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ISOLATION AND CHARACTERIZATION OF LARGE (0.5–1.0 μm) CYTOSKELETON-FREE VESICLES FROM HUMAN AND RABBIT ERYTHROCYTES

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Large (0.5–1.0 μm) cytoskeleton-free vesicles were obtained, by 'budding', from fresh human and rabbit erythrocytes incubated at 45°C and titrated with EDTA and $CaCl_2$. This process occurs without hemolysis. The isolated vesicles maintain their cytoplasmic integrity and normal membrane orientation, and are resistant to hemolysis over the pH range 5.0–11.0 and temperature range 4–50°C. The only membrane proteins detected in vesicles from human erythrocytes were band 3 region polypeptides and bands PAS-1, PAS-2 and PAS-3. Vesicles obtained from rabbit erythrocytes were similarly simple. Because of their size and stability these vesicles are amenable to both kinetic and quantitative analysis using the same experimental techniques employed in studies of synthetic lipid membranes. The results obtained in this study indicate that these vesicles are essentially markedly simplified biological cells, and thus may be useful as a biologically relevant model membrane system for examining the molecular interactions which occur within, across and between cell membranes.

Introduction

An essential prerequisite for understanding the molecular interactions which underlie and govern the biochemical and biophysical properties of biomembranes is the choice, or development, of a simple well-characterized, yet biologically relavent, model membrane system. One approach to meeting this prerequisite, which also emphasizes biological concerns, is the use of 'relatively simple' cells. Of these, erythrocytes are perhaps the most extensively employed example. Although the plasma membranes of erythrocytes are not compositionally defined, they are well-characterized and much has been learned from studies on erythrocytes about the interactions of various membrane components within a cell, including lipid bilayer asymmetry [1–5], cytoskeleton membrane interactions [6–11], and membrane transport [12–15].

Recently, erythrocytes have also been used as a model system for examining cell-cell interactions, including membrane aggregation and fusion [16–28]. These later studies, however, suffer from a number of problems in data interpretation, including: (A) the essentially qualitative nature of the studies, which have been predominantly based upon end product results obtained by light and electron microscopy, (B) the difficulty in distinguishing between phenomena which involve the lipids and/or proteins of the membrane proper, and those which are consequences of cytoskeleton-membrane interactions, and (C) the still excessive

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heterogeneity of the membrane constituents, especially proteins, which make it very difficult to ascertain the roles of specific components in membrane-membrane interactions.

To alleviate these problems we have developed a simple method for obtaining large (0.5–1.0 μm) cytoskeleton-free vesicles from intact human or rabbit erythrocytes. The vesicles maintain their cytoplasmic integrity and normal membrane orientation. In addition to the absence of cytoskeleton associated proteins, these vesicles also have markedly simplified protein compositions. Moreover, because of their size and stability the vesicles are amenable to both kinetic and quantitative analysis using the experimental techniques employed in studies with synthetic lipid membrane systems. These characteristics suggest that these vesicles may be very useful as a biologically relevant model membrane system for examining the molecular interactions which occur within, across, and between cell membranes. The purpose of this paper is to describe the method developed to generate and isolate these vesicles and to biochemically characterize them. Part of the results obtained in this study were reported previously [29].

Materials and Methods

Fresh human blood was kindly supplied by the Veterans Administration Hospital, Buffalo, NY. Fresh rabbit blood was obtained by ear puncture using Sequester-Sol (Cambridge Chemical Products, Inc.) as an anticoagulant. The blood was used within 2–3 h, and not cooled below room temperature before use.

The erythrocytes were repeatedly washed by centrifugation (buffy coat removed) at room temperature in isotonic solution (150 mM NaCl) buffered with 10 mM Tricine (NaT_R^-, pH 7.4), followed by washing in sodium Tricine buffer containing 0.2 mM phenylmethylsulphonyl fluoride (PMSF) as protease inhibitor (NaT_R^+, pH 7.4). The erythrocyte pellets (5–6 ml) were then diluted with NaT_R^+ to 40 ml, and equilibrated to 45°C.

The large cytoskeleton-free vesicles were produced from washed erythrocytes by 'budding'. This was accomplished by titrating the cell suspensions with EDTA and $CaCl_2$. Since the important parameter in this titration procedure was the ratio of blood cells to EDTA and $CaCl_2$ molecules, and not a specific molar salt concentration, the progress of bud formation was monitored microscopically. The titration procedure involved the addition of EDTA (390 mM) and $CaCl_2$ (1.0 M) in increments. Initially 500 μl of EDTA was added, the sample was incubated 15 min, and then 500 μl of $CaCl_2$ was added. Subsequent additions, usually 200 μl, were done simultaneously, separated by 15-min incubation periods. Once approx. 50% of the cells were observed to be forming buds, EDTA and $CaCl_2$ addition was stopped, and the cell suspension was incubated another 15 min. At this point the buds had been pinched off from the cells ('mother cells'), and were free as large vesicles in the suspension. It should be noted that the entire budding and vesiculation process occurs without hemolysis.

Erythrocyte vesicles were separated from the remaining cells by sucrose gradient centrifugation. The vesicle/cell suspensions were layered onto discontinuous 60/40/30% (w/w NaT_R^-) sucrose gradients and centrifuged at 800 r.p.m. (No. 215 head-IEC clinical centrifuge) for 1 h at room temperature. The aqueous and 30% layers, containing vesicles and a few ghosts, were removed and washed twice in NaT_R^+ medium by centrifugation at $10000 \times g$ for 30 min at 4°C (retaining the pellets).

