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Quantitative Composition and Characterization of the Proteins in Membrane Vesicles Released From Erythrocytes by Dimyristoylphosphatidylcholine. A Membrane System Without Cytoskeleton

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Membrane vesicles were prepared by incubation of human erythrocytes with dimyristoylphosphatidylcholine [3] and isolated by isopycnic centrifugation on Dextran density gradients. Protein analyses were carried out with crossed immunoelectrophoresis and dodecylsufate polyacrylamide gel electrophoresis. The right-side-out-oriented membrane vesicles contained membrane and cytoplasmic proteins of the erythrocyte but lacked cytoskeletal components. Comparison of proteins in vesicles and erythrocyte membranes showed that acetylcholinesterase was enriched two to six times in the vesicles relative to both membrane-spanning proteins, band 3, and glycophorin. Two further, hitherto unidentified, sialic acid-containing membrane antigens were found in the vesicles. Both faced the outside of the membranes and were enriched two to seven times.

Ankyrin was not present in the membrane vesicles and spectrin could not be detected by dodecylsulfate polyacrylamide gel electrophoresis. We suggest that the redistribution of proteins in the vesicles reflects differences in their interactions with other membrane components and their relative mobility within the erythrocyte membrane.

Key words: erythrocyte membrane proteins, dimyristoylphosphatidylcholine, vesiculation, crossed immunoelectrophoresis, cytoskeleton

Vesicle release from human erythrocytes can be promoted by various conditions, such as increased intracellular Ca^{2+} concentration [1], metabolic starvation, which results in ATP depletion [2], or incubation of erythrocytes with dimyristoylphosphati-

Abbreviations used: AChE, acetylcholinesterase; CTAB, cetyltrimethylammonium bromide; DMPC, dimyristoylphosphatidylcholine; DOC, deoxylate; SDS-PAGE, sodium dodecylsulfatepolacrylamide gel electrophoresis.

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dylcholine (DMPC) [3]. The Ca^{2+} induced vesiculation is also accompanied by ATP depletion, and furthermore, irreversible crosslinking of membrane proteins by a Ca^{2+}-regulated transglutaminase in the erythrocyte cytoplasma has been described [4]. ATP depletion of the erythrocyte leads to a change in spectrin phosphorylation, which influences the shape of red blood cells [5,6].

Alternatively, a release of vesicles from red blood cells, which is not dependent on ATP depletion, can be induced by incubation of cells with DMPC-liposomes [3]. A transfer of DMPC from the liposomes to the erythrocyte membrane has been described [7]. This lipid uptake might expand the outer half of the membrane bilayer and could be the reason for the observed crenation of the red cell, which is the initial event in the release of vesicles. The lipid composition of these vesicles and qualitative aspects of the protein content have been previously described [3].

The present studies focus on quantitative aspects of the protein composition of erythrocytes and vesicles to provide further insight into the mechanism of DMPC-induced membrane budding. Because of the high hemoglobin content of the vesicles [3], a reliable analysis of the vesicle membrane proteins was not possible by SDS-PAGE. Therefore, crossed immunoelectrophoresis was used as the major analytical tool to define in detail the antigen composition of vesicles and erythrocytes and to quantitate the distribution of proteins. By using crossed-line immunoelectrophoresis, immunochemical reactions of identity (or differences) of individual antigens before and after vesiculation could be detected. The application of cell-surface absorbed antiserum in an intermediate-gel technique allowed to characterize the topography of the vesicle membrane. Finally, charge-shift crossed immunoelectrophoresis was used to distinguish between hydrophilic (cytosolic) and amphiphilic (membrane-bound) proteins. The results obtained provided detailed information on the process of vesiculation and on the redistribution of proteins, which in turn allowed to characterize interactions between several components of the erythrocyte membrane.

MATERIALS AND METHODS

Materials

1,2-dimyristoyl-sn-glycero-3-phosphorylcholine was purchased from Fluka, Buchs, Switzerland. Neuraminidase from Vibrio Comma (Cholerae) and human serum albumin were from Behringwerke AG, Marburg, FRG. Agarose Type HSA, $m_r = -0.13$ was from Litex, Glostrup, Denmark. All other reagents were obtained either from Fluka or Merck.

Biological Material

Fresh human blood from healthy donors of various blood groups (A, O, Rh^+, Rh^-) was supplied by the Central Blood Bank of the Swiss Red Cross. Erythrocytes were sedimented at 30,000g × min at 4°C and washed three times in 10-mM Tris/HCl, 144mM, pH 7.4. The cells were lysed by sixfold dilution with 10-mM Tris HCl, pH 7.4 at 0°C and stirred for 45 min. Membranes were sedimented (6×10^5g × min, 4°C), washed once with the above buffer, and recentrifuged. The pellet contained 7–8 mg of protein/ml of which hemoglobin accounted for about 60%. Spectrin was isolated by EDTA extraction followed by gel filtration on Sepharose Cl 6B [8].

Rabbit anti-human erythrocyte membrane antibody was obtained as an immunoglobulin fraction in 0.1 M NaCl and 15 mM NaN_3 from Dako-Immunoglobulins,

Copenhagen, Denmark. Anti-albumin activity was removed according to the procedure of Bjerrum et al [9].

