

REARRANGEMENTS OF INTEGRAL MEMBRANE COMPONENTS DURING IN VITRO AGING OF SHEEP ERYTHROCYTE MEMBRANES

HANS U. LUTZ, A. J. LOMANT, PAUL McMILLAN, and ERNST WEHRLI

From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545, and Abteilung Cytologie, Institut für Pflanzenbiologie, Universität Zürich, 8006 Zürich, Switzerland. Dr. Lutz's present address is the Biochemistry Department, ETH Zentrum, Swiss Federal Institute of Technology, 8006 Zürich, Switzerland.

ABSTRACT

In vitro aged sheep erythrocytes and sheep erythrocyte ghosts spontaneously release vesicles that consist of long protrusions affixed to flattened headlike structures. The intramembranous particles seen on the protoplasmic face of freeze-fracture electron micrographs of vesicle protrusions are arranged in paired particle rows. On the equivalent fracture face of headlike structures, the particle density is low; if particles are present, they are clustered along the rim of the flattened headlike structure and at the junction with the protrusion. The released vesicles are depleted of the intramembranous particles seen on the exoplasmic face of ghosts but retain almost exclusively particles of the protoplasmic face. Correspondingly, the exoplasmic face of ghosts that have released vesicles reveals a 28% higher density of intramembranous particles than that of fresh ghosts.

Purified vesicles are depleted of spectrin but retain integral membrane proteins, with one of an apparent mol wt of 160,000 accounting for nearly 50% of the total protein (Lutz, H. U., R. Barber, and R. F. McGuire. 1976. *J. Biol. Chem.* **251**:3500-3510). When vesicles are modified with the cleavable cross-linking reagent [³⁵S]dithiobis(succinimidyl propionate) at 0°C, the 160,000 mol wt protein is rapidly converted to disulfide-linked dimers and higher oligomers. Exposure of intact ghosts to the reagent in the same way fails to yield equivalent polymers.

A comparison of the morphological and biochemical aspects of ghosts and vesicles suggests that a marked rearrangement of membrane proteins accompanies the supramolecular redistribution of intramembranous particles during spontaneous vesiculation. The results also suggest that the paired particles of the protoplasmic face of vesicle protrusions are arranged in paired particle helices and contain the 160,000 mol wt protein as dimers.

Lipid-protein and protein-protein interactions in the plasma membrane have great impact on cellular function (4); even slight alterations in the pat-

tern of such complexities can grossly alter the physiology of the cell (2, 7, 20). When the probability of aberrant interactions is increased, as

would occur with a loss of stringent control in the assembly of membrane components, irreversible changes in cell behavior (generally referred to as the aging process) become more likely (3).

One of the simplest systems which exhibits age-dependent irreversible alterations in the organization of membrane-components is the anucleated erythrocyte. These cells, when aged in vitro, release "myelin forms" (1, 18). Such structures, which have been isolated from aged sheep erythrocyte ghosts (10), contain only integral membrane proteins within a preserved lipid bilayer but lack the peripheral membrane protein, spectrin. The preferential retention of integral membrane proteins implies that structural rearrangements of membrane components must precede vesiculation.

In the present report, we describe some aspects of the structural organization of the myelin forms derived from aged sheep erythrocytes as well as their ghosts. Comparison of the arrangement of intramembranous particles (ascertained by electron microscopy of freeze-fractured preparations) and of proteins (deduced through the application of a cleavable cross-linking reagent) within the vesicles to that observed with intact ghosts can provide some insight on the changes in the membrane of senescent cells that must occur before the spontaneous release of vesicles.

MATERIALS AND METHODS

Vesicle Preparation from Sheep Erythrocyte Ghosts

Sheep blood freshly collected in 2 mM EDTA was washed three times in 150 mM NaCl and 16.7 mM NaHCO₃ (pH 7.4). Ghosts were prepared by stepwise lysing the cells in bicarbonate buffer (75, 36, and 16.7 mM NaHCO₃ (pH 7.4)). White ghosts were incubated at 1 mg of protein per milliliter in 16.7 mM NaHCO₃ and 1 mM EDTA (pH 7.4) for 70 h at 2-4°C to release the spontaneously formed vesicles. Vesicles were isolated and washed as previously published (10). Protein was determined according to reference 9.

