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Erythrosomes: Large proteoliposomes derived from crosslinked human erythrocyte cytoskeletons and exogenous lipid

(liposomes/transport protein reconstitution/encapsulation systems/cytoskeletons/erythrosomes)

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ABSTRACT **Large (3-μm diameter) mechanically stable proteoliposomes (erythrosomes) were prepared in good yield by coating crosslinked erythrocyte cytoskeletons with phosphatidylcholine. The erythrosomes consist of the polypeptides designated band 1, 2, 3, 4.1 + 4.2, and 5 (less than 4% of the endogenous lipid) and enough added lipid to form a bilayer coating the surface. Electron microscopy shows only the large proteoliposomes in sealed preparations. The trapping of bovine serum albumin, mannitol, sucrose, glucose, cytosine arabinoside, and sodium in the erythrosomes was demonstrated, yielding an apparent volume of up to 100 liters/mol of phospholipid. This preparation possesses an effective diffusion barrier to glucose, sucrose, and sodium ion with half-equilibration times of 34, 29, and 170 hr, respectively. The results of the present study suggest that erythrosomes may be useful for membrane transport protein reconstitution and encapsulation systems.**

There is much interest in the reconstitution of biological transport into native and model membrane systems. Although transport proteins have been incorporated into intact cells (1), protein degradation, dilution, and other metabolic activity of the cells reduce the usefulness of this approach for many reconstitution studies. Ideally, if model membrane systems are to be used for reconstituted membrane transport, they should be of large volume, tight to the diffusion of small molecules, uniform in size, and available in large quantity. A variety of liposome preparations are available (2–4); while promising, no preparation currently in use possesses all of these attributes. Here we describe a model system in which glutaraldehyde-crosslinked human erythrocyte ghosts are extracted with detergent and used as a support upon which phospholipids are coated by the reverse-phase procedure (5) normally used for the preparation of liposomes. These proteoliposomes, which we term "erythrosomes," are large, stable, and easily prepared in quantity. The tight diffusion barrier and uniform size make them ideal for reconstitution studies of membrane transport protein and for encapsulation of large and small molecules.

MATERIALS AND METHODS

Phosphatidylcholine (PtdCho) was purified from egg yolk as described (5). ^{22}Na, [^{14}C]glucose, [^{14}C]sucrose, [^{14}C]mannitol, [^{14}C]PtdCho, and [^{3}H]Triton X-100 were obtained from New England Nuclear. Bovine serum [^{14}C]albumin was prepared from [^{14}C]acetic anhydride (6). Fluorescein isothiocyanate-conjugated bovine serum albumin and 3,3′-dioctadecylindocarbocyanine dye were obtained from K. Jacobson (Roswell Park Memorial Institute). Glutaraldehyde and Triton X-100 were from Sigma. Polyethylene glycol was from the J. T. Baker Company. Diethyl ether was freshly distilled from sodium bisulfite before use. All other chemicals were of reagent quality.

White ghosts were prepared by the method of Dodge *et al.* (7) from fresh or freshly outdated blood depleted of white cells. The packed ghosts, in 20 mM sodium phosphate (pH 7.4) were crosslinked by the addition of 100 mM glutaraldehyde for 5 min at 25°C and then were mixed with an equal volume of 5% Triton X-100 (50 mg/ml of packed ghosts) in balanced salt solution (BSS) containing 125 mM NaCl/5 mM KCl/3.75 mM $CaCl_2$/2.5 mM $MgCl_2$, and 10 mM Tris·HCl (pH 7.4). The incubation was carried out for 30 min at room temperature, and the mixture was layered over an equal volume of 10% (wt/vol) sucrose in BSS (pH 7.4). The cytoskeletons were pelleted by a 4000 × *g* 5-min centrifugation, and the supernatant and part of the sucrose cushion were aspirated. The pellet was collected and mixed with an equal volume of BSS, and the mixture was reapplied to a sucrose cushion. The crosslinked cytoskeletons then were mixed with an equal volume of 5% (wt/vol) polyethylene glycol 6000 in BSS (pH 7.4). At this stage, the residual Triton X-100 associated with the packed cytoskeletons was 0.08% (0.01 mg/mg of protein) as estimated with [^{3}H]Triton X-100 in the extraction solution. All solutions contained 0.02% sodium azide. This procedure was followed in all cases unless otherwise stated.

