS and 5 mM MalNEt to prevent disulfide formation during freezing and thawing. Slab gels were run as previously described [31] having the following bisacrylamide and acrylamide concentrations: 2.7 and 8%, respectively. Except where indicated all samples were incubated for 30 min at 37°C in 40 mM dithiothreitol prior to electrophoresis. Gels were fixed in 50% methanol and 7—10% acetic acid. The solution was changed and then replaced by one containing only 20% methanol and 7% acetic acid.

Glycoproteins were stained according to Eckhardt et al. [32] with some modifications: 0.5% periodic acid was used instead of 0.7% in 5% acetic acid; dansylhydrazine reacted for 90 min at 60°C instead of 120 min and the gels were stored in acidic dimethyl sulfoxide (Me_2SO overnight rather than 0.1% acetic acid, because otherwise the fluorescence was drastically diminished. After having taken photographs under ultraviolet light from gels kept for roughly 1 h in 0.1% acetic acid to reduce the background, the gels were immersed in 7% acetic acid for the rest of the day. Then they were stained with Coomassie blue. Destained gels were photographed and dried in between cellophan on a gel drier from Pharmacia, Uppsala. Quantitation of both the photographic negative of gels stained for glycoproteins (to assess the amount of glycophorin in reduced and unreduced gels see Tables III and V) and dried Coomassie blue-stained gels was performed on a gel scanner Integraph CH from Bender and Hobein, Zurich.

Results

Selective extraction of glycophorin

When human erythrocyte membranes are extracted at pH 7.4 with 0.5% Triton X-100, the extract contains primarily glycoproteins including protein component 3 [25]. The same protein composition was found in Triton X-100 extracts of membranes incubated for 30 min at 37°C (Fig. 1) and washed as outlined in Scheme 1. However, when the pH of the incubation mixture was lowered to pH 4.5 the Triton X-100 extract contained a prominent protein component that stained weakly with Coomassie blue (Fig. 1, gel pH 4.5). The protein component selectively extracted was shown to be the major sialoglycoprotein (glycophorin) on SDS-polyacrylamide gels stained for glycoproteins (Fig. 2, gel 3). In the experiment shown in Fig. 2, 0.15% Triton X-100 rather than 0.5% was used yielding an even higher selectivity for glycophorin and there was no diffuse protein band between protein component 4.2 and 5. This diffuse band had to originate from protein component 3, because membranes from cells that were freed of platelets and leucocytes as outlined in Materials and Methods did not contain this diffuse protein band, but clearly some protein component 3 upon extraction with 0.5% Triton X-100 (see Fig. 3). Therefore the selectivity of the extraction procedure for glycophorin was analyzed

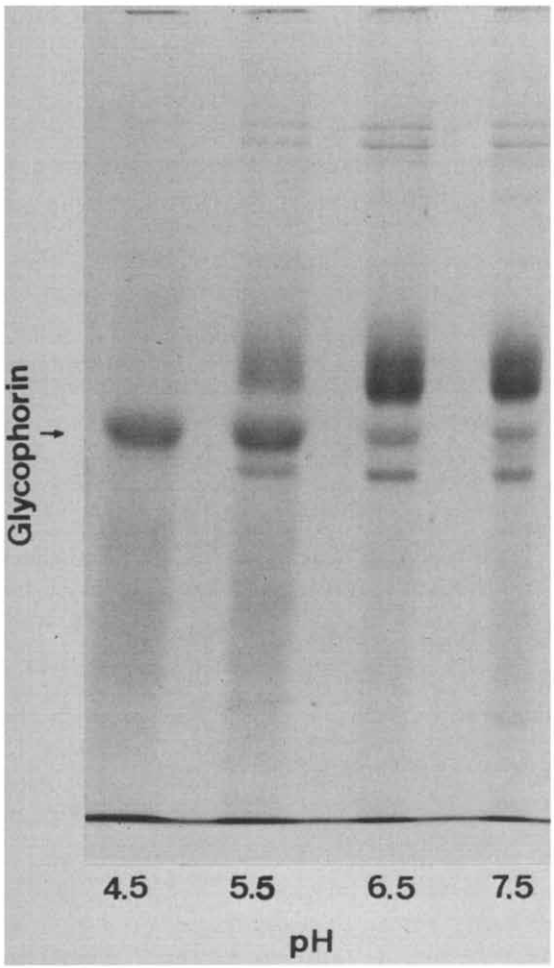


Fig. 1. Triton X-100 extracts from human erythrocyte membranes preincubated at different pH. Membranes were treated as outlined in Scheme 1 except that the pH of the incubation varied as given. All preparations were then washed according to Scheme 1 except that the high salt washing as well as the last washing step were repeated. The membranes were resuspended in one half of the original volume and 0.5% Triton X-100 was added. In this experiment erythrocytes were washed three times instead as given in Materials and Methods.

by measuring the amount of glycophorin and protein component 3 extracted at different Triton concentrations.