Vesicle Preparation from In Vitro Aged Sheep Erythrocytes

Washed sheep erythrocytes (see above) were aged in vitro at 20% hematocrit in the presence of 5 mM NaKHPO₄, 150 mM NaCl (pH 7.4) containing 0.02% sodium azide. The solutions were filtered through a Millipore filter (0.22- μ m pore size, Millipore Corp., Bedford, Mass.), and the cell suspensions were incubated in sterile Nalgene Erlenmeyer flasks (Nalgene

Labware Div., Nalge Co., Rochester, N. Y.). In control experiments, the suspensions were supplemented by 6 mM adenine, 10 mM inosine, and 0.25 g of glucose per liter. After varying lengths of time, the suspensions were centrifuged in order to pellet intact cells (7.5 min, 1,500 rpm in an International centrifuge (International Equipment Co., Boston, Mass.), $r_{\text{bottom}} = 20$ cm). The thus formed supernate was directly layered on a dextran density gradient ranging from $\rho = 1.01$ to $\rho = 1.085$ containing 10 mM NaKHPO₄ and 1 mM EDTA (pH 7.4). Vesicles with the properties given in Results are found at slightly higher densities ($\rho = 1.02$ to $\rho = 1.035$) than those isolated from sheep erythrocyte ghosts (10). In the experiment shown in Results, the total amount of protein found in washed vesicle fractions was 203 μ g after 28 h and 404 μ g after 54 h per milliliter of packed cells present in the incubation mixture. The corresponding control cells that were provided with an energy source yielded less than 10 μ g and 66 μ g, respectively, in the equivalent fractions.

Electron Microscopy

Negative staining with uranyl acetate was performed as published earlier (10). Freeze-etching was done on samples that had been pelleted from 25% glycerol and frozen in Freon 22 onto the gold platelet. For freeze-fracturing, a Balzers BA 360 apparatus (Balzers AG, Balzers, Liechtenstein) was used according to a slightly modified procedure of Moor (11). Micrographs were taken with a Hitachi HU-11E electron microscope. Magnifications were calibrated with a diffraction grating with 2,160 lines per millimeter. Particle sizes were measured vertical to the shadow at its widest part.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to Neville (12), using a lower gel and tray buffer of pH 9.18. The percentages of acrylamide are given in the text, except for the weight ratio of acrylamide to *N,N'*-methylene bisacrylamide that was kept at 80/3.

Cross-Linking of Membrane Preparations

Ghosts, isolated vesicles, and ghosts depleted of vesicles were modified at 0°C for the time indicated with the cleavable protein cross-linking reagent [³⁵S]dithiobis (succinimidyl propionate)¹ (DTSP) (8) in 11 mM NaKHPO₄ (pH 7.4). Acylation of amino groups was terminated by addition of taurine (to a final concentration of 11.5 mM) to quench unreacted active ester. Each reaction mixture contained the same amount and concentra-

¹ *Abbreviations used in this paper:* DMF, dimethylformamid; DTSP, dithiobis (succinimidyl propionate); SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl) aminomethane; and PF/EF, protoplasmic and exoplasmic face in electron micrographs of freeze-fractured samples, respectively.