Vesicle Isolation and Characterization

Membrane vesicles were prepared according to Ott et al [3]. Briefly, 5 ml of washed, packed erythrocytes were incubated with 45 ml of a solution of DMPC-liposomes (0.55 mg/ml in 144 mM NaCl, 10 mM Tris/HCl, pH 7.4), which was previously sonicated for 15 min using an MSE sonicator P. G. 846, with the amplitude set at 18 μ. After 4 hr the erythrocytes were removed by centrifugation (30,000g $\times$ min, 25°C); and the supernatant (9 ml) was layered onto a continuous Dextran T 70 density gradient (26 ml, P = 1.015–1.094 g/ml) in 11 mM phosphate buffer (pH 7.4), 120 mM NaCl, 1 mM EDTA and centrifuged (7.8×10^7g $\times$ min, 4°C). The gradients were then emptied from the bottom, and 40 fractions of 0.0875 ml each were collected. AChE-containing fractions (14–18) were pooled and used for further analysis (cf Fig. 1).

Neuraminidase treatment of vesicles was carried out according to Bjerrum and Bøg-Hansen [10].

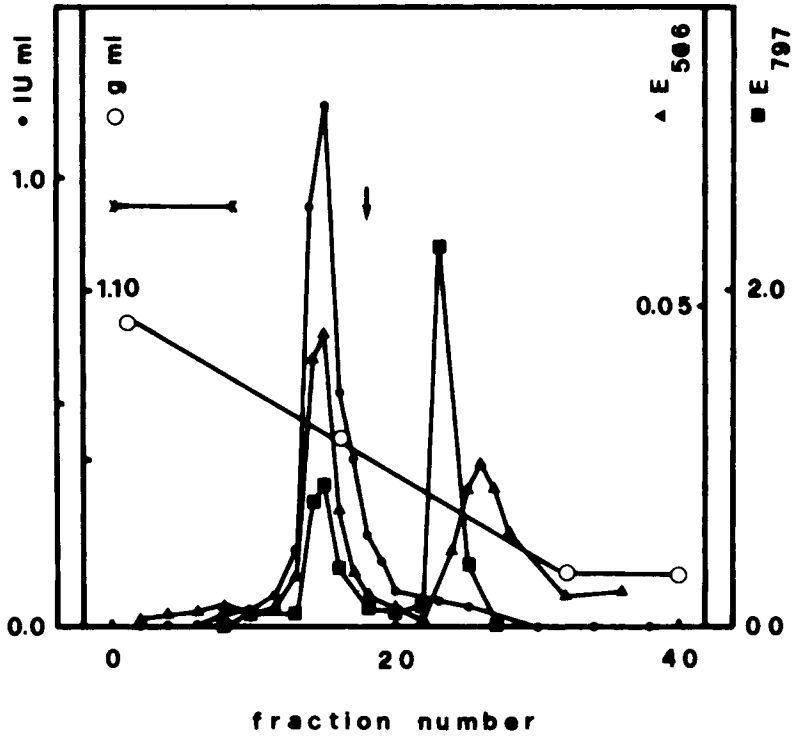

Fig. 1. Profile of Dextran T-70 isopycnic gradient centrifugation. Erythrocytes were incubated with DMPC-liposomes and removed by centrifugation after 4 hr. From the supernatants, 9 ml was layered on top of a linear Dextran T-70 gradient (26 ml) and centrifuged for 16 hr at 81,000g_{av}. The gradient was emptied from the bottom (fraction no. 1) and assayed for AChE activity (●—●), phospholipid phosphorus (■—■), hemoglobin (▲—▲), and density (○—○). In separate experiments, the position in the gradient of native erythrocytes ⩾—⩽ and ghosts (arrow) were determined. For experimental conditions see Materials and Methods.

Polypeptide Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Weber and Osborn [11]. Scans were made from Coomassie brilliant blue R stained gels on a Gelman ACD 15 densitometer at 570 nm. Hemoglobin monomer and dimer were identified on unstained SDS gels on the basis of their molecular weights and their peroxidase activity [12].