Crosslinked cytoskeletons were coated by a modification of the method as described (5). Egg PtdCho (20 μmol in chloroform per ml of packed cytoskeletons) was dried to a film by rotary evaporation. The lipid was dissolved in 2 vol of diethyl ether, and 1 vol of packed cytoskeletons in BSS and 1 vol of 5% polyethylene glycol 6000 in BSS (pH 7.4) were added. Sufficient agitation to produce an emulsion was provided by solvent boiling at reduced pressure with rotation, eliminating the need for the sonication step of the standard reverse-phase procedure. The ether was removed by rotary evaporation, and the aqueous phase containing coated cytoskeletons (erythrosomes) was incubated for up to 24 hr in a large volume excess (50- to 100-fold) of 5% polyethylene glycol 6000 in BSS (pH 7.4). The lipid-coated erythrocyte cytoskeletons were separated from any liposomes at this point by a 5-min 4000 × *g* centrifugation. Lipid not associated with the erythrosomes forms a floating pellet under these conditions, whereas the erythrosomes become tightly packed at the bottom of the centrifuge tube. For the trapping of macromolecules, the cytoskeletons were first incubated 30 min at room temperature with the macromolecule. They were then mixed with 5% polyethylene glycol 6000 in BSS (pH 7.4), and the coating was carried out as usual. This procedure was less satisfactory with small molecules because of the poor diffusion-barrier properties of the erythrosomes immediately

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Abbreviations: BSS, balanced salt solution; PtdCho, phosphatidylcholine.

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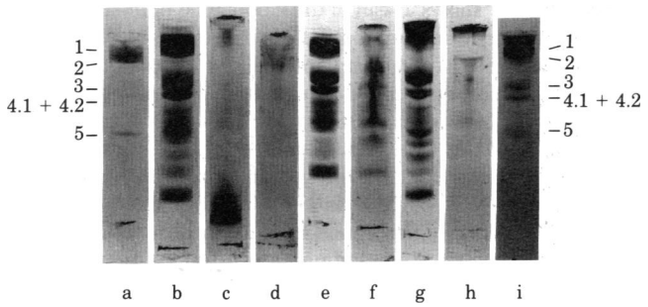

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of Triton shells prepared as described (14). Lanes: a, spectrin, actin, and band 4.1; b, normal erythrocyte ghosts; c, glutaraldehyde-crosslinked ghosts; d, glutaraldehyde cross-linked cytoskeletons; e, normal ghosts without dithiothreitol in the gel system; f, dithiobis(succinimidyl propionate)-crosslinked ghosts without dithiothreitol; g, ghosts crosslinked and reduced (100 mM dithiothreitol; 30 min); h, crosslinked cytoskeletons; i, reduced cytoskeletons. The dithiobis(succinimidyl propionate)-crosslinked cytoskeletons were crosslinked 30 min at room temperature with 20 mM reagent and extracted as if they were glutaraldehyde-crosslinked. All gels represent material derived from $2.5–5 \times 10^8$ cells run on 5.6% polyacrylamide gels (9). After reduction of the crosslinked ghosts, the major bands had identical mobility to that of the major bands of the untreated ghosts.

after coating. Small molecules were allowed to equilibrate after the vesicles were prepared, the uptake being monitored over time.

Erythrosomes were incubated overnight in a 50-fold vol excess of 5% polyethylene glycol 6000 in BSS (pH 7.4). The molecule to be trapped (e.g., sucrose or sodium) was added (final concentration, 0.5 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) to a 25% packed cell volume suspension of the erythrosomes at room temperature. Over time, 0.1-ml aliquots of the mixture were added to 5 ml of the above buffer and centrifuged at 4000 $\times$ *g* for 10 min. The whole pellet and a portion of the supernatant were assayed for radioactivity. Total volume was measured by centrifugation at 8000 $\times$ *g* for 10 min in a heavy-walled Plexiglas microhematocrit tube.

