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MEMBRANE ISOLATION ON POLYLYSINE-COATED GLASS BEADS

ASYMMETRY OF BOUND MEMBRANE

DOUGLAS I. KALISH, CARL M. COHEN, BRUCE S. JACOBSON * and DANIEL BRANTON

Cell and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, Mass. 02138 (U.S.A.)

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Summary

Erythrocyte membranes isolated on polylysine-coated glass beads exhibit many of the properties of the native membrane. Gel electrophoresis indicates that all major protein components of the membrane are retained during membrane isolation. The membrane integrity and accessibility of selected components was tested using non-penetrating probes. In general, membranes on beads displayed accessibility properties typical of inside-out vesicles. The accessibility of membrane acetylcholinesterase to assay reagents, as well as membrane accessibility to the actions of neuraminidase, trypsin and galactose oxidase-NaB^3H_4 demonstrated that the protoplasmic surface of membrane isolated on beads was exposed, while the extracellular surface was inaccessible. The differential accessibility of the membrane surfaces demonstrates the feasibility of investigating asymmetry of membranes isolated on cationic glass beads.

Introduction

Polylysine-coated beads can be used to isolate cell membranes [1,2] because cell surfaces bind tenaciously to polylysine-coated surfaces [3—5] and the portions of the cell not attached to the bead can be sheared away leaving attached plasma membrane on the bead surface.

When intact cells are bound to the beads, the extracellular surface of the plasma membrane is apposed to the bead. Lysis of the cells and removal of unattached membrane should expose the protoplasmic ** surface of the attached

* Current address: Department of Biochemistry, University of Massachusetts, Amherst, Mass. 01002, U.S.A.

** The term "protoplasmic" is used here in the sense defined in Branton et al. [19]. In this context, it is synonomous with "cytoplasmic".

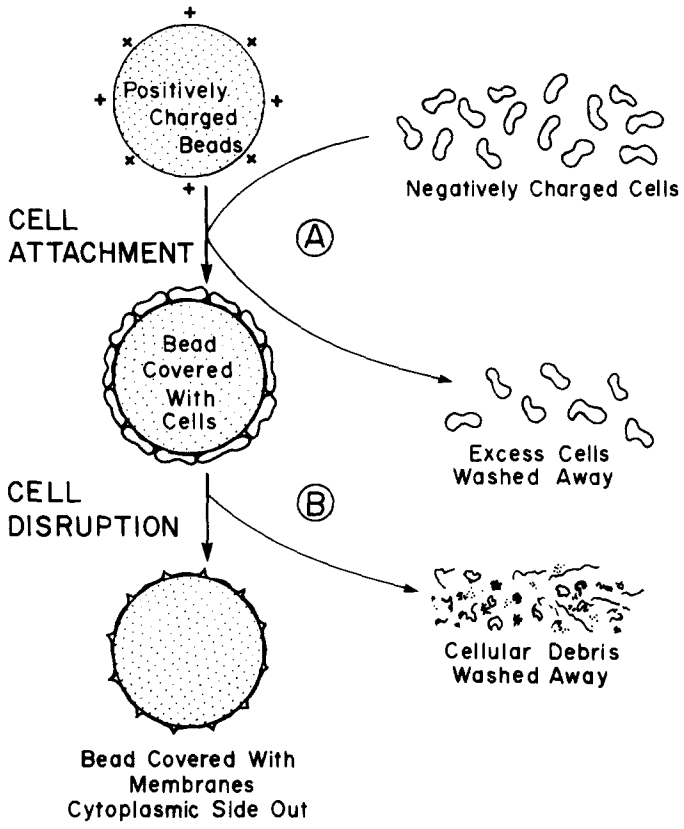

Fig. 1. Schematic illustration of membrane isolation on polylysine-coated glass beads.

membrane (Fig. 1). Assuming that isolation on beads does not destroy the asymmetry of the membrane, exposing the protoplasmic surface should make chemical groups that are normally sequestered in the intact cell susceptible to modification or cleavage by appropriate reagents. Conversely, extracellular chemical groups or enzyme active sites which are exposed in the intact cell may become inaccessible when the cell membrane is apposed to the bead.

The orientation and modified accessibility of extracellular and protoplasmic membrane surfaces following membrane isolation on cationic beads can be verified by exploiting the well-established asymmetry of the red cell membrane [6]. Here, we show that erythrocyte membranes isolated on polylysine-coated glass beads [7] exhibit accessibility properties that are complimentary to those exhibited by the intact cell. This indicates that many aspects of membrane integrity and asymmetry are retained and can be assayed when membranes are isolated on cationic beads.

Methods

Glass beads. Solid soda lime glass beads, (microbeads Class IV A, size number 3200, Cataphote Corporation, Jackson, Mississippi 39205, U.S.A.) were silan-

ized, succinylated and covalently coupled to polylysine using carbodiimide [7].