To remove any remaining ghosts the sample was resuspended in 10 ml of NaT_R^+ medium, layered onto another 60/40/30% (w/w) sucrose gradient, and centrifuged at 1200 r.p.m. (No. 215 head-IEC clinical centrifuge) for 1 h. The purified erythrocyte vesicles formed a red band at the aqueous/30% sucrose layer interface. Any erythrocyte ghosts remaining were found in the 30% sucrose layer. The erythrocyte vesicles were removed and washed twice with NaT_R^+ medium by centrifugation as above. The final erythrocyte vesicle pellet was resuspended in 500–1000 μl of NaT_R^+ and refrigerated.

In developing this procedure a number of parameters were varied. In addition to incubation at 45°C, other samples were tested at 25 and 37°C. Incubations at all three temperatures were also carried out in the presence of EDTA or $CaCl_2$ only. To ascertain if a reduction in pH, which occurs when EDTA and $CaCl_2$ are mixed, was

involved in the budding process, samples were incubated in the presence of $CaCl_2$ at 45°C at pH 5.5. To help determine the importance of using fresh, non-cooled cells equivalent experiments were conducted using 'Dodge' ghosts, old cells (more than 5 h old), and cells previously cooled to 4°C for more than 1 h.

The percentage of hemolysis during the formation of the erythrocyte vesicles was determined spectrophotometrically at 540 nm, with the supernatant obtained from the first wash after the initial sucrose gradient separation. Hemolyzed aliquots of intact erythrocytes were used as standards.

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Fairbanks et al. [30], as modified by Steck and Yu [31]. Gels were stained with Coomassie blue or periodic acid-Schiff reagent as described by Fairbanks et al. [30]. Protein bands were identified according to the nomenclature of Steck [32], using white ghosts obtained according to Dodge et al. [33], and purchased molecular weight marker proteins (Boehringer Mannheim GmbH-Biochemica) as standards. Gels were scanned using a Beckman spectrophotometer equipped with a linear transport system. Peak areas for each band were determined by weighing cut-outs of scan tracings. Protein was measured with the Bio-Rad protein assay [34], using bovine serum albumin as the standard.

Enzymatic modification of the erythrocyte vesicle membrane proteins was carried out with trypsin, EC 3.4.21.4 (Sigma - type XI), and chymotrypsin, EC 3.4.21.1 (Sigma - type II), according to the procedures of Elgsaeter and Branton [6] and Siegel et al. [35], respectively.

Erythrocyte vesicle membrane proteins were distinguished from cytoplasmically trapped proteins by osmotically shocking (diluting, 1 to 4) purified erythrocyte vesicle samples (in NaT_R^+) with T_R^+ (10 mM Tricine + 0.2 mM PMSF, pH 7.4). The diluted samples were stored at 4°C for 3–4 h, centrifuged at 100 000 × g for 1 h at 4°C, to pellet vesicle membranes, and separated into supernatant and membrane samples. To concentrate the supernatant proteins the sample was frozen and then partially thawed. The liquified portion of the sample (approx. 10% by volume), containing the concentrated supernatant proteins, was removed and used directly for SDS-gel electrophoresis.

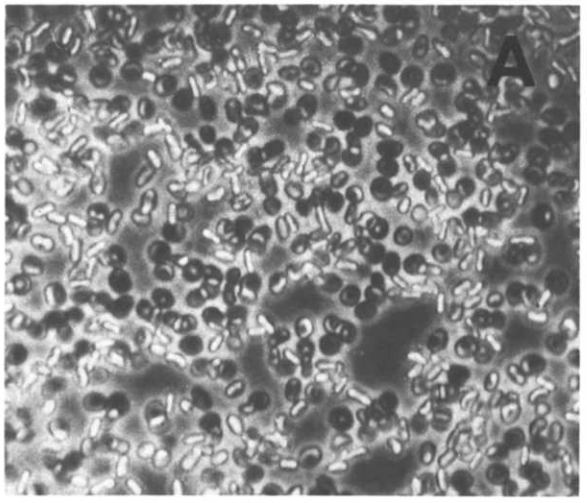
Erythrocyte vesicle and Dodge ghost lipids were extracted with chloroform/methanol (2 : 1, v/v) and the non-lipid contaminants removed according to the method of Radin [41]. Lipids were analyzed by two-dimensional thin-layer chromatography using chloroform/methanol/water (65 : 25 : 4, v/v) in the first direction and chloroform/methanol/acetic acid/water (25 : 15 : 4 : 2, v/v) in the second, on Silica gel G plates. Lipids were detected with I_2 vapor, and the phospholipids identified by comparing their R_f values to those obtained for pure standards chromatographed in the same solvent systems. Phosphate content was measured according to Bartlett [37]. Total cholesterol content was determined spectrophotometrically at 550 nm, according to Kates [38]. Diacylglycerol content of both erythrocyte vesicle and Dodge ghost lipid extracts was examined by thin-layer chromatography using benzene/diethyl ether/ethanol/acetic acid (50 : 40 : 2 : 0.2, v/v) as solvent, according to Allan and Michell [39].

Results

Isolation

When fresh intact human or rabbit erythrocytes are incubated at 45°C and 'titrated' with EDTA and $CaCl_2$ they undergo a series of morphological changes resulting in the release of large (0.5–1.0 μm) vesicles from the cells. This process is illustrated in Fig. 1, A–D. Starting with normal discoid erythrocytes (Fig. 1A) the cells first become spherical (Fig. 1B), and then begin to form 'buds' (Fig. 1C). These buds continue to enlarge and are finally pinched off from the cell bodies to become vesicles, free in solution. The vesicles can then be separated from the 'mother cells', unreacted erythrocytes, and any etythrocyte ghosts by sucrose gradient centrifugation (Fig. 1 D and E). It should be noted that this process of budding does not involve a general fragmentation of the erythrocyte. Usually only one bud was formed per cell, two buds were more infrequent, and three buds per cell were rather rare.