For preliminary determination of sealing, preparations were mixed with 2.5% sucrose/5% polyethylene glycol 6000 in BSS (pH 7.4) at 50% packed cell volume with 0.5 μCi of [^{14}C]sucrose per ml. The erythrosomes were centrifuged at 800 $\times$ *g* for 10 min, and a portion of the packed erythrosomes and the supernatant were sampled for radioactivity. The ratio of the radioactivity in the supernatant to that in the pellet was then used to approximate the sealed volume.

Lipid phosphorous was analyzed as described (8). Phospholipid extraction was carried out with chloroform/methanol, 2:1 (vol/vol). Thin-layer chromatography used chloroform/methanol/acetic acid/water, 52:20:7:3 (vol/vol), on neutral silica gel plates. Lipid spots were developed with sulfuric acid/ethanol, 1:1 (vol/vol), with charring at 180°C. In some experiments, [^{14}C]PtdCho was used as a tracer for phospholipid incorporation.

$NaDodSO_4$/polyacrylamide gel electrophoresis was carried out and the erythrocyte membrane polypeptide profile was numbered as described (9). Protein was estimated with the Bio-Rad protein assay (10).

RESULTS

When the proteins of the human erythrocyte ghost were crosslinked by treatment with glutaraldehyde and the ghost was extracted with high concentrations of Triton X-100 (50 mg/ml of ghost), crosslinked cytoskeletons were obtained lipid-free and in good yield. The cytoskeletons prepared by our procedure

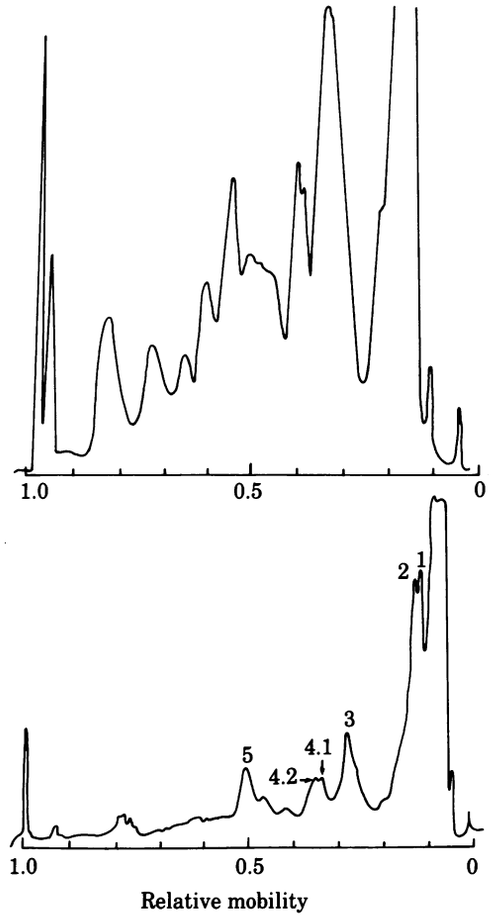

FIG. 2. Scans of Coomassie blue-stained 5.6% acrylamide gels of control ghosts (*Upper*) as in Fig. 1, lane b and of dithiobis(succinimidyl propionate)-crosslinked, 100 mM dithiothreitol-reduced cytoskeletons as in Fig. 1, lane i (*Lower*). Assuming a stain intensity proportional to mass for each of the bands and using the known values for the M_r of each of the polypeptides (9), we found the bands 1, 2, 3, 4.1 + 4.2, and 5 to be present in the mol ratio 1:1:1:1:2.

contained less than 4% of the native phospholipid (by Bartlett phosphorus determination); the remainder was in the Triton extract. This is consistent with studies (11) showing 2–8% of the ghost phospholipid becomes covalently attached to protein upon difluorodinitrobenzene or suberimidate treatment. Steck has shown cholesterol to be removed similarly by Triton treatment (12). The cytoskeletons contained 8 mg of protein per ml of packed cell volume.