Crossed Immunoelectrophoresis

Membrane solubilization and subsequent crossed immunoelectrophoresis were performed according to Bjerrum and Bøg-Hansen [8]. Vesicles and erythrocyte membranes were solubilized by sonication of a suspension of 3 mg protein/ml in 100 mM glycine, 38 mM Tris, pH 8.7 (electrophoresis buffer) and 1% (v/v) Triton X-100 for 2–5 sec. Nonsolubilized material (less than 5% of total protein) was removed by centrifugation (6×10^6g $\times$ min). The gels, made of 1% agarose in electrophoresis buffer containing 0.5% (v/v) Triton X-100 were moulded onto glass plates (7 $\times$ 10 cm and 10 $\times$ 10 cm) in a thickness of 1.5 mm. First dimension electrophoresis was carried out at 10 V/cm until bromophenol-blue-stained bovine serum albumin had migrated 4.5 cm. The agarose of the upper 8 cm of the plate was then removed and replaced by a freshly moulded gel, which contained the anti-human erythrocyte membrane antibody. Subsequently, electrophoresis in the second dimension was carried out at 2 V/cm for 17 hr. Charge-shift crossed immunoelectrophoresis was carried out following the procedure of Bhakdi et al [13]. Crossed-line and crossed immunoelectrophoresis with an intermediate gel were performed as described by Bjerrum and Bøg-Hansen [8]. In crossed-line experiments 50–75 μg of vesicle antigens were separated in the first dimension and subsequently run through intermediate gels (7 $\times$ 2 cm) containing 0.8–3.2 mg of erythrocyte antigens. Alternatively 50–100 μg of erythrocyte antigens were applied to the first dimension and subsequently run through intermediate gels containing 0.4 mg–0.5 mg of vesicle antigens. The identification of antigen No. 23 in erythrocytes was carried out with 2 mg of erythrocyte membrane protein in an intermediate gel and 150 μg vesicle protein applied to the first dimension. Anti-human erythrocyte membrane antiserum devoid of antibodies against determinants located on the outside of the erythrocyte membrane for use in the intermediate gel technique was prepared by two subsequent incubations of 1 ml of anti-human erythrocyte membrane antiserum with 20 ml of washed and packed intact human erythrocytes for 15 min at 25°C [14]. Quantification of peak areas of individual protein precipitates was perfomed by weighing paper sheets of five times enlarged precipitate projections. Staining for esterase activity on immunoplates was performed according to Brogren and Bøg-Hansen [15]. Catalase activity was marked by release of oxygen from H_2O_2 [16].

Other Assays

AChE was assayed by the method of Ellman et al [17]. Hemoglobin in density gradients was determined spectrophotometrically by the method of Zijlstra et al [18]. Phospholipids were extracted by the method of Renkonen et al [19] and quantitatively determined according to Rouser et al [20]. Protein was determined by the method of Lowry et al [21] with bovine serum albumin as standard.

RESULTS

Human red blood cells were incubated for 4 hr with DMPC-liposomes and removed from the incubation mixture by centrifugation. The supernatant was separated

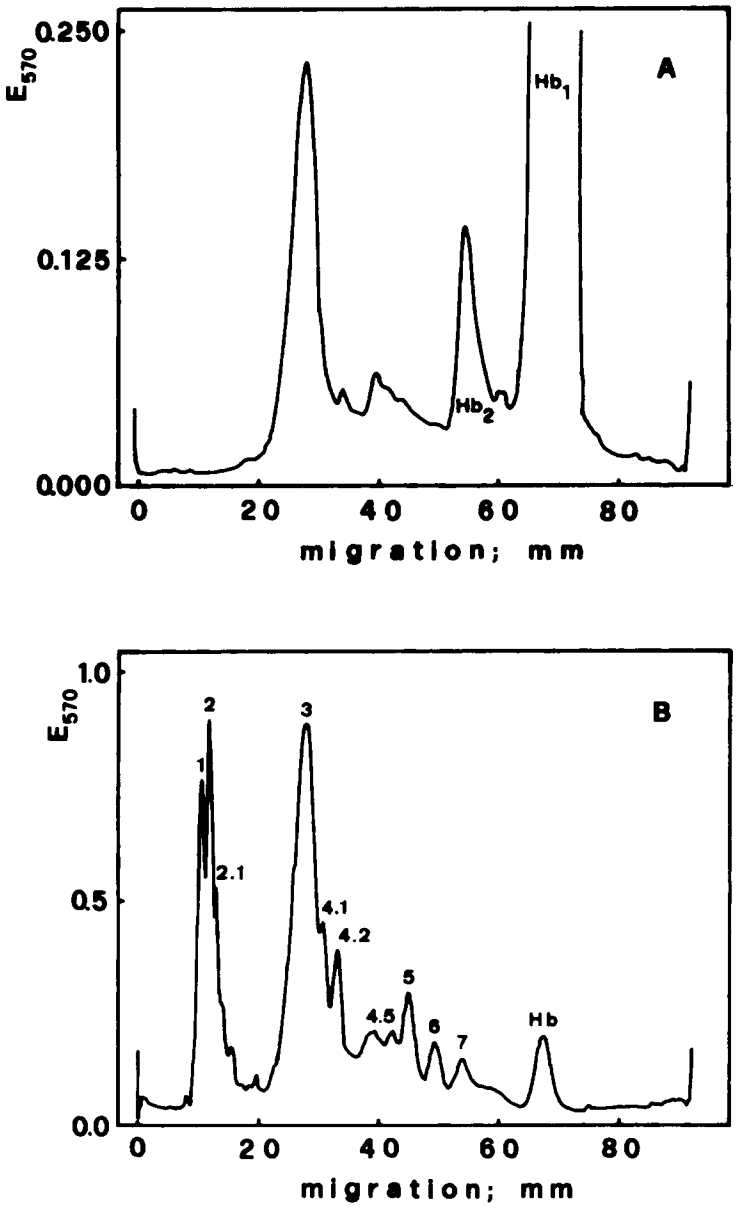

Fig. 2. Densitometric scans of gels from SDS-polyacrylamide gel electrophoresis after Coomassie brilliant blue staining. The following samples were analyzed: A) 127 μg protein of membrane-derived vesicles; B) 35 μg protein of ghosts obtained from erythrocytes before incubation with DMPC-liposomes. Assignment of bands in B is that of Fairbanks et al [21].