The requirements for this process seem to be quite specific, and include the use of fresh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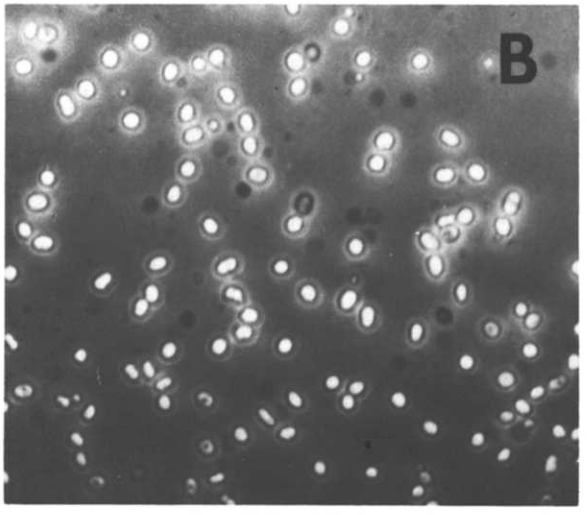
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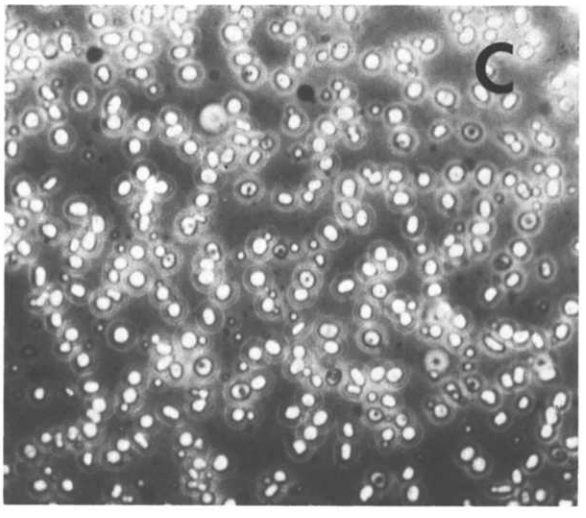
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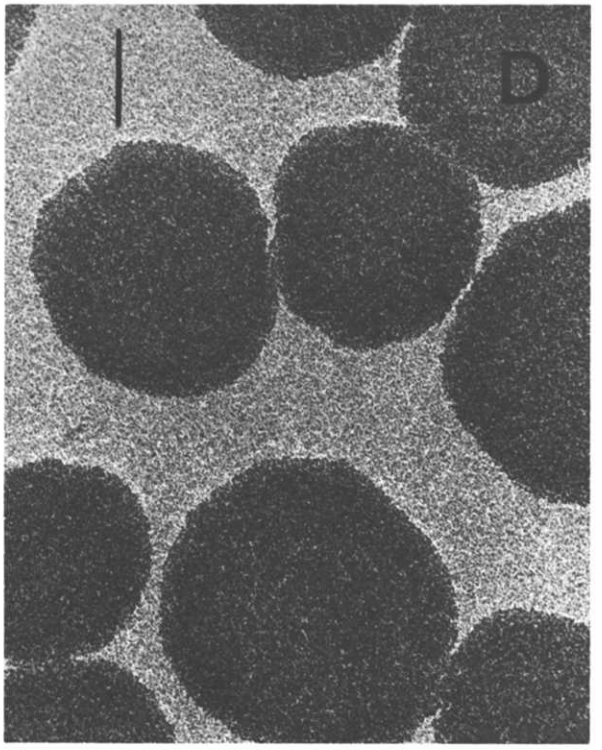
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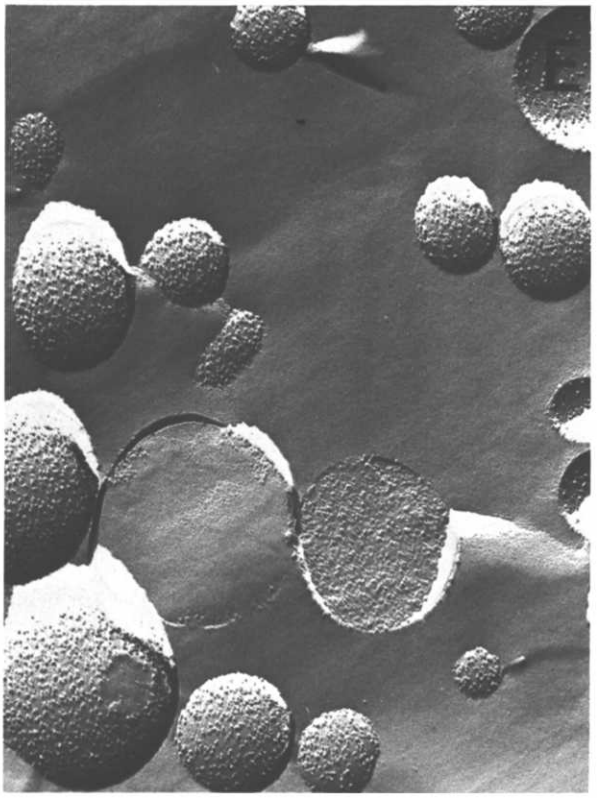
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erythrocytes and the combined presence of EDTA, $CaCl_2$ and a 45°C incubation temperature. Attempts to produce such vesicles using Dodge ghosts, old cells, or cells previously chilled were uniformly unsuccessful, with both old and chilled erythrocyte preparations instead exhibiting massive hemolysis. The incubation temperature was also found to be important. In parallel experiments conducted at 25, 37 and 45°C, erythrocytes maintained their discoid shape (same as Fig. 1A) after a 4-h incubation period at 25 and 37°C, long after vesicles had been formed at 45°C. However, increasing the incubation temperature to 45°C did not itself induce any morphological changes in erythrocyte shape or vesicle formation. Indeed, even when erythrocytes were incubated at 45°C in the presence of either EDTA or $CaCl_2$ only, they maintained their discoid shape and did not form buds or vesicles.