Beads prepared in this manner were produced in large batches, washed in 4 M NH_4Cl, rinsed 5 times in distilled H_2O and lyophilized for storage until used. In some cases, the beads were reclaimed and reused after washing in acetone, 3% sodium dodecyl sulphate and distilled water. No difference was observed between fresh beads and reclaimed beads, but we have not yet used beads reclaimed more than 5 times.

Membrane isolation. All procedures were performed on ice unless otherwise indicated. Erythrocytes from outdated bank blood were used. Intact erythrocytes to be attached to beads were washed twice in 20 volumes of phosphate-buffered saline, twice in 310 mosM phosphate buffer, pH 7.6, and twice in 7 : 3 310 mosM sucrose : 310 mosM phosphate buffer, pH 7.6. Erythrocyte ghosts, prepared by the method of Dodge et al. [8] and inside-out vesicles prepared by the method of Steck and Kant [9] were suspended in 20 mosM phosphate buffer, pH 7.6, prior to attachment to beads.

1 ml of cells or vesicles suspended to 50—90% packed volume in their last washing medium was added to 300—500 mg (dry weight) of beads which had been washed and suspended to approximately 50%, v/v, in the appropriate attachment medium (7 : 3 sucrose : phosphate buffer for erythrocytes; 20 mosM phosphate buffer for ghosts or inside-out vesicles). Beads and cells were gently agitated, then allowed to settle for about 5 min on ice. The supernatant containing unattached cells was removed, 10 ml of 20 mosM phosphate buffer, pH 7.6, was added and the beads were sonicated for 5 s using a Heat Systems probe-type sonifier (20 W output). Beads were sedimented in a clinical centrifuge, the supernatant was removed, and the beads were washed three times, with vigorous vortexing in 20 mosM phosphate buffer.

Protein and phospholipid content of membranes on beads was determined as described in [7]. For electrophoresis on polyacrylamide gels, 100 μl of a gel extraction buffer containing 1.25% sodium dodecyl sulphate, 6.25% β-mercaptoethanol, 10% glycerol, 1 mM sodium ethylenediamine tetraacetate and 0.5% pyronin-y in 10 mM Tris, pH 8.0, was added to 500 mg of beads. The beads were then heated to 50°C for 15 min with intermittent vortexing and 25—100 μl of the supernatant was applied to a polyacrylamide gel prepared as described in Fairbanks, Steck and Wallach [10]. Standard ghost preparations were solubilized in 20 μl of a 5-fold concentrate of the above extraction buffer and treated identically.

Scanning electron microscopy of cells on beads. 1 ml of 4% glutaraldehyde in 7 : 3 310 mosM sucrose : 310 mosM phosphate buffer, pH 7.6, was added slowly to 100 mg of beads and cells in 1 ml of buffer at 4°C, and the mixture was agitated intermittently for 30 min. The beads were then rinsed once in the sucrose:phosphate buffer and resuspended in 1 ml of buffer. An equal amount of 2% OsO_4 in distilled H_2O was added and the beads were agitated at 4°C for 30 min, followed by a rinse in distilled H_2O.

A large (25 cm^2) piece of Al foil was flamed over a bunsen burner in order to remove the gloss. Small pieces of foil were cut and shaped to fit scanning electron microscopy stubs, and then dipped in 3% gelatin heated to 40°C. After excess gelatin was drained off, the foil was placed on ice and enough 8% glutaraldehyde was added to cover the foil. Approximately 250 μl of diluted beads

(50 mg/ml) was then added to the foil and the beads allowed to settle. After 5 min, the unbound beads were washed off by dipping the foil in a large beaker of H_2O. The samples were dehydrated in a graded ethanol series and critical point dried using CO_2 as the transition fluid. The Al foil was mounted on scanning electron microscopy stubs using double-stick tape, coated with Au/Pd, and examined in a scanning electron microscope.

Trypsin treatment. Erythrocytes or inside-out vesicles were isolated on 300 mg samples of beads as previously described. After sonication and washing, the beads were resuspended in 1 ml of 20 mosM phosphate buffer, pH 7.6, with either 0.6 or 0.3 μg/ml of trypsin and agitated continuously for 1 h at room temperature. At the end of the incubation period, the supernatant was removed and the beads were washed twice in 20 mosM phosphate buffer containing 25 μg/ml of phenylmethylsulfonylfluoride. After the beads settled, the supernatant was completely removed and 10 μl of 10 mg/ml phenylmethylsulfonylfluoride in dimethyl sulfoxide was added directly to the pellet. The membranes were then dissolved in gel extraction buffer for electrophoresis.