As the Triton X-100 concentration was raised, the yield of sialic acid in the extract increased and the specific content of sialic acid/mg protein decreased (Fig. 3). Although glycophorin was still preferentially extracted with 0.5% Triton X-100, protein component 3 comprised 55% of the total protein in the extract (determined from bound Coomassie blue). Thus a highly selective extraction of glycophorin is only achieved with 0.1—0.2% Triton X-100 or 1—2 mg Triton X-100/5—7 mg membrane protein. 11 experiments performed with 0.15% Triton X-100 resulted in an average glycophorin extract that was 11.8 ±

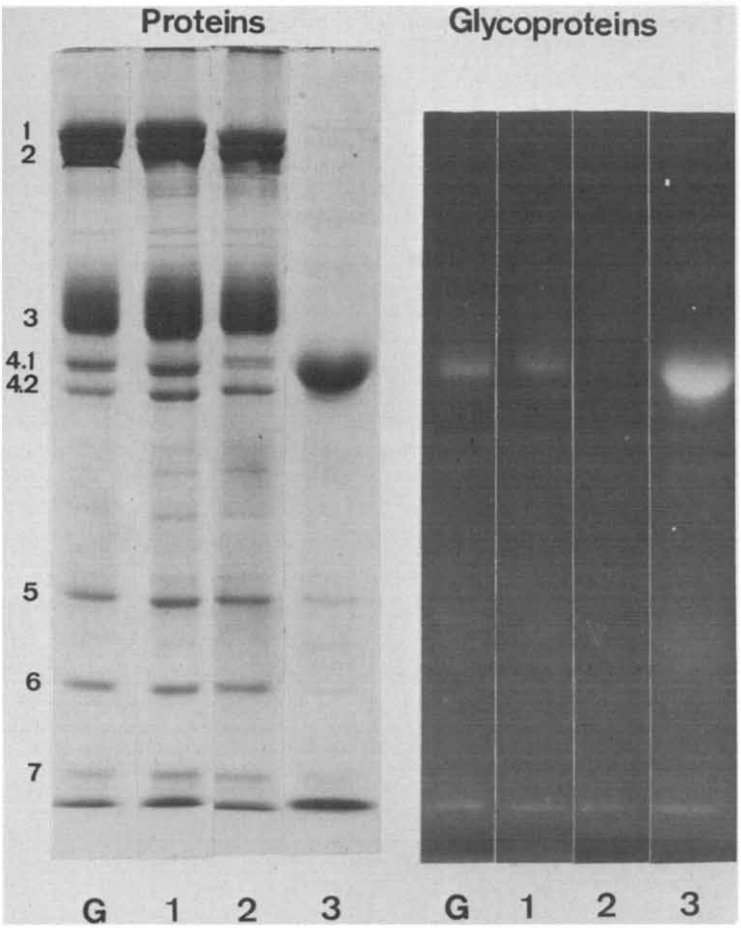


Fig. 2. Protein and glycoprotein composition of extracts obtained with 0.15% Triton X-100 upon incubation of membranes at pH 4.5 and the washing procedure outlined in Scheme 1. Each gel was run with 16.7 μg membrane protein. G, fresh membranes; 1, membranes incubated at pH 4.5 and washed as outlined in Fig. 1 prior addition of Triton X-100; 2, pellet obtained upon removal of the Triton extract treated also with Bio Beads SM 2; 3, Triton X-100 extract after treatment with Bio Beads SM 2. The actual amount of Triton X-100 added was 1.5 mg/5.65 mg protein.

2.4 fold enriched in glycophorin and contained 325 ± 69 μg sialic acid/mg protein representing 61 ± 16% of the total sialic acid in erythrocyte membranes (Table V).

Formation of glycophorin-enriched vesicles from Triton X-100 extracts

One goal of this study was to work out a procedure which should lead to a selective extraction of glycophorin along with endogenous lipids to form glycophorin-enriched vesicles upon removal of Triton X-100. Therefore Triton extracts treated with Bio Beads SM 2 were layered on dextran-density gradients to assess their vesicular nature. From a gradient loaded with an extract obtained with 0.1% Triton X-100 a slightly turbid band ranging from $\rho = 1.007$ to