tion of total protein (2.8 mg/ml) and was diluted 1:1 with DTSP which had been dissolved in dimethylformamid (DMF) and then brought to 4×10^{-4} M in 11 mM NaKHPO₄ (pH 7.4) (cf. reference 8). The final concentration of DMF in the samples was 0.25%. Quenched samples were solubilized in sodium dodecyl sulfate (SDS), and 50- μ g protein aliquots were incubated 30 min at 37°C in the presence or absence of 50 mM DTT before electrophoresis on 4% or 6% polyacrylamide gels. Three identical fractions were cut from unreduced 4% gels according to the stain distribution of a fourth gel. These gel pieces were immersed in 2.0 ml of 1% SDS, 20 mM DTT, 50 mM tris(hydroxymethyl)amino-methane (Tris)-H₂SO₄ buffer (pH 6.1), incubated for 1 h at 37°C, and then dialyzed vs. 5 mM Tris-H₂SO₄ buffer (pH 6.1) for several hours (two changes) at room temperature and then for 24 h at 4°C (two changes). The lyophilized material was taken up in 100 μ l of H₂O and 20 μ l of 5% SDS, 200 mM DTT dissolved in five-fold concentrated upper gel buffer, which also included tracking dye and marker protein from unmodified preparations. After an additional incubation of 30 min at 37°C, the samples were electrophoresed on 6% polyacrylamide gels. Stained gels of both the directly electrophoresed and reelectrophoresed samples were sliced into four pieces longitudinally, and the two 1.5-mm thick center sections were dried and exposed to X-ray films (NS 54 T Kodak) for up to 3 mo. Quantitation of the autoradiographs of gels run from these isolated fractions was performed by scanning the X-ray films with a Helena Quick Scan Instrument (Helena Laboratories, Beaumont, Texas). Areas below the peaks were automatically integrated, and these relative values were used to determine the composition of the original fraction derived from an unreduced gel.

The products of DTSP reaction, which include mercaptandisulfide interchange in addition to acylation, were distinguished by analysis of SDS-gel electropherograms processed with (+) or without (-) the reducing agent DTT. As originally demonstrated by Lomant and Fairbanks (8), label surviving reduction is exclusively a measure of end-on or bifunctional acylation events involving the active ester termini. In the present work, DTSP was applied in small excess over the estimated number of amino groups such that perturbations of the membrane structure were minimized (8). The acylation of component III reaches saturation after 2 min. At saturation, the yield of acylated component III is roughly proportional to the mass of this protein in each preparation (Fig. 1). This result indicates that the number of accessible amino groups in component III of each preparation is nearly constant.

RESULTS

Arrangement of Intramembranous Particles in Vesicles

The spectrin-free vesicles released from *in vitro*

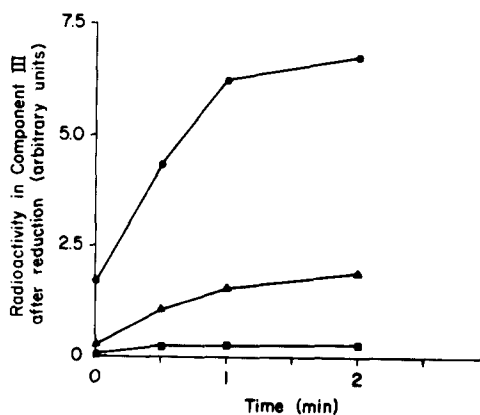


FIGURE 1 Kinetics of [³⁵S]DTSP acylation of protein component III in vesicles, ghosts, and ghosts depleted of vesicles. Radioactivity was measured by quantitation of the densitometric scans from autoradiographs of gels made from reduced preparations that had been modified with [³⁵S]DTSP for the indicated length of time. The conditions for cross-linking were as described in Materials and Methods, except that DTSP was 5×10^{-4} M. The time-course is shown for vesicles (●—●), ghosts (▲—▲), and for ghosts depleted of vesicles (■—■). The mass of protein component III in the three membrane preparations is in percent of the total membrane protein: 48% for vesicles, 20% for ghosts, and less than 11% for ghosts depleted of vesicles. These values were obtained by scanning Coomassie blue-stained electropherograms (10).

aged sheep erythrocyte ghosts are composed of large headlike structures and narrow protrusions of a diameter of 600 ± 50 Å (reference 10, see also Fig. 2). In freeze-fracture electron micrographs, the protoplasmic face (PF) of vesicle protrusions displays a very unique arrangement of intramembranous particles (Fig. 3a and c). Instead of a random distribution, rows of paired particles are evident. These rows are aligned at an angle of 45° to the long axis of the cylindrical protrusion and are spaced 297 ± 27 Å ($n = 30$). The mean diameter of individual particles of the paired particle rows corresponds to that observed on the PF face of ghosts (95 ± 8 Å [$n = 30$] for vesicles; 88 ± 19 Å [$n = 150$] for ghosts). The number of particles per surface area of the protrusion has been calculated² to be $5,600/\mu\text{m}^2$ and is thus higher than that in intact ghosts on the equivalent fracture face ($4,727 \pm 223$ particles/ μm^2).