Unlike cytoskeletons prepared without crosslinking (12–14), these cytoskeletons were very stable. They were not appreciably changed morphologically by centrifugation or by treatment with 0.1 M NaOH (30 min), ionic or nonionic detergents, or organic solvents. Fig. 1 compares the $NaDodSO_4$/polyacrylamide gel electrophoresis patterns of erythrocyte ghosts, glutaraldehyde-treated ghosts, and glutaraldehyde-treated cytoskeletons. The gel patterns of the glutaraldehyde-treated samples showed that most of the protein crosslinked to become high M_r complexes. To determine which proteins are crosslinked by amino-reactive reagents such as glutaraldehyde, ghosts were treated with dithiobis(succinimidyl propionate), a reversible amino-reactive crosslinking agent. The morphological characteristics (under the light microscope) of cytoskeletons obtained with this treatment were similar to those obtained with glutaraldehyde crosslinking. Upon $NaDodSO_4$ gel electrophoresis, proteins of dithiobis(succinimidyl propionate)-treated

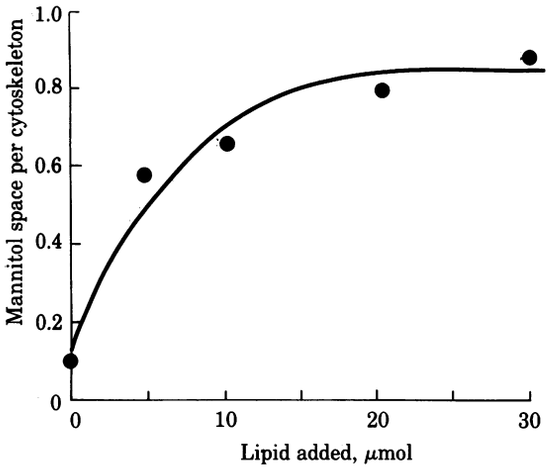

FIG. 3. Mannitol space (ml) per cytoskeleton (ml) as a function of added lipid. Cytoskeletons were incubated with [^{14}C]mannitol (final concentration, 0.07 μCi/ml), coated with phospholipid, and incubated 12 hr at 25°C. The erythrosomes were centrifuged at 650 × *g* for 10 min, and the supernatant was removed by aspiration. The pellet material was then added (100 μl of 50–60% packed cell volume) to 5 ml of 5% polyethylene glycol in BSS (pH 7.4) at 4°C, and centrifuged 8000 × *g* for 5 min. Packed cell volumes were measured in Plexiglas hematocrit tubes by a 5-min 8000 × *g* centrifugation. The data show an apparent saturation of trapped volume of 0.85 ml occurring at 10–20 μmol of phospholipid per ml of cytoskeleton. This value agrees with a value of 10–20 μmol/ml of cytoskeletons required for a bilayer on structures the size of erythrosomes and with surface properties similar to the erythrocyte. In the absence of cytoskeletons or in the presence of excess lipid, the lipid floats in a 4000 × *g* 5-min centrifugation. Bound phospholipid centrifuges with erythrosomes. [^{14}C]PtdCho binding to cytoskeletons is quantitative to 20 μmol of phospholipid per ml of skeleton.

ghosts and cytoskeletons were excluded from the gel, indicating extensive crosslinking. Upon reduction with dithiothreitol, the cytoskeletal gel pattern showed bands 1, 2, 3, 4.1 + 4.2, and 5.

Fig. 2 *Lower* shows a scan of the Coomassie blue-stained gel of the crosslinked and then reduced cytoskeletons of Fig. 1, lane i. By assuming equal staining intensity, an approximate mol ratio of 1:1:1:1:2 was obtained. An additional staining zone near the top of the gel was observed under these conditions. When the mobility of this zone was examined with a series of lower gel concentrations (15), the zone could be resolved into bands of approximately M_r 550,000 and M_r 700,000 and a minor band of M_r >800,000. Similar bands have been observed (16) and shown to be spectrin dimer, trimer, and tetramer, respectively. In our experiments, however, the relative abundance of the M_r 550,000 and M_r 700,000 bands and the apparent lack of other high M_r species, point to the possibility that these bands may also include bands 3, 4.1 + 4.2, and 5. The precise role of each of the proteins in lending structural stability to our crosslinked preparation or to the native membrane is yet to be elucidated.