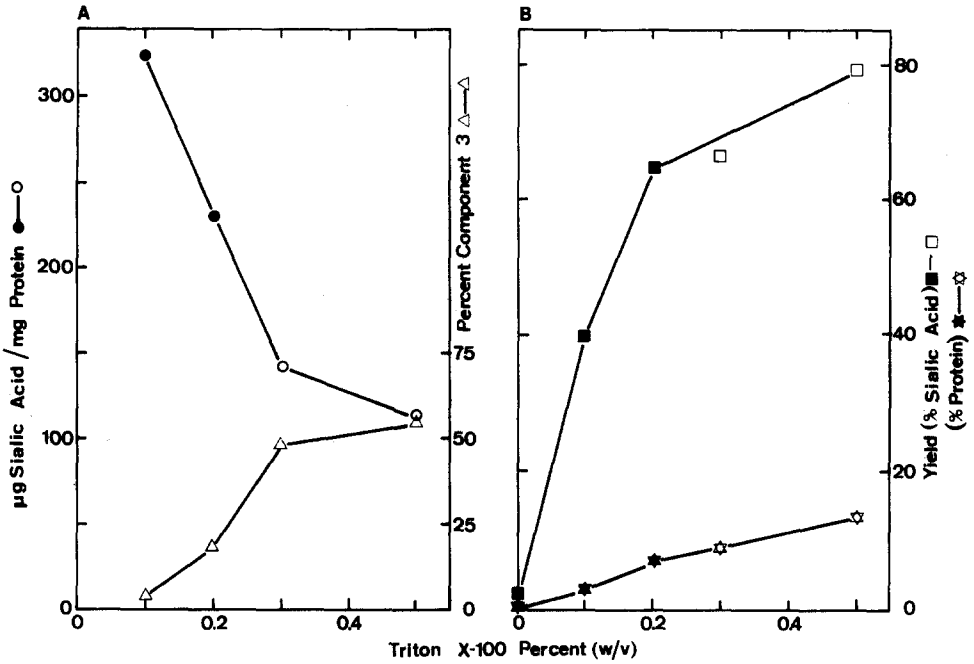


Fig. 3. Effect of Triton X-100 on the selective extraction of glycophorin from membranes incubated at pH 4.5 and washed as outlined in Scheme 1. Filled-in symbols represent averaged values obtained from two independent experiments in which the actual ratio of Triton X-100 to protein was 1 mg/5.3 and 5.6 mg membrane protein, respectively, for 0.1%. Open symbols represent values from one experiment in which 1 mg Triton X-100 was added to 5.6 mg protein for a 0.1% solution. Percent protein component 3 was determined from Coomassie blue-stained gels and is given in percent of total Coomassie blue. Thus the percentages listed are subject of a systematic error, because glycophorin and protein component 3 do not stain to the same extent.

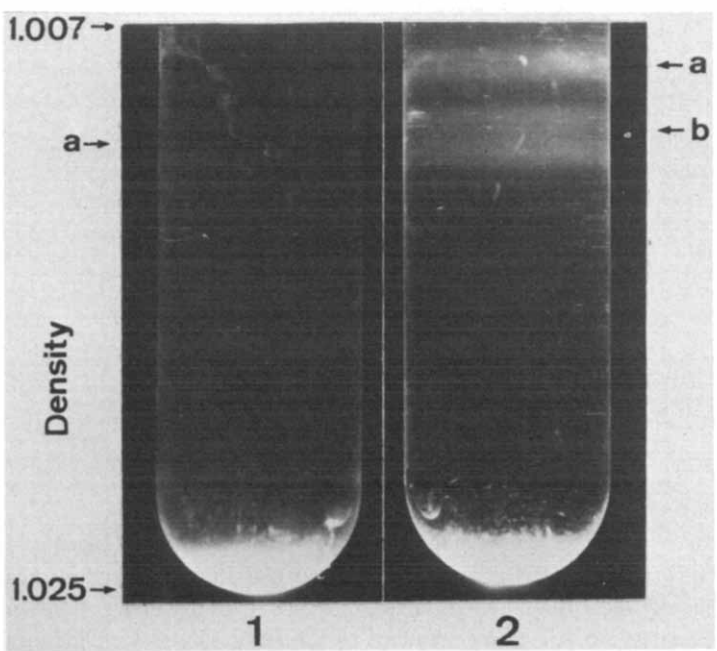


Fig. 4. Isolation of glycophorin-enriched vesicles from Triton X-100 extracts treated with Bio Beads SM 2 by centrifugation on dextran-density gradients. The gradients were loaded with 6 ml of extract and were centrifuged overnight at 100 000 × *g*. 1, extract obtained with 0.1% Triton X-100; 2, extract obtained with 0.2% Triton X-100.