Acetylcholinesterase assay. Acetylcholinesterase was assayed using a modification of the technique of Ellman [11]. 1 g of beads with attached membranes was diluted with 1 ml of the 7 : 3 sucrose : phosphate buffer, pH 7.6. After vigorous vortexing to suspend the beads, a 25 μl aliquot was immediately removed and added to 3 ml of 7 : 3 sucrose : phosphate buffer containing 25 μl of 75 mM acetylthiocholine iodide, and 25 μl of 10 mM dithiobisnitrobenzoic acid containing 1.5 mg/ml $NaHCO_3$ in 100 mosM phosphate buffer, pH 7.6. The tubes were capped and continuously tumbled for the duration of the incubation (0 to 5 min, room temperature). At the end of the incubation time, the tubes were spun in a clinical centrifuge at about 3000 $\times g$ for 10 s to sediment the beads, and the absorbance of the supernatant at 412 nm was determined. Enzymatic activity was computed as the slope of the line obtained by plotting absorbance at 412 nm versus time of incubation. In order to determine total acetylcholinesterase activity on the beads, 25 μl of 10% Triton X-100 was added to the reaction mixture, and the activity measured as described above.

Neuraminidase treatment. 20 μl of 0.1 mg/ml neuraminidase (Type VI from *Clostridium perfringens*, 1.9 units/mg, Sigma Chem. Co., St. Louis, Mo.), containing 0.3 mg/ml bovine serum albumin in 20 mosM phosphate buffer, pH 7.6, was added to 300—500 mg (dry weight) beads with attached membranes in a final volume of 300—400 μl of 20 mosM phosphate buffer, pH 7.6. Incubations ranged from 0 to 4 h at room temperature and were terminated by immersing the tubes in boiling water for 2 min to inactivate the neuraminidase. The incubation conditions vary considerably from other reports of neuraminidase treatment (e.g., 1 h at 37°C, pH 5, [9]) since it was found that these latter conditions led to complete removal of sialic acid of membranes on beads. Total sialic acid of membranes on beads was determined by incubating the beads in 200 μl of 0.4 N H_2SO_4 at 80°C for 1 h. Free sialic acid was determined by the method of Warren [12], correcting for the contribution to absorbance not due to *N*-acetylneuraminic acid. Control experiments showed that the presence of beads had no effect on the assay.

Galactose oxidase labeling. Ghosts or membranes on beads were labeled by a

modification of the method of Steck and Dawson [13]. Galactose oxidase (*Polyporus circinatus*, spec. act. 85 units/mg protein, Sigma Chemical Co., St. Louis, Mo.) was stored frozen as a 28 unit/ml solution in 50 mM phosphate buffer, pH 7.6 after having been heated to 50°C for 30 min to inactivate proteases. Tritiated $NaBH_4$ of specific activity 1 mCi/mg was purchased from New England Nuclear (Boston, Ma.).

20 μl of NaB^3H_4 in 0.01 M NaOH (5 μCi/ml, freshly prepared), plus either 0 or 25 μl (0.7 units) of galactose oxidase stock, was added to 500 mg of beads with attached membranes or to ghosts containing an equivalent amount of protein (40—60 μl packed ghosts), in a final volume of 300 μl of 20 mosM phosphate buffer, pH 7.6. After a 1 h incubation at room temperature, the beads or ghosts were washed 3 times in 10 ml of 20 mosM phosphate buffer, pH 7.6. To the beads was added 100 μl of the gel extraction buffer, while the ghosts received 25 μl of the gel extraction buffer, while the ghosts received 25 μl of 5-fold concentrated gel extraction buffer. Both samples were heated to 50°C for 10 min. 100 μl samples were removed from the ghost suspension and bead supernatant of which 20 μl were used for counting and the rest for electrophoresis. The rest of the tube was flushed with Aquasol and also counted so that the total incorporated counts could be determined.

For electrophoresis, 15—30 μg of protein was loaded on to each 7.5% polyacrylamide gel, prepared and run as described above. Gels were sliced into 2-mm thick sections and placed in scintillation vials containing 4 ml of scintillation fluid composed of 50 ml Protosol, 40 ml Liquiflor, made up to 1 l in toluene. The slices were incubated overnight at 37°C and counted for ^3H.

Results

Integrity of bead-isolated membranes. When erythrocytes and beads were mixed together in a low ionic strength buffer, the cells covered the beads so that the only bead surfaces left uncovered were too small to accommodate additional cells (Fig. 2a). Maximal coverage required excess cells and low ionic strength. We found that a buffer containing 7 parts 310 mosM sucrose and 3 parts 310 mosM phosphate buffer, pH 7.6, was ideal. Too low an ionic strength (9 : 1 sucrose : buffer) caused the cells to clump.