The cytoskeletons were coated with lipid by evaporation of ether containing phospholipid in contact with an aqueous phase containing cytoskeletons. Triton X-100 is insoluble in the presence of polyethylene glycol 6000 (unpublished observation). Polyethylene glycol was routinely included in this study to precipitate residual Triton X-100.

Fig. 3 shows the apparent mannitol space as a function of added PtdCho. Saturation of the mannitol space occurred between 10–20 μmol of phospholipid per ml of cytoskeleton. The incorporation of PtdCho was quantitative under the same conditions up to ≈20 μmol/ml of cytoskeletons. In phase microscopy at high phospholipid, protein ratios showed the presence of some liposomes in addition to the erythrosomes.

The fluorescent lipid analogues, including dioctadecylindocarbocyanine, have received a great deal of attention recently for the measurement of lipid mobility in model membrane systems and in intact cells (17, 18). The dye is soluble only in organic phases and only fluoresces when in solution. When sealed erythrosomes coated with PtdCho were incubated with the dye, the erythrosomes became highly fluorescent (Fig. 4 *Left*). The specific association of the dye with the boundary of the erythrosome suggests that the surface of the cytoskeleton was

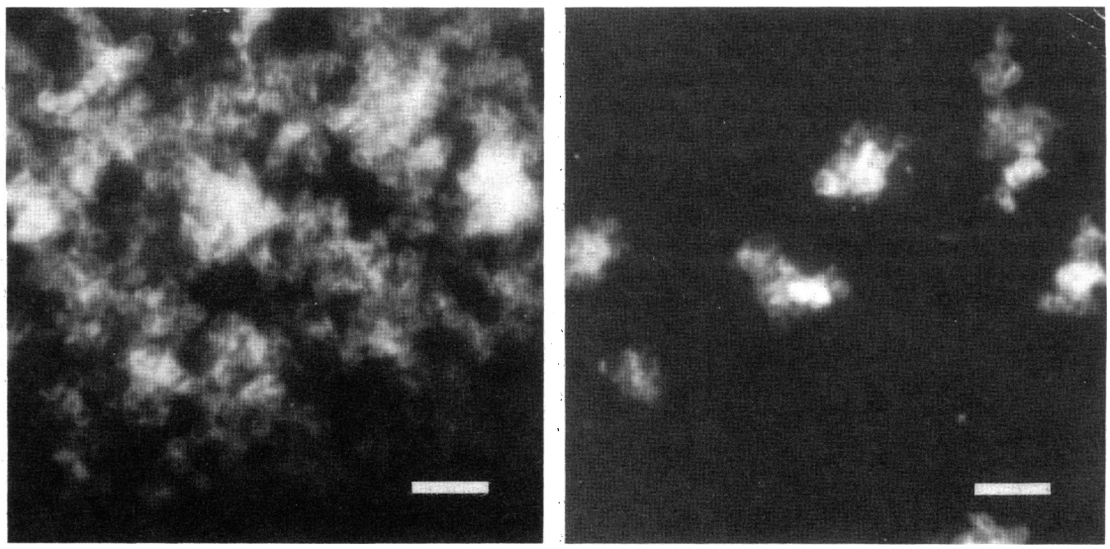

FIG. 4. (*Left*) Fluorescence microscopy of dioctadecylindocarbocyanine dye binding to sealed erythrosomes shows a highly fluorescent boundary, the dye associated only with the erythrosomes. Control experiments show some dye binding to cytoskeletons, but lipid coating greatly enhanced the dye binding. (*Right*) Erythrosomes were equilibrated with fluorescein isothiocyanate-conjugated bovine serum albumin (1 mg/ml) in the presence of radioactive albumin prior to the coating with PtdCho. After two washes in 5% polyethylene glycol in BSS followed by three washes in BSS, the structures remained highly fluorescent, whereas cytoskeletons treated similarly exhibited only the autofluorescence of crosslinked cytoskeletons. Of the radioactive albumin, 55% remained associated with the erythrosomes, while less than 1% remained associated with the uncoated cytoskeletons. (Bar = 10 μm.)