by isopycnic centrifugation on a Dextran T 70 gradient. Figure 1 shows the distribution of phospholipid phosphorus, AChE activity, and hemoglobin within the gradient. AChE was found mainly in one peak (P = 1.060 g/ml), which also contained the vesicles. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 2) showed that membrane-derived vesicles contained proteins in the regions of band 3, 4.5, and 7 as well as hemoglobin. Bands 4.1 and 4.2 were diminished, while spectrin and band 2.1–2.5 proteins were virtually absent. To further define the other bands observed in Figure 2A a sample of cytosolic proteins devoid of ghosts was subjected to SDS-PAGE.

The results indicated that the proteins seen in the region between band 4.5 and hemoglobin monomer partially originate from cytosolic proteins. One large peak could be identified as hemoglobin dimer. No differences of the protein patterns of ghosts obtained from erythrocytes before and after vesiculation could be discovered by SDS-PAGE.

Since the vesicles contained considerable amounts of cytosolic proteins and were resistant to lysis, either by hypotonic shock or repeated freeze-thawing cycles, it was not possible to obtain further information on individual membrane proteins by SDS-PAGE. However, this could be achieved by crossed immunoelectrophoresis. The method involves a two-dimensional electrophoretical separation of erythrocyte membrane proteins solubilized to an extent of more than 95% in 1% (v/v) Triton X-100 at pH 8.7 and low ionic strength. Incorporation of a polyspecific rabbit antibody raised against membranes into the second dimension gel permits precipitation of each antigenic entity as individual immunoprecipitate in a highly reproducible manner [22]. The various modifications of this technique allowed to define the molecular properties and the quantitative distribution of the vesicle proteins, their association to the membrane, as well as the topography of the membrane itself.

The precipitates observed in crossed immunoelectrophoresis of vesicles (Fig. 3A) and erythrocytes before (Fig. 3B) and after incubation with DMPC (Fig. 3C) were numbered according to the reference pattern published by Bjerrum and Bøg-Hansen [9]. In these patterns, the following precipitates have been correlated to proteins characterized by SDS-PAGE [23]: No. 5 corresponds to ankyrin (band 2.1), No. 6 to spectrin (bands 1 and 2), No. 15 to hemoglobin, No. 16 to band 3 (in complex with other proteins), No. 20 to AChE and No. 21 to glycophorin [9, 10, 24]. The assignment of precipitates was made by comparison of position, morphology, shape, and staining intensity [22]. A further precipitate corresponding to catalase (Cat) was identified by its enzymatic activity (Fig. 3D).

Furthermore, crossed-line immunoelectrophoresis allowed to establish immunochemical identity between proteins of the native erythrocyte membrane and the vesicle membrane. Solubilized erythrocyte membrane protein was incorporated in an intermediate gel between the first dimension gel and the upper antibody-containing gel (reference gel). Identity to vesicle antigens was established by observation of an upward displacement of these precipitates in the reference gel where they fused with line precipitates of the erythrocyte membrane protein. Experiments with reversed application of the antigens were also performed [25, 26]. In this way, the presence of band 3, glycophorin, AChE, No. 19, hemoglobin, and catalase in the vesicles was verified (results not shown).

The vesicle protein composition differed from erythrocytes as follows (Fig. 3): vesicles did not contain ankyrin (No. 5) and precipitate No. 16 was symmetrically bell shaped in contrast to the more blunt appearance in erythrocytes. This probably reflects the loss of components of the band 3 antigen complex (e.g., ankyrin) during the vesiculation process. Furthermore, a precipitate that had not been described previously was found (No. 23). This hitherto undiscovered antigen was found by crossed-line immunoelectrophoresis to be present in erythrocyte membranes, but in a lower concentration than in vesicles. Only traces of spectrin could be discovered in vesicles. In crossed-line immunoelectrophoresis, this vesicle antigen showed only partial identity [25, 26] with purified spectrin (results not shown). A precipitate (X) found in erythrocytes did not appear in vesicles. Because it was neither present in white ghosts, it obviously represents a protein that is loosely associated with the erythrocyte membrane.