Osmotic lysis of the bound cells freed the membranes of most intracellular material and left collapsed membranes on the beads (Fig. 2b). Subsequent vigorous vortexing or sonication removed membrane not directly apposed to the beads. Although beads treated in this way (Fig. 2c) were indistinguishable from virgin beads, (presumably because the thickness of the bound membrane could not be resolved by our scanning electron microscope) protein and lipid assays demonstrated the presence of membrane components. The phospholipid to protein ratio of membranes on beads was 0.52 mg phospholipid/mg protein in good agreement with the ratio of 0.49 mg phospholipid/mg protein we determined for erythrocyte ghosts. Electrophoresis in sodium dodecyl sulphate demonstrated that the polypeptide composition of membranes on beads was similar to that of ghosts (Fig. 3) although the reason for increased background staining in the region between bands 3 and 5 is unclear. Further, the amount of hemoglobin associated with the beads depended on how many times the beads

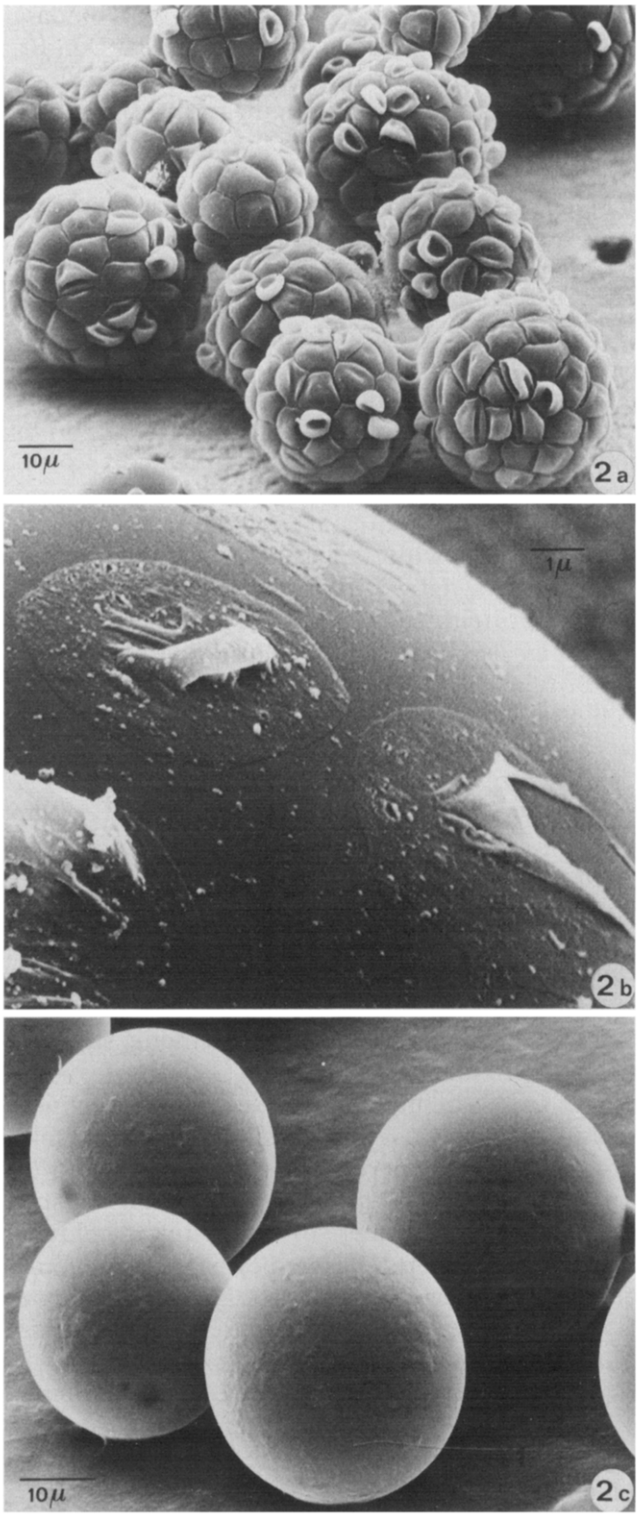

Fig. 2. For legend, see opposite page.

were washed to remove unbound cells before the bound cells were lysed. As with ghosts in solution, bands 1, 2 and 5 could be selectively eluted [10] from membranes on beads by incubation at low ionic strength (Fig. 3).

Although we have not overcome technical difficulties which prevent electron microscopic observation of membranes on beads, we have removed membranes from beads by salt treatment (Table I). The resultant sheets and vesicles were easily visible in a phase contrast light microscope (not shown) and examination by freeze-fracture revealed the presence of particles whose degree of aggregation was similar to that observed in native membranes (Fig. 4). Some of the fracture faces had high particle densities characteristic of protoplasmic faces whereas others had low particle densities characteristic of extracellular faces. Only a few of the fracture faces (approx. 1%) showed membranes without particles. The similarities between membrane released from the beads and native membranes suggests that attachment to the beads does not perturb the membrane morphology. Direct observation of membranes attached to polylysine-coated glass slides [14] supports this conclusion.

Electrophoresis in sodium dodecyl sulphate-polyacrylamide gels (not shown) showed that the polypeptide content of the membrane released from beads by salt treatment was similar to that of ghosts subject to the same salt treatment. Band 6, known to be eluted at high ionic strength was absent from the desorbed membranes, as it was from ghosts treated with high salt concentration [15].