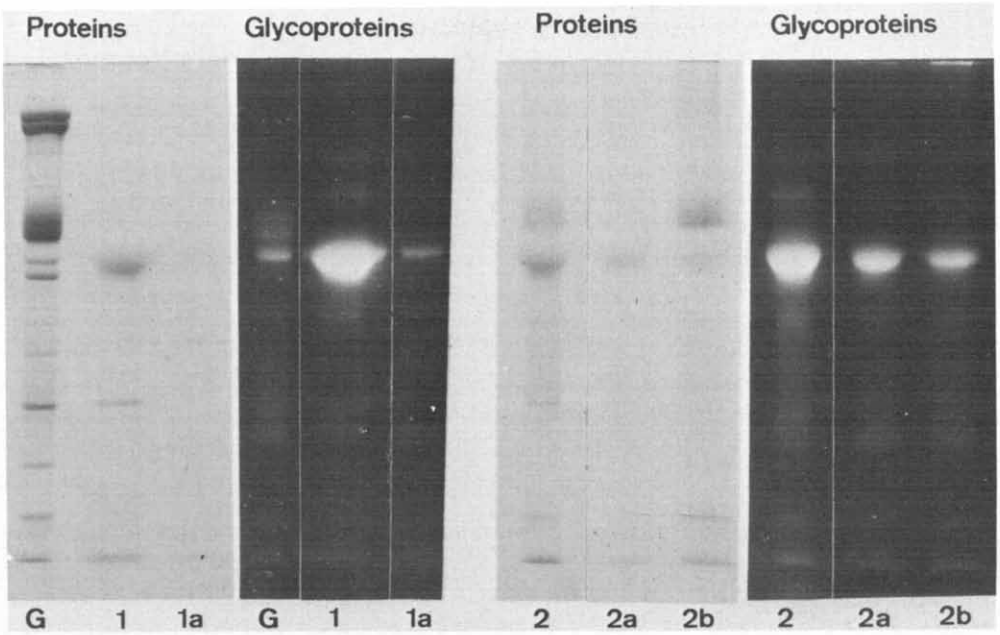


Fig. 5. Protein and glycoprotein composition of glycophorin-enriched vesicles recovered from dextran-density gradients. 1, glycophorin extract obtained with 0.1% Triton X-100, 8 μg protein added on gel; 1a, glycophorin-enriched vesicles recovered from band a of a gradient loaded with extract 1; 2, glycophorin extract obtained with 0.2% Triton X-100, 20 μg protein added on gel; 2a, material recovered from band a (see Fig. 5) of a gradient loaded with extract 2; 2b, material recovered from band b (see Fig. 4) of a gradient loaded with extract 2. G, 20 μg protein from fresh membranes.

ρ = 1.012 (Fig. 4, Expt. 1) could be isolated that had the same protein composition as the extract (Fig. 5, gel 1a), but only 13% of sialic acid was recovered in this fraction. Another gradient was loaded with an extract obtained with 0.2% Triton X-100 (Fig. 4, Expt. 2) containing glycophorin and protein component 3 (Fig. 5, gel 2). Following centrifugation two light-scattering bands were detectable (Fig. 4, Expt. 2). The upper band ranging from ρ = 1.007 to ρ = 1.010 contained almost exclusively glycophorin (Fig. 5, gel 2a), whereas the lower band (2b) was comprised of glycophorin and protein component 3 (Fig. 5, gel 2b). The recovery of sialic acid in both bands together (30%) indicates the formation of glycophorin-enriched vesicles from a third of the total glycophorin in the extract.

Furthermore, glycophorin extracts obtained with 0.15% Triton X-100 reveal 36% of the total sialic acid in pelleted vesicles when treated with Bio Beads SM 2 and spun for 3 h at 100 000 × g (Table I). The phospholipid content of these glycophorin-enriched vesicles was 20 μg phosphorus/mg protein and their sialic acid content was comparable to that determined in the extract. When M-antigens were quantified by hemagglutination inhibition assays in whole extracts and glycophorin-enriched vesicles, 50% were detected in vesicles (Table I). This number is higher than expected from total sialic acid content and may originate from an underestimation of the antigens that were not reconstituted in vesicles and thus tend to aggregate. In addition, the accessibil-

TABLE I

EFFECT OF ADDED ERYTHROCYTE LIPIDS ON THE FORMATION OF GLYCOPHORIN-ENRICHED VESICLES

Addition of lipid extract (μg phosphorus/ml extract)	Recoveries in vesicles in percent of total *			Sialic acid in vesicles (μg sialic acid/mg protein)	Phospholipid in vesicles (μg phosphorus/mg protein)	Number of experiments
	Protein	Sialic acid	M-Antigens			
0	35	36	50	384	20	2
0 **		57	75			2
2.5	63	62		329		3
10	82	87		351	55	2

* The data are listed in percent of total material added and the recoveries in both the pellet and the supernatant ranged from 79 to 98% for protein and sialic acid.

** Glycophorin-enriched vesicles were frozen before analysis.

ity of glycophorin to trypsin digestion in glycophorin-enriched vesicles was 67 ± 7% (Table II).

In conclusion, a third of the selectively extracted glycophorin molecules is associated with vesicles formed from coextracted lipids upon removal of Triton X-100. The fraction of glycophorin associated with lipids can further be increased by (i) freezing the extract after removal of Triton X-100 or (ii) by adding exogenous lipids from erythrocytes (Table I).

Structural aspects of glycophorin-enriched vesicles

Electron micrographs of negatively stained extracts after removal of Triton X-100 reveal vesicles with diameters from 25 to 200 nm (Fig. 6A). In addition to vesicles smaller particles and rod-like structures are seen. The smaller particles are absent in freeze-fractured pellets of these extracts (Figs. 6B—6D).