While examining the variables involved in erythrocyte vesicle formation a reduction in the pH of the incubation medium to 5.5 was observed as a consequence of EDTA and $CaCl_2$ addition. Control experiments were therefore conducted at 45°C in the presence of $CaCl_2$ only with erythrocyte suspensions previously adjusted to pH 5.5. The erythrocytes did not form buds or vesicles under these conditions, indicating that vesicle formation was not due to the presence of Ca^{2+} at low pH.

Analysis indicated that the cells and vesicles did not become leaky to hemoglobin during the process of bud and vesicle formation. In addition, the isolated vesicles were resistant to hemolysis over the temperature range 4–50°C and pH range of 5.0–11.0.

The relative yield of vesicles from an erythrocyte preparation, determined by comparing the average phospholipid content of six combined vesicle samples to that obtained for equivalent Dodge ghost preparations was 4.9%. It should be noted that this value was obtained for the purified vesicle preparations and dos not include losses incurred during the sucrose gradient and washing steps. The theoretical maximum yield calculated assuming every erythrocyte formed one vesicle, and no loss during isolation, was between 6 and 7%.

Characterization

The protein composition of the isolated vesicles from human and rabbit erythrocytes was examined by SDS-gel electrophoresis. Fig. 2 illustrates the reference gel pattern, demonstrating the results obtained with Dodge ghosts of human erythrocytes stained with Coomassie blue or periodic acid-Schiff reagent. The corresponding results obtained with human erythrocyte vesicles are shown in Fig. 3. Only four major protein bands and two minor components were observed with Coomassie blue (bands A–F). The distinguishing characteristic of the human erythrocyte vesicles is, however, the marked absence of proteins associated with the cytoskeletal network, specifically spectrin (bands 1 and 2) and actin (band 5). Of the proteins present the most abundant was hemoglobin (Band F_H), followed by band 3 protein (band A_H). The relative percentages of the protein components (excluding hemoglobin), stained with Coomassie blue, were: band A_H, $56 \pm 4\%$; band B_H, $8 \pm 2\%$; band C_H, $3 \pm 1\%$; bands $D_H + E_H$, $32 \pm 3\%$. The relative percentages of glycoproteins PAS-1, PAS-2, and PAS-3 were: 68%, 20%, and 12%, respectively.

The reference gel pattern observed for rabbit erythrocyte ghosts, stained with Coomassie blue, is illustrated in Fig. 4. The results obtained for rab-

Fig. 1. Photomicrographs and electron micrographs illustrating the process of bud and vesicle formation in human erythrocytes. The intact erythrocytes (A), first become spherical (B), and then begin to bud-off large vesicles (C). These vesicles can be isolated by sucrose gradient centrifugation to yield a vesicle preparation as shown in (D) and (E). (Note: The erythrocytes do not hemolyze during this process). Photomicrographs: A, B, C: 280×. (D) Electron micrograph of human erythrocyte vesicles obtained from the aqueous/30% sucrose interface, before washing by centrifugation. Human erythrocyte vesicles were negatively stained with 2% PTA in absolute ethanol, after being fixed with 4% glutaraldehyde for 24 h at 4°C. Bar = 1 μm. (E) Electron micrograph of a freeze-fractured sample of a 'purified' preparation (after repeated washing and both sucrose gradient centrifugation separations) of human erythrocyte vesicles, indicating the presence of proteins in the vesicle membrane. Freeze-fracturing done without cryoprotectant. Note: There is some reduction in the size of the vesicles in the final preparation due to the washing procedure. Magnification: 49455×.