Asymmetry of bead-isolated membranes. The active site of acetylcholinesterase is located exclusively on the extracellular surface of the erythrocyte membrane [16]. If the native asymmetry of membranes is preserved, the accessibility of assay reagents to acetylcholinesterase should be restricted when membranes of intact cells are isolated on beads. This hypothesis was confirmed when the ensyme activity of membranes on beads was compared before and after disruption of membrane integrity by Triton X-100. The acetylcholinesterase accessibility (expressed as the percent enzyme activity of membranes on beads relative to Triton-treated membranes on beads) of membranes on polylysine-coated glass beads was only 60%. On amino glass beads [7], which appear to bind membranes less tightly, the accessibility to acetylcholinesterase reagents was 80%.

Using similar reasoning, we also investigated the accessibility of membrane components to enzymic treatments capable of modifying cell surface components.

Steck and Dawson [13] demonstrated that all of the erythrocyte glycoprotein and glycolipid labeled by the galactose oxidase-NaB^3H_4 procedure is present only on the extracellular erythrocyte membrane surface. Thus, glycopro-

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Fig. 2. Scanning electron micrograph of intact erythrocytes attached to polylysine-coated glass beads. (a) Cells were attached in a buffer composed of 7 parts 310 mosM sucrose and 3 parts of 310 mosM phosphate buffer, pH 7.6 and unbound cells were washed away. (b) Erythrocytes were attached as in (a). After unbound cells were washed away, 20 volumes of 20 mosM phosphate buffer, pH 7.6, was added, the beads suspended by inversion, and washed twice in buffer. (c) After lysis of the cells as in (b), beads were diluted in 2 ml of 20 mosM phosphate buffer, pH 7.6, sonicated for 5 s with a Heat-Systems probe type sonicator, and then washed twice in buffer. In all these figures, beads were fixed in an equal volume of 2% glutaraldehyde, rinsed and fixed in OsO_4, rinsed again and prepared as described in Methods.

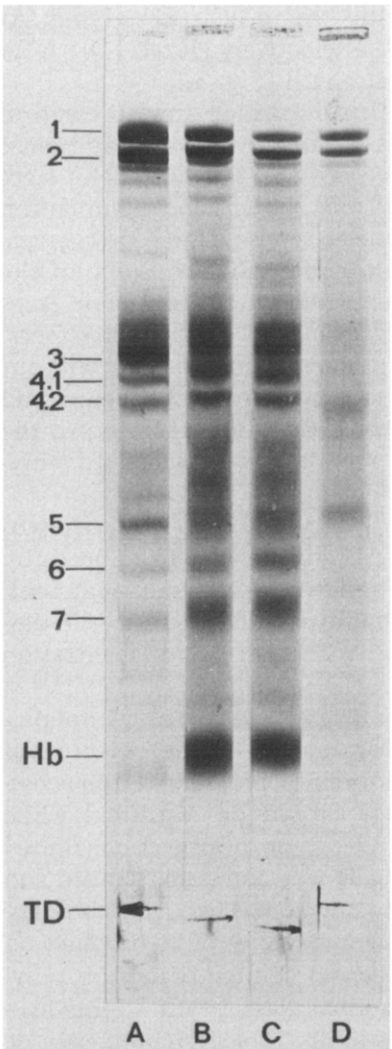


Fig. 3. Sodium dodecyl sulfate-polyacrylamide gels of erythrocyte membrane proteins, stained with Coomassie Blue. A, Erythrocyte ghost; B, erythrocyte membrane isolated on polylysine-coated glass beads; C, erythrocyte membrane on beads from which spectrin was eluted by incubation in 0.5 mM phosphate buffer, pH 7.6, for 40 min at 37°C; D, the supernatant from C which had been centrifuged for 1 h at 100 000 × *g* in order to sediment membrane fragments and then lyophilized and solubilized in 25 μl of 5-fold concentrated gel extraction buffer. Equal amounts of protein were initially present on the beads in gels B and C. Note the relative decrease in bands 1 and 2 on gel C. Extraction of virgin polylysine-coated beads with gel extraction buffer indicated that no polylysine is eluted from the beads. Hb, hemoglobin; TD, tracker dye.

teins and glycolipids in sealed, inside-out vesicles prepared from ghosts were not accessible to galactose oxidase. Similarly, we have found that when erythrocyte membranes were isolated on beads their glycoproteins were less accessible to galactose oxidase (Fig. 5) than those of free ghosts, whose labeling pattern was qualitatively similar to that described by Steck and Dawson [13]. The total label incorporated into membranes on beads was only 23% of that in an

TABLE I

RELEASE OF ERYTHROCYTE MEMBRANE ISOLATED ON BEADS

1 ml of salt solution at the concentration indicated was added to 1 g of erythrocyte membranes on beads. Following vigorous vortexing, the beads were allowed to settle for 2 min, the supernatant was removed and the operation repeated with an additional ml of salt. Release of membrane was determined by assaying the combined supernatants for phospholipid, as described in Methods. The percent release shown has been corrected for any losses in the volume trapped between the beads.