The electron micrographs of freeze-fractured pellets from glycophorin-enriched vesicles obtained from extracts with 0.15% Triton X-100 show plasmatic faces that are smooth (Figs. 6C and 6D). However, the plasmatic fracture face of vesicles from extracts obtained with 0.5% Triton X-100, which contain protein component 3 as shown in Fig. 3, occasionally reveal intramembranous particles (Fig. 6B). Thus vesicles of a 'pure' glycophorin extract are devoid of intramembranous particles as shown for glycophorin-enriched vesicles obtained from fresh membranes and spectrin depleted membranes (Figs. 6C and 6D). It was noted that glycophorin-enriched vesicles prepared by adding exogenous

TABLE II

ACCESSIBILITY OF GLYCOPHORIN IN GLYCOPHORIN-ENRICHED VESICLES

Experiment	Total sialic acid (μg/ml)	Trypsin-digestable sialic acid (μg/ml)	Percent sialic acid accessible	
1	42	27	64	(67 ± 7, S.D.)
2	60	45	75	
3	76	46	61	

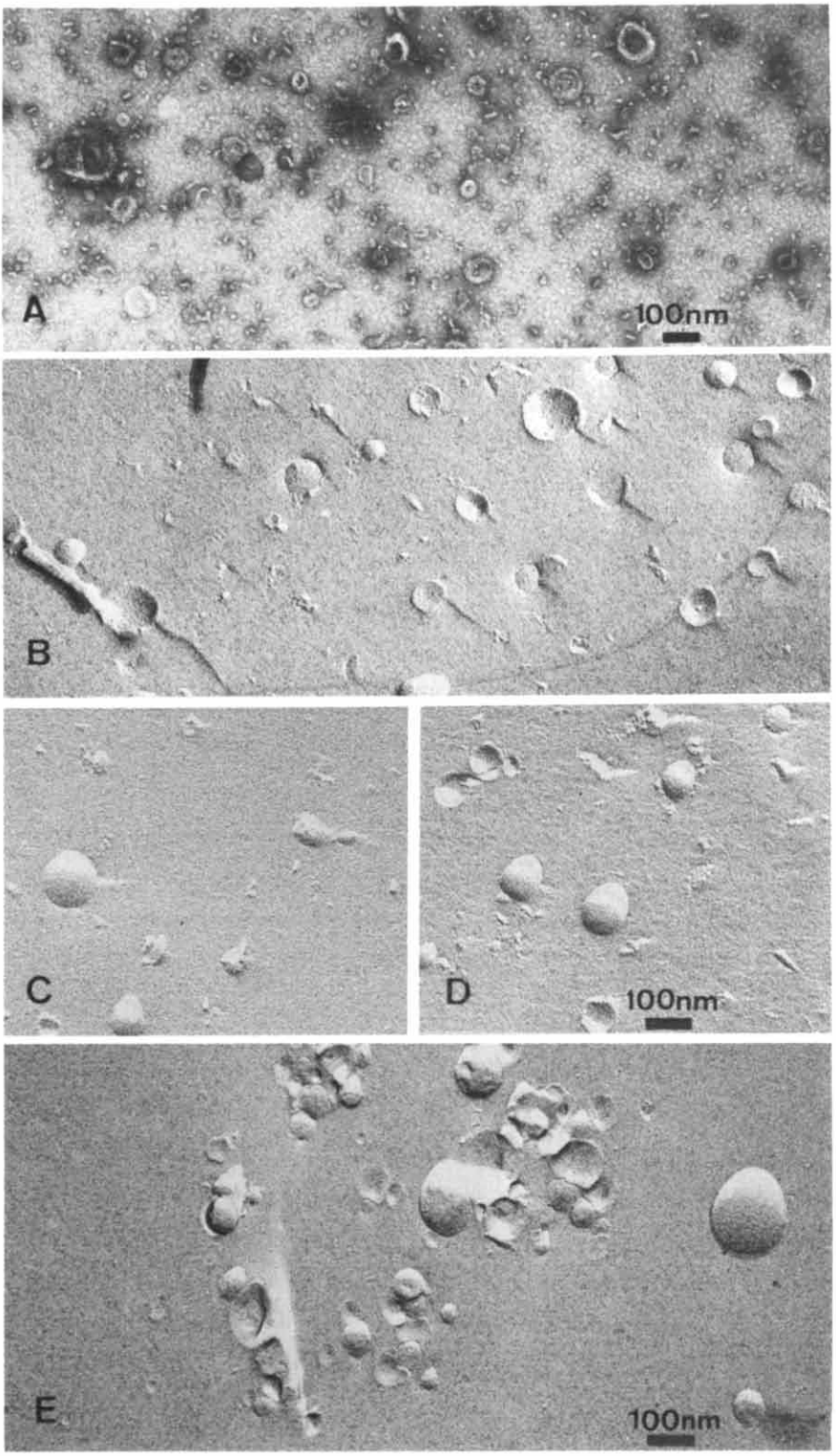


Fig. 6. Electron micrographs of glycophorin-enriched vesicles. (A) Glycophorin extract obtained with 0.15% Triton X-100 following treatment with Bio Beads SM 2, negative staining, ×50 000. (B—D) Freeze-fractured pellets of glycophorin-enriched vesicles obtained with endogenous lipids, ×80 000. B, preparation obtained with 0.5% Triton X-100. C, preparation obtained with 0.15%. D, preparation obtained with 0.15% from spectrin-depleted membranes. (E) Freeze-fractured pellets of glycophorin-enriched vesicles supplemented with exogenous lipids, ×70 000. The glycophorin-enriched vesicles had the properties listed for '10 μg phosphorus added' in Table I.