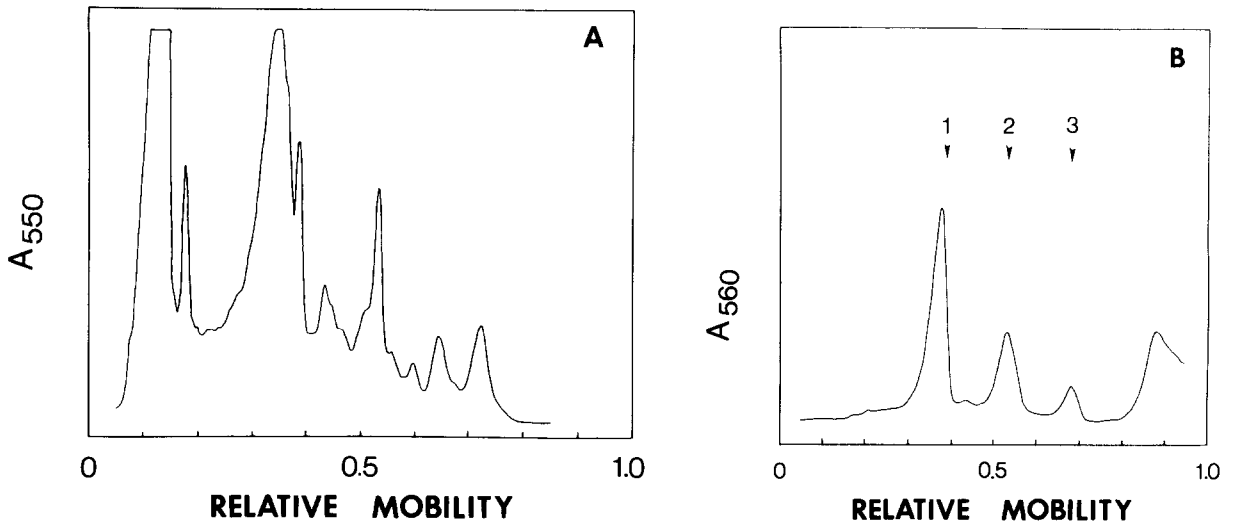

Fig. 2. 'Reference' SDS-gel electrophoresis recording of absorbance vs. relative mobility obtained for Dodge ghost proteins. (A) Densitometric scan of a representative gel stained for protein with Coomassie blue. Absorbance measured at 550 nm. (B) A similar gel stained for carbohydrate with periodic acid-Schiff reagent and measured at 560 nm. Peaks numbered 1, 2, 3 according to Steck [32].

bit erythrocyte vesicles are shown in Fig. 5. As is the case for human erythrocyte vesicles, the gel pattern obtained for rabbit erythrocyte vesicles stained with Coomassie blue, is markedly simplified in comparison with that observed for rabbit erythrocyte ghosts. Moreover, the rabbit vesicles are also characterized by the absence of proteins associated with the cytoskeletal network. The most abundant protein present was again hemoglobin (band E_R), followed by band 3 protein (band A_R). The relative percentages of the Coomassie blue-stained proteins in the rabbit

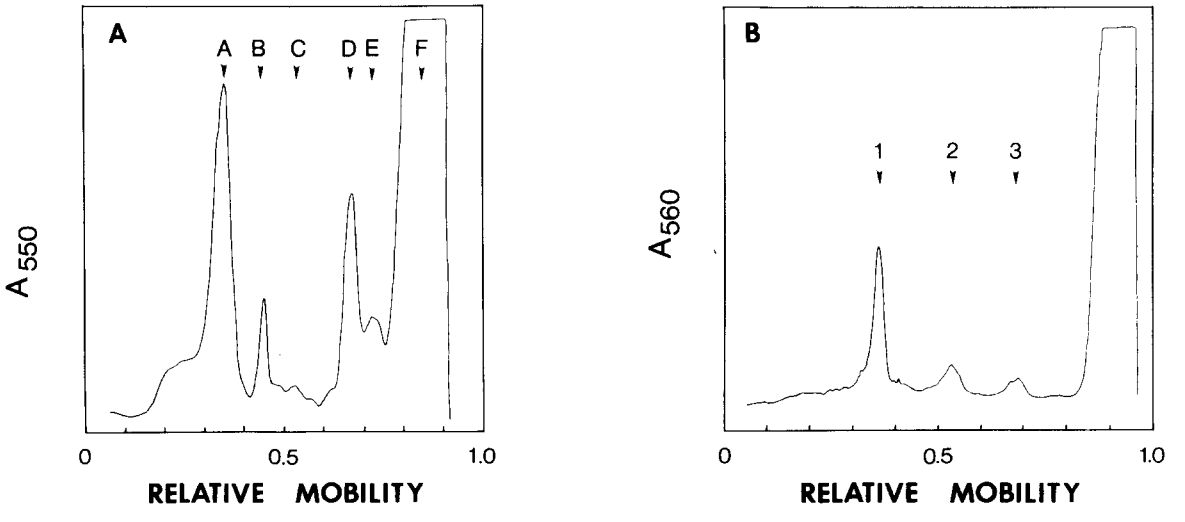

Fig. 3. SDS-gel electrophoresis recording of absorbance vs. relative mobility obtained for the isolated human erythrocyte vesicle proteins. (A) Densitometric scan of a representative gel stained for protein with Coomassie blue, and measured at 550 nm. (B) A similar gel stained for carbohydrate with periodic acid-Schiff reagent and measured at 560 nm. In (A) peak A = band 3 proteins; peak F = hemoglobin and tracking dye. In (B) peaks numbered 1, 2, 3 according to Steck [32] (Compare to Fig. 2).

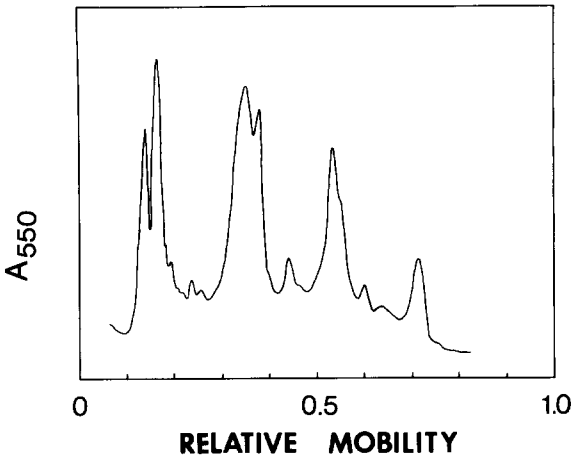

Fig. 4. 'Reference' SDS-gel electrophoresis recording of absorbance vs. relative mobility obtained for rabbit erythrocyte ghost proteins. The gel was stained for protein with Coomassie blue and measured at 550 nm.

vesicles (excluding hemoglobin) were: band A_R, $71 \pm 3\%$; band B_R, $5 \pm 3\%$; band C_R, $17 \pm 3\%$; and band D_R, $7 \pm 2\%$. Three periodic acid-Schiff reagent-positive bands were clearly distinguishable (Fig. 5b), with a possible fourth band being detected between R_M (relative mobility) 0.1 to 0.2. Their relative percentages were not calculated because of the excessive background noise.