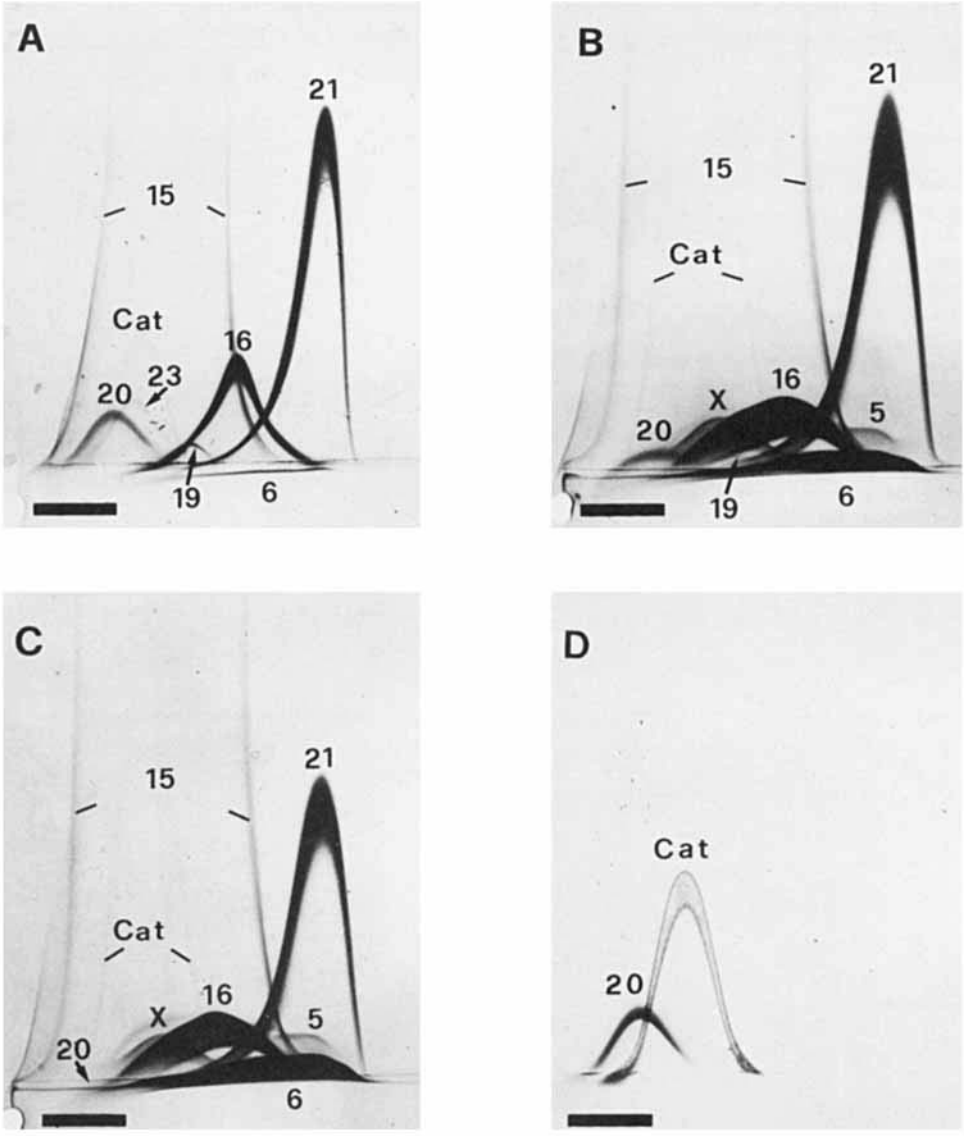

Fig. 3. Crossed immunoelectrophoresis of human erythrocyte membrane proteins solubilized in Triton X-100 at pH 8.7. The patterns represent A) membrane derived vesicles (96 μg of protein), B) partially washed erythrocyte membranes before incubation with DMPC-liposomes (25 μg of protein), C) membranes after the incubation (20 μg of protein). Plates A, B, and C were stained for protein with Coomassie and for AChE activity with α-naphthylacetate and Fast Red; D) shows the same experiment as in A (100 μg of protein) stained for AChE and catalase activity. Precipitates are labelled according to the procedure of Bjerrum et al [9]. Note the decrease in precipitate size of AChE (No. 20) from A through C, simultaneous with the increase of spectrin precipitate (No. 6). Band 3 (No. 16) has a different shape in A where ankyrin (No. 5) is absent. Nos. 19 and 23 are most apparent in A. Electrophoresis in the first dimension was performed at 10 V/cm until the bromophenol blue-stained albumin marker had migrated to a distance of 4.5 cm toward the anode on the right. Electrophoresis in the second dimension was carried out at 2 V/cm overnight, with the anode on top. The gels contained 0.5% Triton X-100. The rabbit anti-human erythrocyte membrane antibody content in the second dimension was 5.4 $\mu l/cm^2$. The bar = 1 cm.

TABLE I. Charge Shifts Observed in Crossed Immunoelectrophoresis of Proteins of Dimyristoylphosphatidylcholine-Induced Vesicles From Human Erythrocytes

Protein (No. of precipitate)	Charge shift in mm[a]	
	Cetyltrimethylammonium bromide	Deoxycholate
Band 3 (No. 16)	16	25
Glycophorin (No. 21)	6	35
AChE (No.20)	6	14
No. 19	6	9
No. 23	7	16
Spectrin (No. 6)	16	14
Catalase (Cat)	1	3
Hemoglobin (No. 15)	0	0

Electrophoresis was carried out according to Bhakdi et al [12] with membrane derived vesicles solubilized in 1% (v/v) Triton X-100. Charge shifts were obtained by the addition of 0.0125% (w/v) cetyltrimethylammonium bromide or 0.2% (w/v) deoxychlolate to the gels containing 0.5% (v/v) Triton X-100 and electrophoresis buffer. Electrophoresis in the first dimension was run until hemoglobin had migrated 20 mm. Other conditions were as in Figure 3.