TABLE III

PERCENT OF PROTEIN COMPONENTS UNDERGOING DISULFIDE CROSS-LINKAGE

Equal amounts of total protein from fresh erythrocyte membranes or from membranes incubated at pH 4.5 and washed as outlined in Scheme 1 were incubated with or without dithiothreitol and run on the same slab gel. The gel was directly stained with Coomassie blue. Each band pattern was scanned and the absorbance at 560 nm determined for the listed protein components (numbered according to Fairbanks et al [35]). The difference in absorption between reduced and unreduced gel is given in percent of the absorbance of the protein component in the reduced gel. The data represent an average from two independent experiments. For glycophorin the same samples were run (from one experiment only) on an additional gel that was stained for glycoproteins. Instead of measuring fluorescence on the gel a photographic negative was scanned as given in Materials and Methods. GAP-DH 6, glyceraldehyde 3-phosphate dehydrogenase.

Protein component	Fresh membranes	Membranes incubated at pH 4.5 and washed as outlined in Scheme 1
Spectrin 1	7	58
Spectrin 2	0	54
Anion transport protein		
3	—4	20
4.1	—1	28
4.2	5	41
Actin 5	—4	23
GAP-DH 6	—6	23
Glycophorin	0	0

lipids and thus containing up to 87% of the total glycophorin (Table I) lack intramembranous particles as well (Fig. 6E).

Conditions leading to a selective extraction of glycophorin

As evident from Fig. 1 the selective extraction requires the erythrocyte membranes to be pretreated at low pH. These conditions induce aggregation of intramembranous particles [33] and disulfide-bonded polymers [24]. Membranes incubated at pH 4.5 and washed as outlined in Scheme 1 show a small fraction of their protein in SDS and dithiothreitol uncleavable protein aggregates on top of the gel (Fig. 2, gel 2). The amount of protein irreversibly aggregated was roughly 5% of the total protein *. In addition, these membranes contain over 50% of spectrin but only 20% of protein component 3 in disulfide-bonded polymers (Table III). Thus chemical cross-linking cannot fully explain the selective extraction of 61% of glycophorin and hardly any protein component 3 at 0.15% Triton X-100.

Furthermore, it is unlikely that aggregation of intramembranous particles prevents protein component 3 from being coextracted, because these particles are incompletely aggregated when fresh membranes are incubated at pH 4.5 (Fig. 7A). Depletion of spectrin is known to enhance aggregation of intramembranous particles upon treatment at low pH [34] (Fig. 7B) and yet when these membranes are extracted with Triton X-100 the enrichment of glycophorin in

* The amount of irreversibly aggregated protein was determined from SDS-polyacrylamide gels run with equal amounts of reduced protein from fresh membranes and membranes treated at pH 4.5. The Coomassie blue-stained and dried gels were scanned at 560 nm and the difference of the total absorbances in all protein bands (except the top of the gel) was given in percent of the total absorbance recorded for fresh membranes.

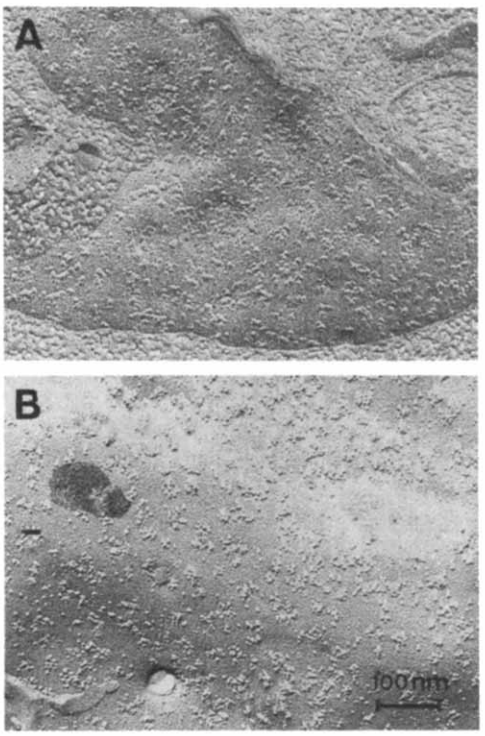


Fig. 7. Electron micrographs from freeze-fractured membranes incubated at pH 4.5 and washed as outlined in Shcme 1. (A) Fresh membranes incubated at pH 4.5. (B) Spectrin-depleted membranes incubated at pH 4.5. ×80 000.

the extract is comparable to that found for fresh membranes (Table IV). The specific content of sialic acid was even lower in extracts from spectrin-depleted membranes because of a 10—15% loss of sialic acid/mg protein during spectrin depletion.