Given the absence of cytoskeleton associated proteins, specifically band 1, 2, and 5, in both the human and rabbit erythrocyte vesicles the first question which arises concerns the fate of these proteins during vesicle production. One possibility is that these proteins were selectively segregated from that portion of the erythrocyte membrane which formed the bud and vesicle. A second possibility is that the absent proteins have been degraded during the vesicle-forming process. To help distinguish between these two possibilities the protein composition of the 'mother cell' fraction was analyzed by SDS-gel electrophoresis. The 'mother cell' is the major portion of the erythrocyte remaining after a vesicle was pinched off. If protein degradation is responsible for the absence of the cytoskeletal proteins, they should also be absent in the 'mother cell' fraction. If, on the other hand, their absence is due to their selective segregation during bud and vesicle formation they should be present in the 'mother cell' fraction. The results

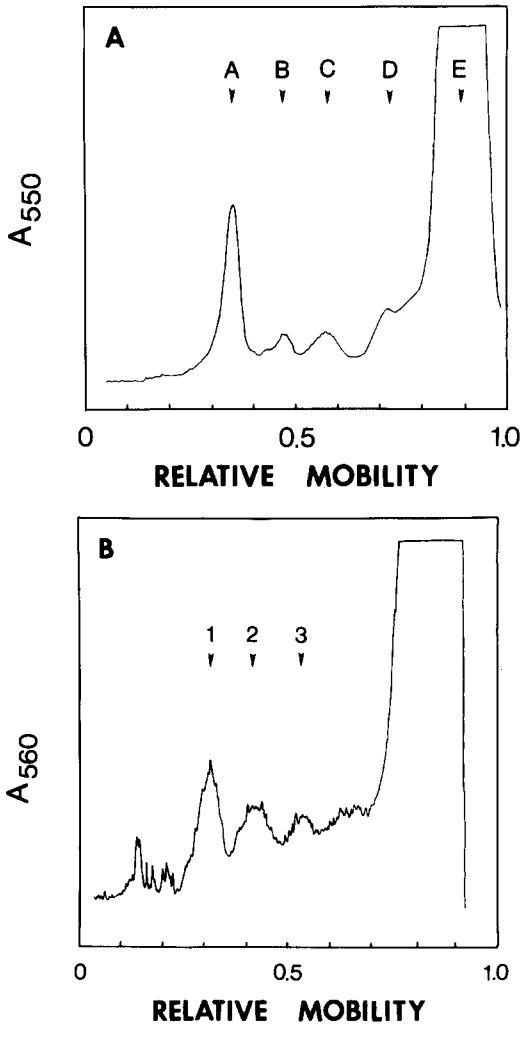

Fig. 5. SDS-gel electrophoresis recording of absorbance vs. relative mobility obtained for the isolated rabbit erythrocyte vesicle proteins. (A) Densitometric scan of a representative gel stained for protein with Coomassie blue, and measured at 550 nm. Peak A = band 3 region proteins; peak E = hemoglobin and tracking dye. (B) A similar gel stained for carbohydrate with periodic acid-Schiff reagent and measured at 560 nm. The three major glycoproteins detected are numbered 1, 2, and 3. A possible fourth glycoprotein may also be present between R_M 0.1 and 0.2. (Note: The periodic acid-Schiff reagent-stained gel pattern obtained for rabbit erythrocyte ghosts was the same as that shown in this figure for rabbit vesicles).

are shown in Fig. 6. The cytoskeleton associated proteins, bands 1, 2, and 5 are clearly evident, as are the other membrane protein bands observed in Dodge ghost samples. However, there is a marked reduction in the relative quantity of the band 3

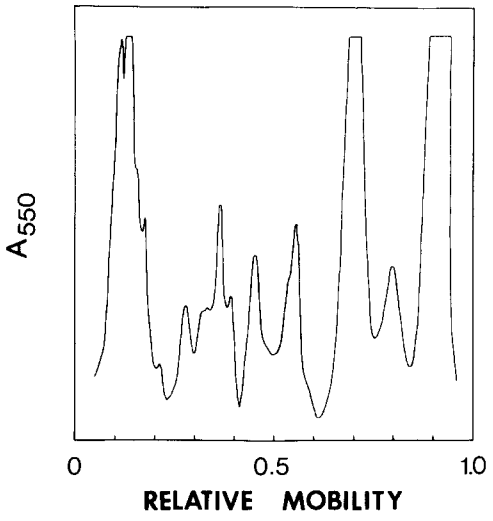

Fig. 6. SDS-gel electrophoresis recording of absorbance vs. relative mobility obtained for the 'mother cell' fraction of human erythrocyte proteins. The gel was stained for protein with Coomassie blue and measured at 550 nm. Note the presence of the cytoskeleton associated proteins, bands 1, 2, and 5 according to Steck [32] and the marked reduction in the percentage of band 3 region proteins present. Cytoplasmic proteins, including hemoglobin, are also present in this cell fraction.