[a]Difference between the migration in presence of Triton X-100 and in presence of the charged detergents.

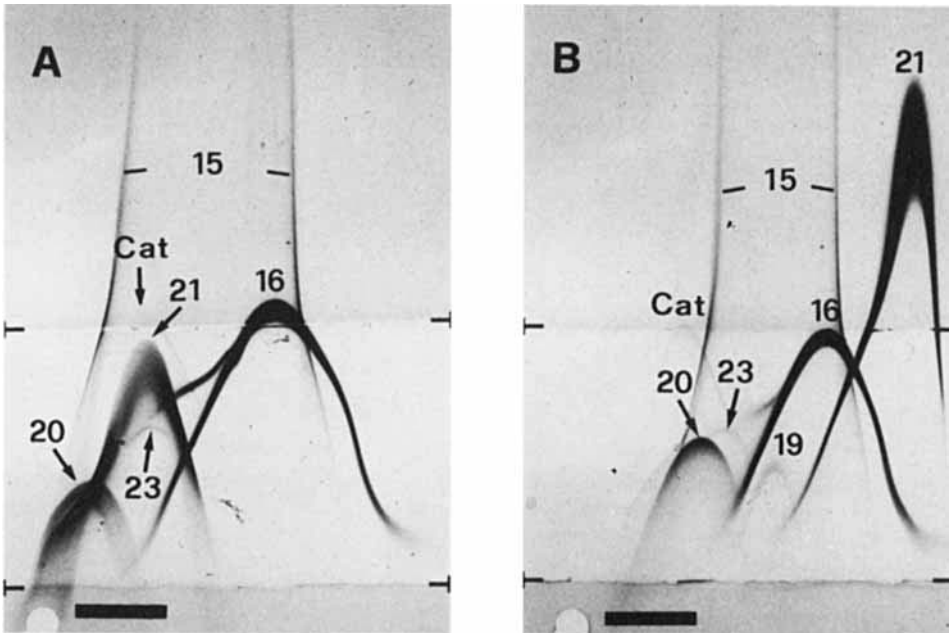

Fig. 4. Sialic acid-containing antigens of the vesicles. Crossed immunoelectrophoresis of 12.6 μg neuraminidase-treated vesicles solubilized in Triton X-100 is shown in A. A control of untreated vesicles (9.5 μg protein) is shown in B. To increase the resolution in the second dimension an intermediate gel (between the markings) containing 1 $\mu l/cm^2$ of the same antibody was used. Conditions of electrophoresis and designation are as described in Figure 3. The bar = 1 cm.

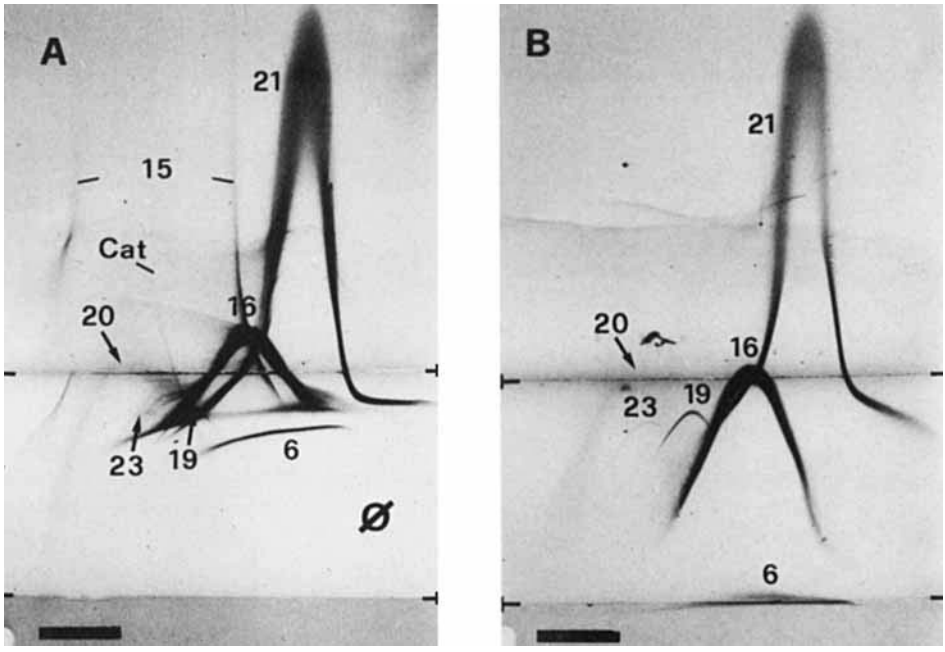

Fig. 5. Topography of the vesicle antigens. Vesicles solubilized in Triton X-100 at pH 8.7 were electrophoresed in the bottom gel (anode to the right). Second dimension electrophoresis was performed through an intermediate gel (between the markings), which in A was free of antibody and in B contained 19 $\mu l/cm^2$ of anti-human erythrocyte membrane antibody that had been absorbed with intact erythrocytes. The upper reference gels in A and B contained anti-human erythrocyte membrane antibody (8.6 $\mu l/cm^2$). Totally retarded antigens are located on the inner surface, partially retarded antigens span the membranes, whereas undisturbed precipitates represent antigens on the outer surface. Conditions of electrophoresis and designation are as described in Figure 3. The bar = 1 cm.