To study whether glycophorin segregates from other integral membrane proteins upon incubation at pH 4.5 we searched for spontaneous release of glycophorin-enriched vesicles from these membranes. A spontaneous release of glycophorin-enriched vesicles requires a preceding segregation. When erythro-

TABLE IV

EFFECT OF SPECTRIN DEPLETION ON THE SELECTIVE EXTRACTION OF GLYCOPHORIN

Control and spectrin-depleted membranes were treated as shown in Scheme 1. Glycophorin was extracted with 0.15% Triton. Spectrin was extracted from erythrocyte membranes according to Ref. 35.

Pretreatment of erythrocyte membranes	Percent of protein in extract	Sialic acid content (μg sialic acid/ mg protein)	Enrichment of glycophorin	Content of protein component 3 in percent of total Coomassie blue-stained proteins
None	4.5	373	11.6	3.6
Spectrin depleted	8.6	331	13.2	3.7

cyte membranes were incubated at pH 4.5 and layered on dextran-density gradients without further washings, a negligible amount of protein was found at a low density of $\rho = 1.007$ with glycophorin not being enriched (Table V, Expt. a). When membranes pretreated at pH 4.5 were washed as given in Table V, Expt. c, they released spontaneously a small fraction of the total protein as glycophorin-enriched vesicles. These vesicles were 7 fold enriched in glycophorin and had a similar protein composition as those obtained by extraction with 0.15% Triton X-100 (Fig. 8). Although the amount of protein found in spontaneously released vesicles comprised only 1% of total glycophorin, the release occurred under the same conditions that favor the selective extraction of glycophorin by Triton X-100 (Table V).

On the other hand, when fresh erythrocyte membranes were centrifuged, no vesicles were obtained. When membranes were incubated at pH 7.4 and washed

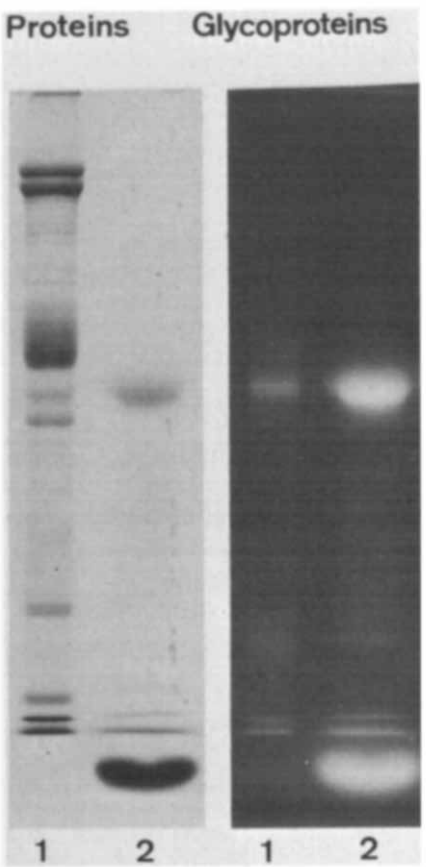


Fig. 8. Protein and glycoprotein composition of spontaneously released glycophorin-enriched vesicles. Membranes were incubated at pH 4.5 as outlined in Scheme 1 and were washed as listed in Table V, row c. 13 ml of these pretreated membranes were layered on a dextran-density gradient ($\rho = 1.01$ to $\rho = 1.04$). The major vesicle fraction with an average density of $\rho = 1.012$ was washed and analyzed. 1, fresh membranes; 2, spontaneously released glycophorin-enriched vesicles.

TABLE V

CONDITIONS FAVORING RELEASE AND SELECTIVE EXTRACTION OF GLYCOPHORIN FROM HUMAN ERYTHROCYTE MEMBRANES INCUBATED AT pH 4.5

Human erythrocyte membranes were incubated at pH 4.5 as outlined in Materials and Methods and were washed as indicated prior to be layered on dextran-density gradients (ρ = 1.007 to ρ = 1.04) to analyze for spontaneous release of glycophorin-enriched vesicles, or to be extracted with Triton X-100 (0.5%) to analyze for selective extraction of glycophorin. Enrichment of glycophorin is given as the ratio of sialic acid content in the appropriate sample over that measured in erythrocyte membranes. n.d., not determined.

Washing procedure	Spontaneous release of glycophorin-enriched vesicles		Triton X-100 extract		
	Percent of protein in vesicles	Enrichment of glycophorin *	Percent of protein in extract	Enrichment of glycophorin *	Yield of sialic acid
(a) None	0.01	1 *	0.8	3.5 *	
(b) None, except pH adjusted to 7.4	n.d.	n.d.	3.2	4.8 *	
(c) (1) EDTA 0.1 mM (pH 7.4) (2) 0.5 M NaCl, 5 mM phosphate (pH 7.4)	0.1—0.13	7.1 (6.4 *)	7.7	7.0 (5.8 *)	61
(d) As in (c) plus (3) 5 mM phosphate (pH 7.4)	n.d.	n.d.	13	5.7	79
(e) As in (d) but Triton X-100 0.15% (average from 11 experiments, S.D.)	n.d.	n.d.	5.2 ± 0.4	11.8 ± 2.4	61 ± 16

* Enrichment of glycophorin was determined from relative absorptions of glycophorin on gels stained for glycoproteins (see Materials and Methods).

in 0.1 mM EDTA and 0.5 M NaCl, 5 mM phosphate (pH 7.4), they released a small amount of protein with the same composition as found in whole membranes (data not shown).