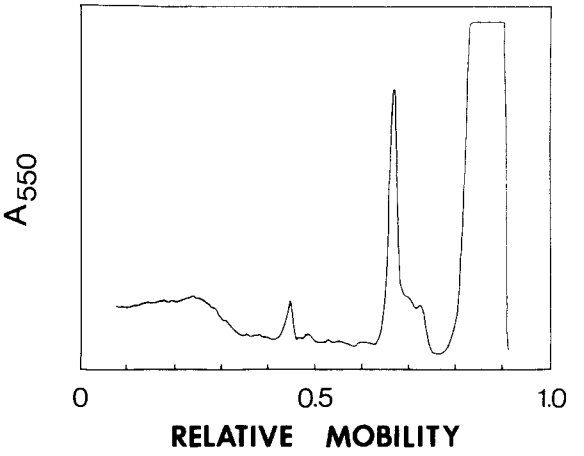

Fig. 7. SDS-gel electrophoresis recording of absorbance at 550 nm vs. relative mobility observed for the human erythrocyte vesicle proteins released by hemolysis, and stained with Coomassie blue. Compare with Fig. 3.

region proteins present in this cell fraction. Together these results strongly support a selective segregation hypothesis.

Human and rabbit erythrocytes, and consequently their vesicles, contain a number of cytoplasmic proteins in addition to hemoglobin, as well as membrane proteins [40,41]. To distinguish between these two protein classes human erythrocyte vesicles were osmotically shocked and the hemozylate supernatant analyzed by SDS-gel electrophoresis. Fig. 7 shows the gel pattern obtained for the released proteins. The results indicate that in addition to hemoglobin, band F_H, the proteins represented by bands B_H, C_H, D_H and E_H are also cytoplasmic in origin. Control experiments using the equivalent mother cell fraction gave the same results. The molecular weights of these proteins also match those obtained for the cytoplasmic proteins of normal erythrocytes [40,41].

During the process of formation and isolation the erythrocyte vesicles maintained their cytoplasmic integrity, at least with respect to hemoglobin and larger proteins, and therefore their natural membrane orientation. It is still possible, however, that the orientation of the proteins within the membrane, band 3 and bands PAS-1, PAS-2 and PAS-3 had changed during isolation, either as a consequence of the absence of the cytoskeletal network, or the 'movement' of the proteins into the vesicles. To examine this possibility, the susceptibility of band 3 proteins in human erythrocyte vesicles to enzymatic attack by trypsin and chymotrypsin was measured. If the human vesicle membrane is impermeable to trypsin and chymotrypsin (molecular weights 24 000 and 25 000, respectively) only the exterior surface of the vesicles will be accessible to enzymatic attack. The cleavage site for trypsin on band 3 protein, however, is located on the cytoplasmic side of the plasma membrane [42]. Consequently, trypsin should have no effect on band 3 protein molecules which maintain their native orientation. Chymotrypsin, in contrast, does cleave band 3 protein at the exterior surface of the membrane, which should, for those protein molecules that maintain their native orientation, result in the appearance of an approx. 65 000 molecular weight fragment at the expense of the original 95 000–100 000 molecular weight protein [42]. Thus, by examining the results obtained with these two enzymes, the orientation of the band 3 protein molecules could be studied. Unfortunately, this

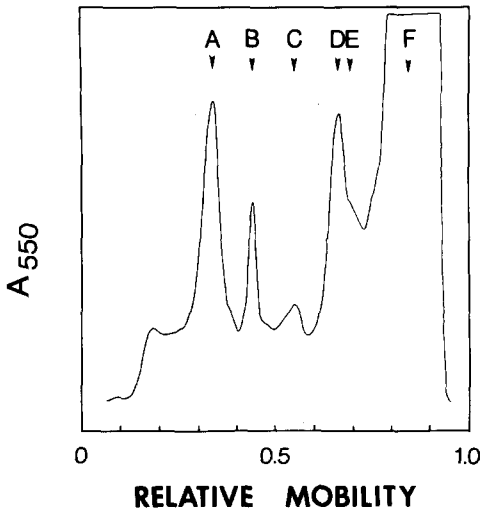

Fig. 8. SDS-gel electrophoresis recording of absorbance at 550 nm vs. relative mobility obtained for human erythrocyte vesicle treated with trypsin, and stained with Coomassie blue. Trypsin treatment was carried out as described in Materials and Methods. Nomenclature of peaks same as Fig. 3 (Compare to Fig. 3.).

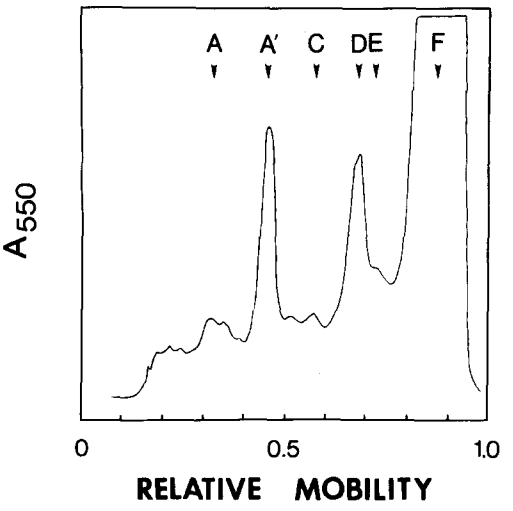

Fig. 9. SDS-gel electrophoresis recording of absorbance at 550 nm vs. relative mobility obtained for human erythrocyte vesicle treated with chymotrypsin, as described in Materials and Methods, and stained with Coomassie blue. Nomenclature of peaks A, C, D, E, F same as Fig. 3. Peak A′ is actually composed of two protein bands, peak A′, corresponding to the chymotryptic fragment of peak A, and peak B which is burried underneath (Compare to Figs. 9 and 3.).

experimental procedure could not be used for glycophorin A, band PAS-1, since it is susceptible to attack by both enzymes at the exterior surface.