Table 1. Diffusion barrier properties of erythrosomes*

Properties	$t_{1/2}$, hr	Apparent volume, liters/mole
Time course		
[^{14}C]Sucrose (uptake)	29	85
^{22}Sodium (uptake)	170	90
L-[^{14}C]Glucose (efflux)	34	100
Trapping during preparation†		
Bovine serum [^{14}C]albumin (erythrosomes)		55
Bovine serum [^{14}C]albumin (cytoskeletons)		<1
[^{14}C]Sucrose (erythrosomes)		21
[^{14}C]Sucrose (cytoskeletons)		<1
[^{14}C]Mannitol (erythrosomes)		30
[^{14}C]Mannitol (cytoskeletons)		<1
[^{3}H]Cytosine arabinoside (erythrosomes)		35

* The efflux study is shown in detail in Fig. 5.

† Trapping of bovine serum [^{14}C]albumin was measured after five washes. Trapping of small molecules was measured after three washes over a 2-hr period.

coated with phospholipid. No structures of less than 0.5 μm were visible. Cytoskeletons treated similarly were only slightly fluorescent because of the dye binding to the lipid-free cytoskeletal proteins.

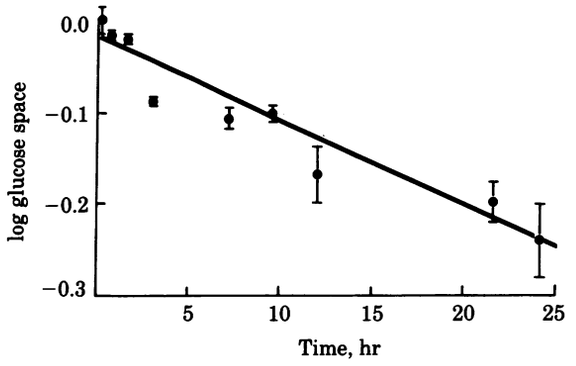

FIG. 5. L-Glucose flux from erythrosomes. Erythrosomes prepared from 10 μmol of PtdCho per ml of cytoskeletons were preincubated 24 hr in the presence of a 100-fold vol excess of 5% polyethylene glycol in BSS. The erythrosomes were incubated 48 hr with 0.05 μCi of L-[^{14}C]glucose (1 mM) per ml at 30% hematocrit. At the indicated times, 50 μl of the equilibrated suspension was added to 5 ml of 5% polyethylene glycol in BSS (pH 7.4). The mixture was centrifuged 8000 × *g* for 5 min; 1.0 ml of the supernatant was mixed with 1.0 ml of the buffer, and radioactivity was assayed in 10 ml of the scintillation fluid. The radioactivity associated with the supernatant and the pellet were compared with that of the packed cell volume. The L-glucose space is the ratio of the apparent L-glucose space (ml) (from radioactivity) to the packed cell volume (ml). A value of 1.0 was obtained, with a equilibration half-time of 34 hr.