² The number of particles on the protoplasmic face of vesicle protrusions has been calculated for a cylindrical surface with a diameter of 600 Å and the number of particles in one paired particle row (44).

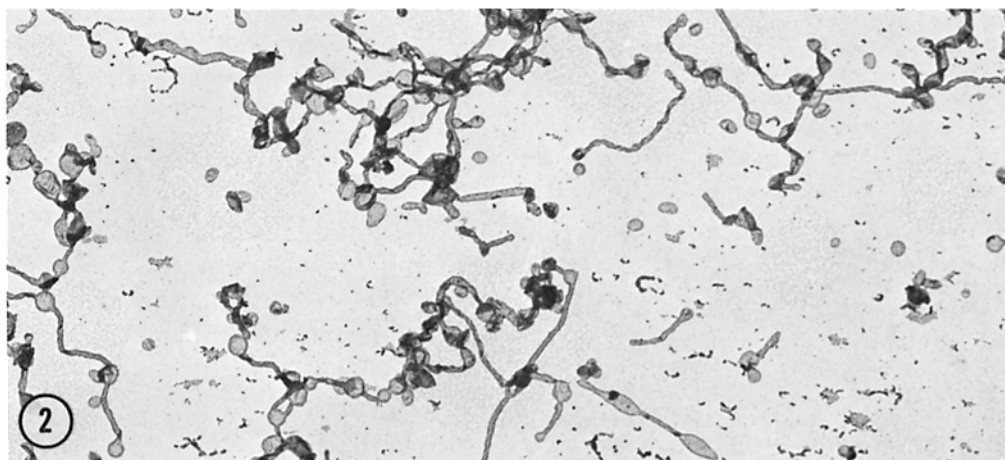


FIGURE 2 Electron micrograph showing vesicles from in vitro aged sheep erythrocyte ghosts stained with uranyl acetate. $\times 9100$.

The freeze-fracture electron micrographs suggest that the paired particles are arranged in a helix around the protrusion, although an alternate arrangement in rings cannot be excluded.

On the protoplasmic face (PF) of vesicle headlike structures, the particle density is low; if particles are present, they are clustered along the rim of the flattened headlike structure and at the junction with the protrusion (Fig. 3*b*).

The exoplasmic face (EF) of the entire vesicle is almost free of intramembranous particles (Fig. 3*a* and *b*). The particle density is at least fivefold lower than on the corresponding face of intact ghosts (varying between 0 and 140 particles/ μm^2).

The protoplasmic face (PF) of whole ghosts is randomly covered with particles (mean diameter $88 \pm 19 \text{ \AA}$) (Fig. 4*a*). There is no evidence for patches; pairs of particles may exist but cannot be distinguished from single particles because of the high density of the particles ($4,727 \pm 223$ particles/ μm^2). The exoplasmic face of ghosts has fewer particles (615 ± 113 particles/ μm^2 ; mean size $119 \pm 22 \text{ \AA}$, $n = 188$) than the protoplasmic face (Fig. 4*b* and reference 14 for human erythrocytes). If the equivalent fracture face (EF) is studied from ghosts depleted of vesicles, the number of particles per unit area is 28%³ higher than in whole ghosts (Fig. 4*c*). From these data, it is

³ The mean particle densities have been determined from 40 measurements each of arbitrarily chosen fields and are 615 ± 113 particles/ μm^2 for ghosts and 790 ± 130 for ghosts depleted of vesicles. The difference in the standard means is significant at the 5% confidence level.

concluded that in vitro aging of sheep erythrocyte ghosts causes intramembranous particles of the exoplasmic face to be unequally distributed so as to become enriched in ghosts that have released the vesicles and to be depleted in vesicles. Thus, the released vesicles contain intramembranous particles almost exclusively on the protoplasmic face in an arrangement entirely different from that in the original ghosts. The unique new organization of intramembranous particles implies a drastic redistribution of the randomly organized particles in both fracture faces during spontaneous vesiculation of sheep erythrocyte ghosts.