Salt	Concentration	% Release
NaCl	600 mM	6
NH_4Cl	600 mM	8
$NaOOCCH_3$	600 mM	5
NH_4OOCCH_3	600 mM	10
Na_2HPO_4	600 mM	75
K_2HPO_4	600 mM	82
K_2SO_4	600 mM	65
Polyphosphate (degree of polymerization = 15)	100 mg/ml	81
Polyglutamate (M_r = 98 000)	10 mg/ml	1

equivalent amount of ghosts (Table II). The diminished labeling cannot be attributed to non-specific inhibition of the enzyme by beads since 1 : 1 mixtures of free ghosts and membranes on beads resulted in additive labeling (Table II).

Accessibility and membrane asymmetry were also evaluated with neuraminidase and trypsin. While virtually all the membrane sialic acid, which is on the

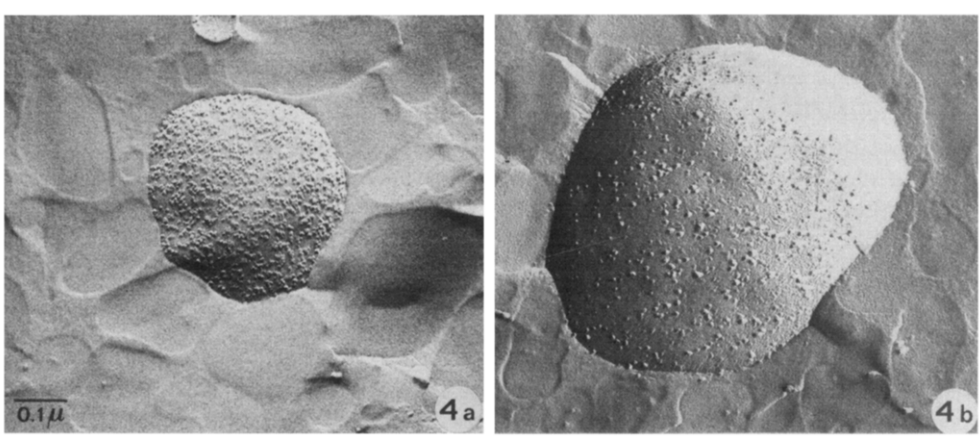


Fig. 4. Freeze-fracture of membrane eluted from beads. Membrane isolated on beads as in Fig. 2c was removed by placing 1 g of beads in 1 ml of 1 M KH_2PO_4, followed by vigorous vortexing. The beads were allowed to settle for 2 min and the supernatant was removed. The operation was repeated with an additional ml of 0.5 M KH_2PO_4 and the supernatants combined, diluted to 10 ml with 20 mOsM phosphate buffer, pH 7.6, and sedimented at 100 000 × *g* for 1 h. After removal of the supernatant and resuspension of the pellet, the wash was repeated. Samples of the translucent pellet were mixed with 10 μl of 20% glycerol, applied to copper hats and frozen in liquid N_2-cooled Freon for freeze-fracture. a, P face; b, E face.

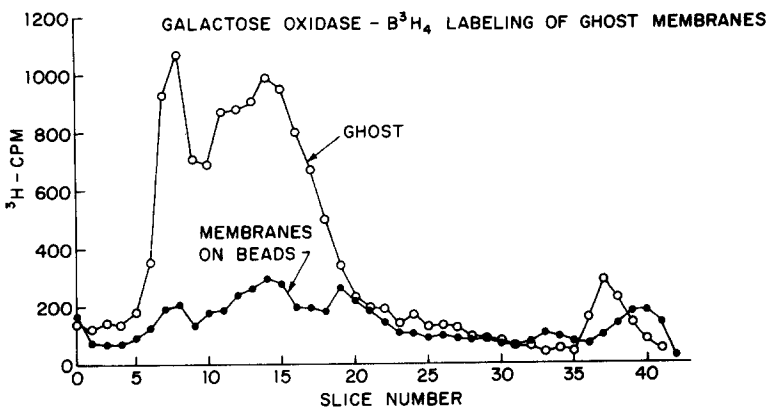


Fig. 5. Galactose oxidase and NaB^3H_4 labeling of ghosts and membrane on beads. 25 μl of 28 units/ml galactose oxidase and 20 μl of 5 μCi/ml NaB^3H_4 was added to either 500 mg of membranes on beads or an equivalent amount of ghost protein (210 μg) in a final volume of 300 μl 20 mosM phosphate buffer, pH 7.6. After a 1 h incubation at room temperature, followed by three washes in cold buffer, the membranes were solubilized and aliquots containing approximately 15 μg of protein were run on a 7.5% polyacrylamide gel, sliced and counted as described in Methods.

extracellular surface of the erythrocyte membrane [17], was removed from ghosts by a 2 h incubation in neuraminidase, only 20—25% was removed from membranes attached to beads (Fig. 6). This difference cannot be ascribed to a selective loss of sialic acid during membrane isolation on beads because the total sialic acid to protein ratio of membranes on beads was identical to that of ghosts (80 nmole sialic acid/mg membrane protein). The diminished hydrolysis also cannot be attributed to a non-specific inhibition of the enzyme because, as with galactose oxidase, neuraminidase in the presence of a 1 : 1 mixture of free ghosts and membranes on beads gave additive results (Table III). Furthermore, when inside-out vesicles rather than erythrocytes are attached to beads, sonicated, and washed as usual, 77% of the total sialic acid was removed (Table III). The remaining sialic acid, roughly 20% of the total, corresponds to the percentage of right side-out vesicles which contaminated the inside-out vesicle preparation (as determined by neuraminidase accessibility). Sialic acid present on these vesicles would be apposed to the beads and, therefore, not accessible to neuraminidase.