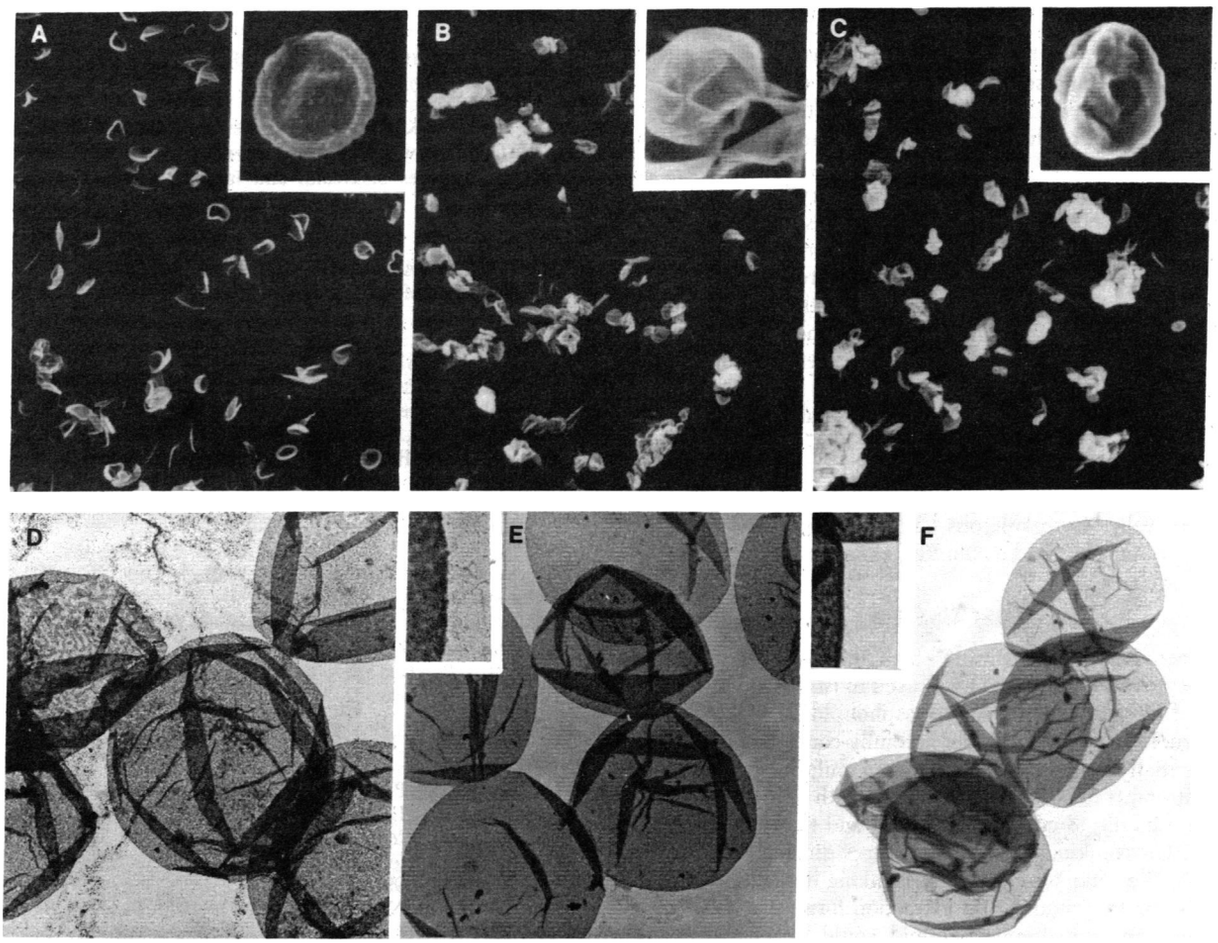

FIG. 6. Scanning electron micrographs of ghosts (*A*), cytoskeletons (*B*), and erythrosomes (*C*) prepared by critical point drying and gold coating of structures that had adhered to polylysine-coated coverslips. (×900.) (*Upper Insets*) Individual cells that were prepared by the same procedures after adhesion to coverslips that had not been coated with polylysine. (×4500.) Distinct reticula are present in each of the structures. Erythrosomes have obvious biconcave shape and rough surface properties. Transmission electron micrographs of ghosts (*D*), cytoskeletons (*E*), and erythrosomes (*F*) that were negatively stained with 1% uranyl acetate (pH 4.5) after adhesion to carbon-coated grids. (×4500.) (*Lower Insets*) Magnified view of structures from representative areas: a granular boundary on the cytoskeletons (*E*) and a smooth boundary on the erythrosome (*F*) indicating a lipid coating. (×16,000.)

When erythrosomes were formed in the presence of [^{14}C]bovine serum albumin, an apparent capture volume of 0.55 ml/ml of erythrosome was maintained after five washes. Cytoskeletons treated the same way had no radioactivity over background (Table 1). In the same experiment, fluorescein isothiocyanate-conjugated albumin also was included. The erythrosomes were highly fluorescent (Fig. 4 *Right*). Cytoskeletons treated similarly exhibited no fluorescence over the autofluorescence of the crosslinked cytoskeletons. A relatively heavy staining at the perimeter was evident, most likely caused by the biconcave-disc shape of the erythrosome. We have not ruled out the possibility that some binding of bovine serum albumin to phospholipid occurs. $NaDodSO_4$ treatment (2%) resulted in the loss of fluorescent bovine serum albumin from the erythrosomes. Sucrose, mannitol, and cytosine arabinoside were also trapped under the same experimental conditions, albeit with lower trapping efficiencies (Table 1).