Spontaneous vesiculation also occurs in sheep erythrocytes that are deprived of energy sources and thus undergo ATP-depletion (17). When intact sheep erythrocytes age in vitro, vesicles are released that reveal the same paired particle rows on the protoplasmic face of the vesicle protrusions (Fig. 5). The protein composition of these vesicles is the same as that of the vesicles released from in vitro aged ghosts. They contain primarily components III, IV, and hemoglobin (Fig. 6) but decreased amounts of the minor Coomassie blue components seen in vesicles from ghosts. Thus, the paired particle rows found in vesicle protrusions are not just an artifact observed with ghosts but would seem to represent a final state of the membrane when the intact cell ages in vitro.

The highly ordered supramolecular structure in vesicle protrusions should have its molecular correlate. In an attempt to detect an altered molecular organization of integral membrane proteins, we have studied the cross-linking of these proteins

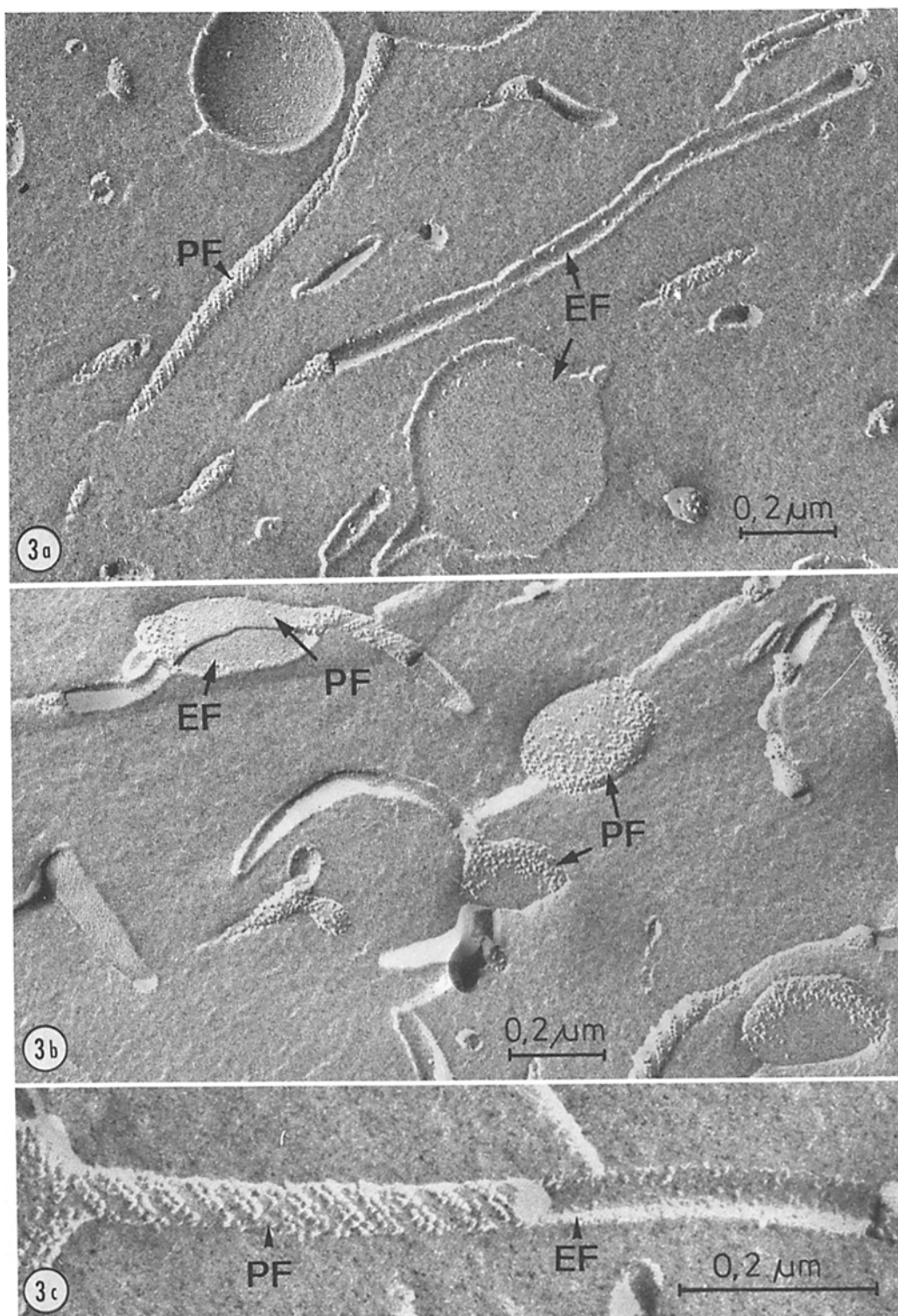


FIGURE 3 Electron micrographs of freeze-fractured samples from vesicles of in vitro aged sheep erythrocyte ghosts. (a and b) $\times 70,000$; and (c) $\times 150,000$.

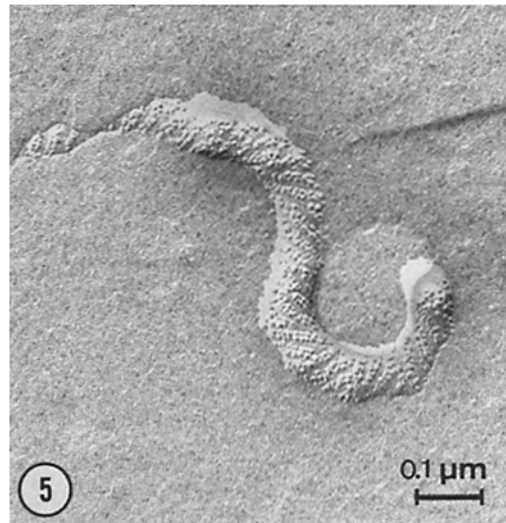
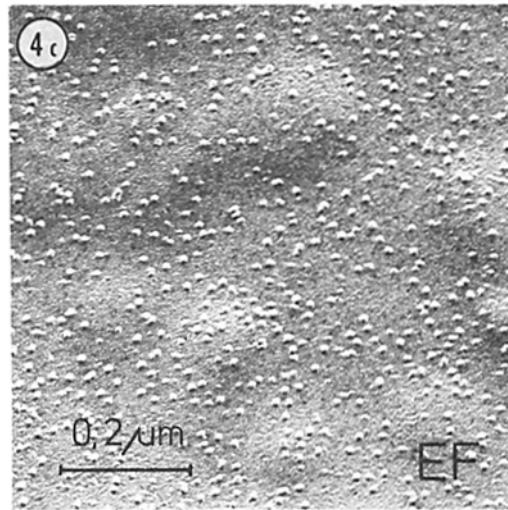
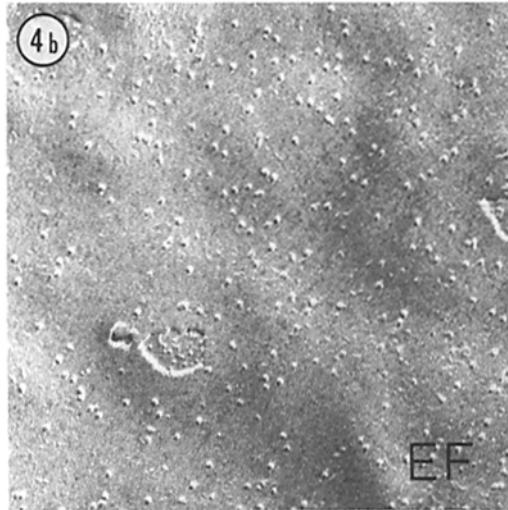
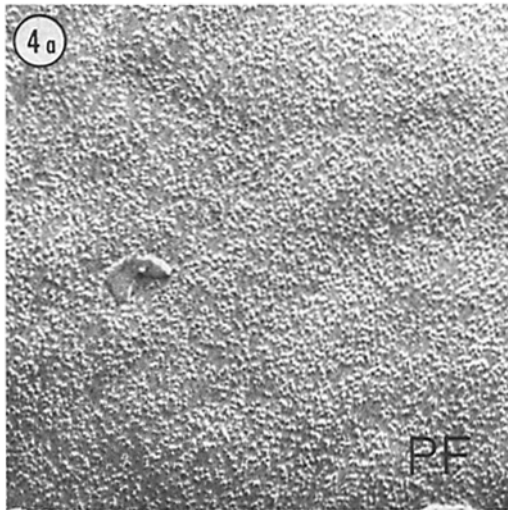


FIGURE 5 Electron micrograph of freeze-fractured vesicles released from *in vitro* aged (54 h) sheep erythrocytes. $\times 85,200$.

in vesicles, ghosts, and in vesicle-depleted ghost counterparts.