TABLE II

GALACTOSE OXIDASE SPECIFIC ACTIVITY OF LABELING

Specific activity of labeling with galactose oxidase and NaB^3H_4. 500 mg of erythrocyte membranes on beads or an equivalent amount of ghost protein (210 μg) were labeled as described in Fig. 7. After washing and solubilization, a 100 μl sample was removed for gels and the rest of the tube was flushed with Aquasol and counted. Protein was determined as described in Methods on parallel tubes.

	cpm/mg	%
Ghosts	433 666	100
Ghost membrane isolated on beads	99 812	23
1/2 Ghosts + 1/2 membranes on beads	208 582	
(Prediction: 433 000/2 + 99 000/2) =	255 000	

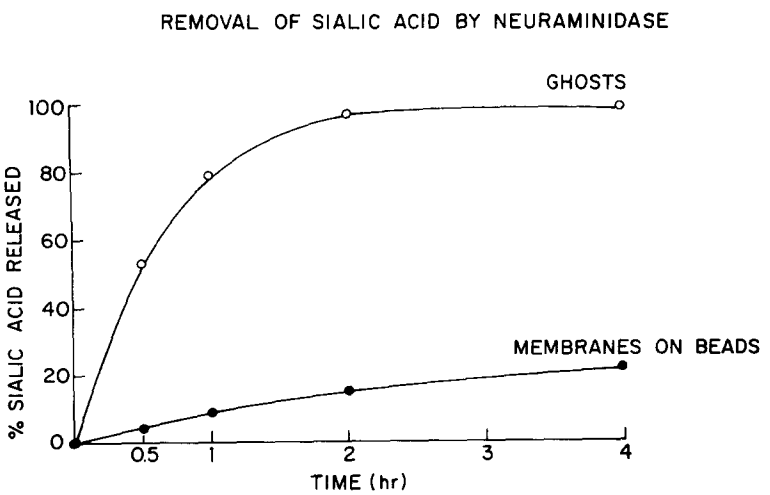


Fig. 6. Removal of sialic acid from ghosts and erythrocyte membrane isolated on beads by neuraminidase. 20 μl of 0.1 mg/ml neuraminidase was added to either 700 mg of membrane on beads or to an equivalent amount of ghost protein (275 μg) in a total volume of 200 μl of 20 mosM phosphate buffer, pH 7.6. Incubations were performed at room temperature as described in Methods. Total sialic acid was determined on parallel samples. All points are the means of three determinations.

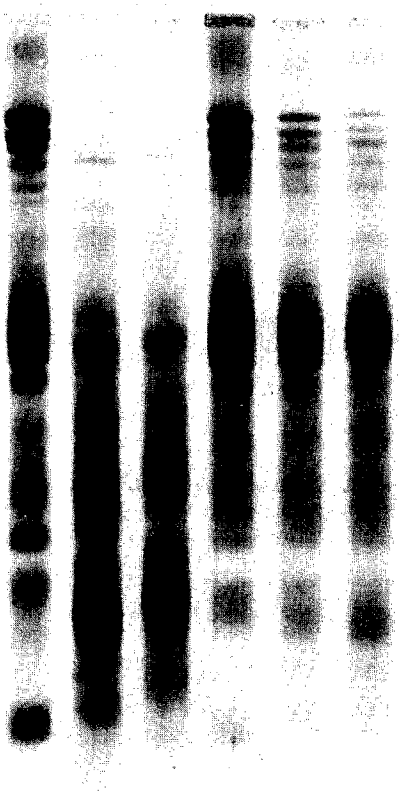
Differential assessibility was also demonstrated with trypsin. All major erythrocyte proteins are cleaved when trypsin is applied to inside-out vesicles [18]. Band 3 and spectrin are particularly sensitive to proteolysis at the protoplasmic surface of the membrane [18]. When erythrocytes were attached to the beads and their membranes isolated and incubated in 0.3 μg/ml trypsin, nearly all of the band 3 and spectrin polypeptides were cleaved (Fig. 7, A—C). In contrast, when inside-out vesicles were attached to beads and their membranes isolated and incubated under identical conditions, only 25% of band 3 and 60% of spectrin was cleaved (Fig. 7, D—F). (Although we used phenylmethylsulfonyl-

TABLE III

REMOVAL OF SIALIC ACID

Removal of sialic acid from 50 μl packed ghosts, 20 μl inside-out vesicles and 350 mg of beads with attached membrane (isolated from either ghosts or inside-out vesicles). 20 μl of 0.1 mg/ml neuraminidase was added to the membrane sample in a total volume of 200 μl of 20 mOsM phosphate buffer, pH 7.6. Samples were incubated for 2 h at room temperature and then the tubes were immersed in boiling water for 2 min. Total sialic acid present was determined by adding 200 μl of 0.4 M H_2SO_4 to parallel samples and heating to 80°C for 1 h.