Early experiments showed that the time course for the trapping of small molecules extends 12–24 hr for the maximal trapped volume. Immediately after lipid coating, only 20% of the maximal sealing was observed. Over time, sealing efficiency increased to a maximum of 85–100% of the packed cell volume. This increased trapping efficiency, over time, was seen only with small molecules and possibly reflects a slow organization of the lipid into a bilayer on the erythrosome surface. Fig. 5 shows the efflux of L-[^{14}C]glucose from the erythrosomes. The erythrosomes were incubated for 48 hr in buffer containing 5% polyethylene glycol and L-[^{14}C]glucose prior to the efflux experiment. The $t_{1/2}$ for the efflux of L-glucose was 34 hr. The uptake of sucrose and sodium exhibited a $t_{1/2}$ of 29 and 170 hr, respectively (Table 1). These data indicate that the erythrosomes are as tight to the diffusion of small molecules as intact cells are.

Fig. 6 compares the structure of human erythrocyte ghosts, crosslinked cytoskeletons, and erythrosomes. Scanning electron micrographs of erythrosomes show a structure similar to that of the ghost, with biconcave shape and rough surface. No discontinuities are evident in the structure at this magnification. Transmission micrographs of cytoskeletons show an outer edge with a granular appearance. In contrast, the erythrosomes possess sharply defined borders, suggestive of a lipid bilayer.

When erythrosomes were subjected to analysis by a Coulter particle counter, we obtained a mean size of 27 μm^3 and a counting efficiency of >95% (when compared to manual counting methods). The size compared favorably with the size estimated microscopically. Cytoskeletons had a size distribution similar to erythrosomes but had a counting efficiency of only 5%.

DISCUSSION

The selective extraction afforded by Triton X-100 treatment of erythrocytes and ghosts has been used to isolate structures depleted of integral proteins and phospholipid (12–14). The limiting structures obtained under carefully controlled conditions are essentially lipid-free. We have been uniformly unsuccessful in our attempts to coat these structures with lipid because they are quite fragile to centrifugation (12) and to the coating procedure. Crosslinking of these structures did not improve the stability. We report here that crosslinking human erythrocyte ghosts prior to Triton X-100 extraction formed a stable cytoskeleton upon which phospholipid could be coated. These crosslinked cytoskeletons are also essentially lipid-free and yet are quite resistant to centrifugation damage, ionic and nonionic detergent, 0.1 M NaOH, and organic solvent. The recovery of protein is high. The observed stoichiometry between the isolated proteins suggests the existence of specific complexes in the membrane composed of the protein bands 1, 2, 3, 4.1 + 4.2, and 5, although reassociation studies of the individual proteins will be required to prove this point.

The cytoskeletons presumably organize phospholipid in the native membrane (19). The binding of PtdCho by the cytoskeletal proteins confirmed this possibility. The time course for sealing to small molecules, as opposed to macromolecules, suggests a complex mechanism for the formation of a tight bilayer structure. The coating process may involve fusion of vesicles and interaction of micelles with the cytoskeleton. It is clear that the crosslinked cytoskeletons provide structural support for the lipid association with the erythrosome. The behavior of the lipid probe (dioctadecylindocarbocyanine dye) in fluorescing only at the boundary of the erythrosome, calculations of lipid:surface area ratios, scanning and transmission electron microscopy, and the diffusion-barrier properties all support the argument that the lipid in the erythrosome exists in a bilayer form, coating the entire cytoskeleton.

The solute-trapping volume of erythrosomes, 100 liters/mol of phospholipid, is at least 4 times higher than that of available liposomes (0.23–22.5 liters/mol of phospholipid) (5, 20).

The utility of the erythrosome as a model system for the reconstitution of membrane transport should await protein incorporation. However, the large internal volume (small surface-to-volume ratio that allows quantitative flux measurement), the tight diffusion barrier, uniformity in size, stability, and ease of preparation make erythrosomes attractive for such studies. These properties and the potential ability to modify the surfaces of the erythrosome also make them useful for encapsulation systems for *in vitro* and *in vivo* use.

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