Membrane specificity of the vesicle antigens was estabished by performing crossed immunoelectrophoresis with membrane depleted hemolysate in an intermediate gel. Reaction of identity was found only for hemoglobin and catalase, indicating the membrane origin of the other vesicle antigens.

The proteins of the vesicles were further characterized by charge-shift electrophoresis, which allows to distinguish between amphiphilic and hydrophilic membrane constituents. The former bind detergents and change their migration velocity in the first dimension of the electrophoresis depending on the charge of the detergent [13, 27]. Proteins that show a bidirectional charge shift of more than 5 mm have to be considered as amphiphilic molecules [12]. Table I summarizes the results obtained with vesicle antigens and shows that all vesicle antigens except catalase and hemoglobin are amphiphilic.

Neuraminidase treatment of vesicles resulted in reduced migration velocities of sialoglycoproteins in the first dimension of crossed immunoelectrophoresis. Figure 4A shows that glycophorin (No. 21), AChE (No. 20), and antigen No. 23 migrated only 28, 31, and 72%, respectively, as compared with the migration in untreated control (Fig. 4B). Precipitate No. 19 could not be detected after neuraminidase treatment, and the migration velocity of band 3 was only slightly reduced (93% of the control).

To localize the antigenic determinants of individual proteins within the membrane, crossed immunoelectrophoresis with an anti-human erythrocyte membrane antiserum that had been absorbed with intact red cells was performed (Fig. 5). This treat-

TABLE II. Ratio of Precipitate Areas in Crossed Immunoelectrophoresis of Triton X-100 Solubilized Vesicles, Erythrocytes Before and After Incubation With Dimyristoylphosphatidylcholine-Liposomes

	Area ratio			
Source	AChE Band 3 ± SD	No. 19 Band 3 ± SD	No. 23 Band 3 ± SD	Glycophorin Band 3 ± SD
Vesicles[a]	1.0 ± 0.5	0.11 ± 0.05	0.47 ± 0.24	2.32 ± 0.33
Erythrocytes[b]				
before incubation	0.21 ± 0.03	0.038 ± 0.007	0.13[c]	2.32 ± 0.11
after incubation	0.041 ± 0.049	ID	ID	2.22 ± 0.30

[a]Six immunoplates from three different experiments

[b]Fourteen immunoplates from five different experiments.

[c]Determined as ratio between the distances from the baseline to the lines of No. 23 and band 3 in crossed-line immunoelectrophoresis [25]. This electrophoresis was performed with human erythrocyte membrane antigens applied to the intermediate gel using vesicles as antigens in the first dimension, whereby the corresponding lines of band 3 and No. 23 could be identified.

ID = indeterminable.

ment removes antibodies directed against antigenic determinants facing the outside of the cell membrane. If this modified antiserum is incorporated into an intermediate gel of the second dimension, only the antigens facing the inside of the membrane will be precipitated or retarded in migration by the antibodies in the intermediate gel [24]. On the other hand, those antigens facing the outside of the membrane will migrate to the reference gel, which contains the complete antiserum. Figure 5 shows that antigens No. 19, No. 20, No. 21, and No. 23 were exposed to the outside of the erythrocyte. Precipitate No. 16 (band 3 complex) was partially retarded and had long "feet," typical for membrane spanning proteins. No. 6 (spectrin) precipitated in a position expected for a protein confined to the inner surface of the erythrocyte membrane.

In crossed immunoelectrophoresis, the area under a precipitate is proportional to the amount of antigen applied, which allows the quantification of antigens [28]. Band 3 and glycophorin appeared in the same ratio in membrane-derived vesicles as in erythrocyte membranes before and after incubation with DMPC. In vesicles, AChE and the precipitates No. 19 and No. 23 were enriched compared with band 3 and glycophorin: the enzyme twofold to sixfold, No. 19 threefold to sevenfold and No. 23 twofold to fivefold. After incubation with DMPC, the amount of AChE was strongly reduced in DMPC-treated erythrocytes, whereas No. 19 and No. 23 could no longer be discovered (Table II). The results were confirmed by determination of the specific activity of AChE in erythrocytes and vesicles. The ratio of AChE to precipitate No. 19 and No. 23 in vesicles varied from one experiment to the other, which indicated that these antigens were sequestered independently.

DISCUSSION

Several theories on the mechanism of vesiculation have been proposed. These include disulfide bond formation [29–31], formation of γ-glutamine-ϵ-lysine crosslinks [4] and changes in phosphorylation of membrane proteins [32]. From our results obtained with crossed immunoelectrophoresis, such possibilities could be ruled out as the

patterns showed neither splitting nor fusion of any precipitate [25,26]. This was confirmed by SDS-PAGE where no indications for the presence of a high molecular weight complex could be found. In addition, the DMPC-induced vesiculation is not accompanied by ATP-depletion of the erythrocytes [3], an observation that supports recent results by Patel and Fairbanks [33] who described shape changes of erythrocyte membranes that were not accompanied by changes in spectrin phosphorylation. It has been suggested that interactions of the exogenous lipid with the bilayer of the red cell membrane and changes in the lipid composition of the bilayer can induce the vesiculation [3, 34]. This hypothesis is supported by the bilayer-couple theory of Sheetz and Singer [35] and other recent results [36–38.].