The results obtained after trypsin treatment are illustrated in Fig. 8. The gel pattern, stained with Coomassie blue, is almost the same as that observed for untreated human vesicles (see Fig. 3). The results obtained after chymotrypsin treatment are shown in Fig. 9. In contrast to treatment with trypsin, chymotrypsin treatment of the human vesicle preparations caused a major change in the observed gel pattern. Peak A_H, representing band 3 proteins, has been greatly reduced, and a new peak A'_H has appeared. The molecular weight calculated for band A'_H, 66000–68000, is essentially the same as that predicted above. From the fact that the sum of $A_H + A'_H$ (Fig. 9) is equal to the sum of $A_H + B_H$ (Fig. 8) it is also clear that band A'_H is actually composed of two superimposed components, the band 3 protein fragment and the underlying band, B_H.

The lipid composition of the erythrocyte vesicles was examined by comparing the results obtained for human erythrocyte vesicles and Dodge ghost lipid extracts using two-dimensional thin-layer chromatography. The results indicated that the lipids present in the human erythrocyte vesicles are qualitatively identical to those present in Dodge ghost lipid extracts. However, analysis indicated an 11% (mol/mol) increase in the cholesterol-phospholipid ratio for human vesicles (0.94) relative to Dodge ghost lipid extracts (0.83). No diacylglycerols were detected in either human erythrocyte vesicles or Dodge ghost lipid extracts by thin-layer chromatography, even at very high lipid concentrations (240–785 μg lipid per spot).

Discussion

In this paper we describe a procedure for generating and collecting large cytoskeleton-free vesicles from intact human and rabbit erythrocytes. The vesicles maintain their cytoplasmic integrity and native membrane orientation, and are obtained without hemolysis. The isolated vesicles are resistant to hemolysis from pH 5.0–11.0 and 4–50°C. As noted earlier the conditions necessary for generating these vesicles are simple, but rather specific. They are: (1) the erythrocytes must be fresh and unchilled (Dodge ghosts could not substitute for intact cells), and (2) the combined presence of both EDTA and $CaCl_2$ and a 45°C incubation temperature.

Both human and rabbit erythrocyte vesicles were found to have markedly simplified protein compositions, relative to those obtained for the corresponding erythrocyte ghosts. Analysis indicated that the only membrane proteins present were band 3 region polypeptides and bands PAS-1, PAS-2, and PAS-3 in human vesicles, and band 3 region polypeptides and three or four glycoproteins in rabbit vesicles. The results obtained with human erythrocyte vesicles to ascertain the orientation of the membrane proteins in the isolated vesicles indicate that the band 3 proteins have retained their native orientation, in the absence of a cytoskeletal network. It should be noted, though, that we could not definitively exclude the possibility that minute amounts of band 4.5 region proteins were present in the human erythrocyte vesicles, but buried under the peaks B_H and C_H. However, if band 4.5 region proteins were present, they comprised a miniscule fraction of the total protein observed.

The results obtained under a variety of conditions indicate that the formation and simplified protein compositions of the human and rabbit erythrocyte vesicles, especially the absence of the cytoskeleton associated proteins, bands 1, 2, and 5 are not a consequence of proteolytic degradation. They include: (1) the presence of the protease inhibitor PMSF during vesicle formation and isolation, (2) the finding that vesicles were not formed in the presence of high Ca^{2+} concentrations at low pH, even at 45°C, (3) the absence of vesicle formation in the presence of both EDTA and $CaCl_2$ at 25 or 37°C (preliminary experiments using $CaCl_2$ plus the ionophore A23187 at 45°C were also unsuccessful) and, (4) the fact that vesicles were only formed when fresh unchilled erythrocytes were used. In addition, the clearest evidence eliminating proteolysis is the presence of the cytoskeleton associated proteins in the 'mother cells', which during vesicle formation is cytoplasmically continuous with the vesicle.

The possibility that vesicle formation is a consequence of a destabilization of the membrane's bilayer structure by diacylglycerols [39], or ATP depletion [43,44] is also quite unlikely. In contrast to the reported production of diacylglycerols by Ca^{2+} plus the ionophore A23187 in micro-vesicles released from echinocytes [39,45–48], no diacylglycerols were detected in human erythrocyte vesicles. Similarly, the required use of fresh unchilled erythrocytes is contradictory to that expected if a depletion of ATP were necessary.

It is clear from the shape changes which occur previous to budding and vesicle release, and the absence of bands 1, 2 and 5 in the isolated vesicles, that the erythrocyte's cytoskeleton is involved in the vesiculation process. Although the molecular mechanisms responsible remain unknown, the fact that only one vesicle was usually formed per erythrocyte suggests that the molecular forces driving the formation of these vesicles could be compensated for by changes in a localized area, which becomes a bud and eventually a large cytoskeleton-free vesicle. Such a result would seem to imply a role for mechanical and/or osmotic forces in vesicle formation, perhaps similar to that reported by Elgsaeter et al. [7]. In addition, the presence of band 3 proteins coupled with the absence of cytoskeleton-associated proteins in the isolated vesicles indicates that any potential molecular linkage between these two components must be broken during vesiculation. Finally, the need for fresh unchilled erythrocytes suggests that the erythrocytes have to be in a particular physical-chemical and/or biochemical state for vesicle formation. This state would be modified in some way when chilled or old erythrocytes are used, or erythrocytes are first converted into ghosts.

The goal of this study was to develop a model membrane system which: (1) retains many properties of a plasma membrane, (2) has a simple and well characterized chemical composition, (3) allows one to distinguish between phenomena which involve the lipid and/or proteins of the membrane proper, and those which are consequences of cytoskeleton/membrane interactions, and (4) is amenable to quantitative and kinetic methods of analysis. The large cytoskeleton-free vesicles isolated from erythrocytes in this study fulfill these criteria and therefore may prove to be an excellent model system for studying membrane phenomena, including membrane aggregation and fusion.

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