Discussion

Glycophorin has been extracted with the non-ionic detergent Triton X-100, but the pretreatment of human erythrocyte membranes at pH 4.5 is a prerequisite for the selective extraction of glycophorin. The Triton X-100 extracts are 11.8 fold enriched in glycophorin and thus 73% of the total protein in the extract consists of glycophorin. This represents a considerable purification of glycophorin by solubilizing red cell membranes by a non-ionic detergent without further purification. This extract could serve as a convenient starting material for isolation of chemically pure glycophorin in high yields. However, the major aim of this study was to obtain glycophorin-enriched vesicles with coextracted, endogenous lipids rather than chemically pure glycophorin. The conditions we have worked out lead to vesicles containing a third of the extracted glycophorin incorporated in membranes or up to 87% when supplemented with exogenous lipids.

The most interesting aspect of these vesicles is their lack of intramembranous particles despite they contain glycophorin associated with lipids and have 67% of the glycophorin molecules facing outwards. Intramembranous particles have been reported to occur in all reconstitutions of chemically pure glycophorin isolated by ionic detergents or organic solvents [11—13]. Although some authors find them to be only half the size observed in intact erythrocyte membranes, these particles are large aggregates of glycophorin. In our preparations high molecular weight aggregates of glycophorin are hardly detectable on SDS-polyacrylamide gels, but are apparent at molecular weight ranges of 110 000 and 160 000 when glycophorin is isolated with ionic detergents. The absence of intramembranous particles and thus the lack of highly aggregated glycophorin in glycophorin-enriched vesicles may be due to coextracted lipids. Hence, we have clearly demonstrated that glycophorin can be reconstituted in glycophorin-enriched vesicles and does not form large aggregates appearing as intramembranous particles.

When Triton X-100 is added to fresh erythrocyte membranes, the extract contains glycophorin and in addition a large portion of protein component 3 as originally shown by Yu et al. [25]. Thus Triton X-100 alone does not preferentially remove glycophorin but all integral membrane proteins together. Its capability to selectively remove glycophorin must therefore be due to molecular or supramolecular alterations in the membrane. Due to the incubation of membranes at low pH, proteins containing SH groups undergo disulfide polymerization, except for glycophorin that does not contain SH groups [26]. As a consequence of these cross-linkages the low ionic strength wash with 0.1 mM EDTA following the incubation at pH 4.5 hardly removes any spectrin (Fig. 2, gel 1), although this treatment is known to extract 90% of spectrin from fresh erythrocyte membranes [35]. The failure of this treatment to extract spectrin is conceivably because 58% of spectrin 1 and 54% of spectrin 2 appear in disulfide-bonded polymers (Table III).

On the other hand, disulfide cross-linking alone can only partially explain why hardly any protein component 3 is coextracted with Triton X-100, because only 20% of this protein undergo disulfide polymerization. If intramembranous particles were mainly due to protein component 3, a non-covalent aggregation of these particles could prevent protein component 3 from being coextracted. However, the intramembranous particles on fresh membranes treated at pH 4.5 show an incomplete aggregation. Extraction of these membranes yields the same enrichment of glycophorin as obtained from spectrin-depleted membranes with heavily aggregated intramembranous particles. Thus neither covalent nor non-covalent constraints appear sufficient to explain the retention of protein component 3 in the membrane. One possible explanation of the selective extraction of glycophorin calls for segregation of glycophorin from protein component 3 upon incubation at low pH. While the two proteins may be associated in fresh membranes [36], they seem to segregate into different domains following a treatment at low pH close to the isoelectric point of protein component 3 (pH 4.2, ref. 23) without revealing heavily aggregated intramembranous particles. A segregation of glycophorin from other integral membrane proteins within the plane of the membrane is difficult to study, but a segregation resulting in a spontaneous release is easy to

monitor and although it involves only 1% of total glycophorin is a strong indication for such a segregation to precede the spontaneous release. The segregation of glycophorin from protein component 3 appears to contradict earlier indirect evidence for a concomitant aggregation of both proteins [37,38]. However, in those studies aggregation was caused by incubating membranes at pH 5.5 or by treatment with antispectrin.

Gahmberg [39] has recently reported that intramembranous particles of EN (a-) erythrocyte membranes that lack glycophorin aggregate more readily than those from normal membranes. Similarly, our results suggest a segregation of glycophorin from protein component 3 to preceed aggregation of intramembranous particles when erythrocyte membranes are treated at pH 4.5.

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