	Total sialic acid (nmol)	Sialic acid removed by neuraminidase (nmol)	Percent sialic acid removed by neuraminidase
Ghosts	12	10	83
Ghost membrane on beads	12	3	25
1/2 Ghosts + 1/2 membranes on beads	12	6	50
Prediction: (10 + 3)/2		6.5	57
Inside-out vesicles	10	2	20
Inside-out vesicles on beads	13	10	77



A B C D E F

Fig. 7. 5% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of erythrocyte (A, B, C) or inside-out vesicle (D, E, F) membranes on beads, subjected to proteolysis by trypsin. 300 mg of beads containing 50 μg ghost protein or 42 μg inside-out vesicle protein were treated with trypsin at a concentration of 0 μg/ml (A, D), 0.3 μg/ml (B, E) or 0.6 μg/ml (C, F). Incubation and extraction for gels performed as described in Methods.

fluoride, some of the spectrin cleavage may be due to residual trypsin activity. We have noted that spectrin is particularly sensitive to proteolysis during dissolution in sodium dodecyl sulfate if even trace amounts of trypsin are present.)

Discussion

Our observations indicate that erythrocyte membranes isolated on polylysine coated beads retain many of the structural features usually associated with the native membrane. Although some extrinsic proteins may be selectively lost during extended sonication (not shown), the biochemical and morphological

evidence suggests that relatively large intact patches of membranes, not just selected membrane components, remained attached to the bead during the isolation process. Furthermore, many of the protein components appear to retain the same asymmetric distribution manifest in intact cells or ghosts. As a consequence, proteins that are usually located at the extracellular surface show reduced accessibility when this surface is apposed to a bead, whereas proteins that are located at the protoplasmic surface in the intact cell become fully accessible. Whether the lipids also retain their asymmetric distribution when the membrane is attached to the bead remains to be determined.

It is remarkable that the usual membrane impermeant probes that detect asymmetry in closed semipermeable vesicles can detect diminished accessibility in the presumably open-edged membrane patches that are isolated by the bead technique. Although some sealing may occur as a result of fusion between the torn edges of neighboring patches, simpler mechanisms may explain limited accessibility. For example, close association of the membrane with the bead may restrict diffusion of substrates or probing reagents so that passage to their target site under the membrane is prevented or retarded. Alternatively, there may be no restrictions at all on diffusion, but rather the enzyme itself may be inhibited in the immediate proximity of the positively-charged polylysine-coated bead. However, the latter alternative does not explain the observation that the acetylcholinesterase activity of erythrocyte membranes attached to polyacrylamide beads prepared as described in ref. 1, was equal to the activity of free ghosts (not shown). These beads contain a comparable amount of bound polylysine but, unlike glass, are porous to the acetylcholinesterase substrates. Further work is, therefore, required to determine why apposition to the bead diminishes probe accessibility. For the purpose of investigating membrane asymmetry the source of diminished accessibility is of little consequence. As long as the two membrane surfaces exhibit differential reactivity, this differential can be exploited to study membrane asymmetry.

Preliminary experiments suggest that the accessibilities we have investigated here can be further diminished by filling in the spaces between the membrane patches on beads with negatively-charged sonicated lipid vesicles. Increasing the size of the probing molecule should also diminish its access to the bead-apposed membrane surface. Indeed, we have noted that the accessibility to the extracellular membrane surface of the acetylcholinesterase reagents which are relatively small is nearly twice as great as the accessibility of the larger molecules galactose oxidase and neuraminidase. We are currently investigating a range of variables so as to optimize the use of beads for analysis of membrane asymmetry.

Isolation on cationic beads minimizes several problems inherent in plasma membrane isolation. Typically, the plasma membrane represents a small fraction of the total cell membrane, differing only slightly in density from the other membranes. Bead isolation does not depend on the inherent density differences between cellular membranes, but rather specifically isolates plasma membrane because it is the only membrane which is exposed in the intact cell. Thus, the defining property of the membrane (that it is the external enclosing membrane) is the basis for its specific isolation. Furthermore, the orientation of the isolated membrane is known. Membranes isolated using other techniques may vesiculate or break up into fragments, leading to a mixture of inside-out

and right side-out membrane vesicles. Membrane which has been isolated on cationic beads is attached in one orientation, with the cytoplasmic surface exposed. Membrane isolation on beads should therefore be a useful adjunct to standard isolation methods.

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