The present study shows that the overall qualitative composition of the antigens in vesicles is similar to that in erythrocytes. Both antigen populations show the same properties with respect to immunochemical behavior, sialic acid content, topography, detergent binding, and enzymatic activities. On the other hand, the quantitative distribution of proteins is different. Spectrin, the major cytoskeletal component, is almost completely absent in vesicles. Furthermore the spectrin found in vesicles is only partially identical to the spectrin complex observed in the erythrocyte membrane and may possess different molecular properties. This could be the reason why it was not detected by SDS-PAGE [2, 3]. There are several reasons for the appearance of spectrin in vesicles. Specific interactions via ankyrin to band 3 have been demonstrated by Bennet et al [39, 40], but the absence of ankyrin in vesicles indicated that spectrin probably was not taken up into the vesicles because of this interaction. It also possesses some amphiphilic properties, e.g., the bidirectional charge shift (cf. Table I and [12]) and binding of hydrophobic ligands have been demonstrated [41]. This makes a direct interaction with the lipid bilayer possible, thus explaining its presence in the vesicles. Additionally, it has to be considered that any free spectrin, which might be present in the cytosol of the erythrocyte, could be transferred to the vesicles by simple entrapping. The only partial identity of vesicle spectrin with erythrocyte membrane spectrin and the lack of ankyrin indicate that vesicles have no intact cytoskeleton. The appearance of band 3 and glycophorin in vesicles is in accordance with the occurrence of intramembraneous particles in freeze-fracture electron microscopy [3]. This might indicate that the specific interaction of band 3 with the cytoskeleton of the erythrocyte (via ankyrin) is abolished prior to vesiculation. Alternatively, two populations of band 3 proteins have been described [42], one, which is bound to the cytoskeleton and comprises about 10–15%. This population would not appear in the vesicles due to an attachment to spectrin. The other population protrudes into the interstices of the spectrin-actin network without specific binding to the cytoskeleton but is restricted in long range lateral mobility by trapping in the network "frames" [42, 43]. This latter portion would either preexist in those patches of the membrane from which vesicles are formed or would appear mobile enough to diffuse into those regions. The assumption that band 3 of the population, which is not bound to spectrin, is being taken up into the vesicles is strongly supported by the absence of ankyrin in the vesicles. Band 3 and glycophorin appear in vesicles at the same ratio as in erythrocytes before and after vesiculation. This suggests that they are directly associated with each other in the membrane, irrespective of the cytoskeleton, which is consistent with recent findings of Nigg and Cherry [44].

The enrichment of AChE relative to band 3 and glycophorin in the vesicles indicates that this protein is neither associated to any of these nor bound to the cytoskeleton. Similar properties were found for antigen No. 19 and No. 23. Both represent am-

phiphilic membrane sialoglycoproteins located on the outer cell surface. Furthermore No. 19, No. 23, and AChE do not contain major antigenic determinants on the cytoplasmic side of the membrane as shown with absorbed antibody in crossed immunoelectrophoresis. Although we cannot exclude penetration of the three antigens through the lipid bilayer, a cytoplasmic part of these proteins would probably be small. These antigens would not be restricted as band 3 and glycophorin in their lateral mobility due to the lack of interactions with the cytoskeleton. This is consistent with the fact that they are all enriched in vesicles relative to band 3 and glycophorin. The independent enrichment of each of these three sialoglycoproteins, however, might also indicate an independent mobility in the native erythrocyte membrane.

Our results can be summarized by the following mechanistic model for the vesiculation process in erythrocytes: after the initiation of the shape change the interactions of the cytoskeleton with other membrane components are proceedingly abolished. When this process reaches a certain extent, protein segregation begins [45]. The most mobile components of the membrane preferentially diffuse into destorted membrane areas, whereas components which are restricted in their lateral mobility, eg, by interaction with the cytoskeleton, are retarded. The latter will only be found in the membranes of the vesicles after being released from their restrictive interactions.

This model is based on differences in the lateral mobility of the erythrocyte membrane glycoproteins. Even though the majority of these proteins does not seem to be directly attached to spectrin [46, 47], it has been shown that the state of spectrin aggregation controls their lateral mobility and distribution within the membrane [48–50].

The results presented further show that the vesiculation of the erythrocyte induced by DMPC provides an additional tool to distinguish between three categories of membrane proteins: 1) those of high lateral mobility which become enriched relative to the typical membrane spanning proteins (the surface sialoglycoproteins AChE, No. 19, and No. 23), 2) those proteins of restricted mobility which are membrane-spanning and, in addition, appear at a constant ratio to each other probably indicating association (band 3 and glycophorin), 3) those that remain with the erythrocyte during vesiculation (spectrin and ankyrin).

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