Cross-Linking Experiments

The spectrin-free vesicles are enriched in the 160,000 mol wt component shown to be a glycoprotein (10). When vesicles are modified with the cleavable cross-linking reagent [^{35}S]DTSP (8), a protein aggregate with a mobility slightly less than that for spectrin I is detectable on SDS-polyacrylamide gels (Fig. 7, see asterisk). This aggregate, as well as that formed which does not enter the gel, disappear nearly completely if the cross-linked preparation is reduced with DTT (cleavage of cross-linking reagent) before applying it to the gel (Fig. 7). Both types of aggregates contain primarily protein component III, the 160,000 mol wt protein, as shown by reelectrophoresis of the isolated aggregates (fractions *A* and *B*, Fig. 8) in the presence of DTT. Therefore, the aggregate found in fraction *B* with a molecular weight slightly higher than that of spectrin I must consist of dimers of component III, whereas the aggregate in *A*, according to its higher molecular weight, is a

FIGURE 4 Electron micrographs of freeze-fractured ghosts and ghosts depleted of vesicles. $\times 85,200$. (a) protoplasmic face of whole ghosts; (b) exoplasmic face of whole ghosts; and (c) exoplasmic face of ghosts depleted of vesicles.

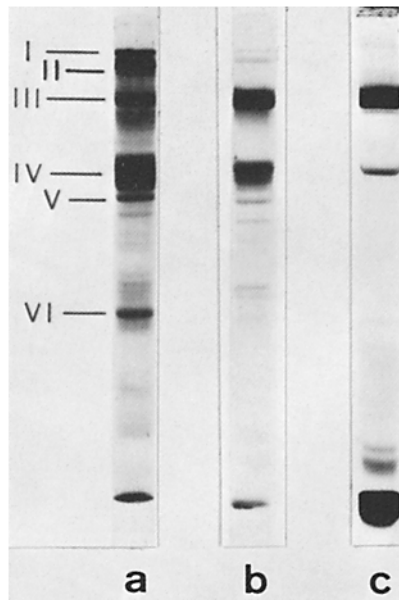


FIGURE 6 SDS-polyacrylamide gels stained with Coomassie blue from spontaneously released vesicles from sheep erythrocytes and their ghosts. (a) ghosts, 50 μg of protein; (b) vesicles released from in vitro aged sheep erythrocyte ghosts after 70-h incubation (as outlined in reference 10) at 2–4°C, 50 μg of protein; and (c) vesicles released from in vitro aged sheep erythrocytes after 54-h incubation at 37°C, 50 μg of protein including hemoglobin at the tracking dye front of the gel.

more complex multimer of component III. Fraction C represents an aggregate including components III and IV, while D contains modified component III that does not alter its molecular weight upon reduction (end-on labeled and/or cross-linked to small molecules such as hemoglobin and aminolipids).

Although protein component III is only 1.3 times more prevalent in vesicles than in ghosts (10) when its amount is referred to that of phospholipid phosphorus, dimers, and higher aggregates of component III are only formed readily in vesicles that are modified with DTSP. When ghosts are modified (Fig. 7), radioactivity with a mobility of that of component III-dimer (region of spectrin I) is largely retained after reduction. Furthermore, the ratio of the radioactivity detectable in the region of spectrin I over that in spectrin II remains constant upon reduction. Aggregates with properties as found in fractions A and B of vesicles (dimers and multimers of component III) are undetectable in modified ghosts (see Fig. 9). Instead, spectrins I and II account for the bulk of A, B, and

C. The composition of fraction B indicates that in intact ghosts no detectable component III-dimer has been formed upon DTSP modification. Similarly, modification of ghosts depleted of vesicles yields no dimers involving the small amount of component III present (not shown).

DISCUSSION

When sheep erythrocytes or their ghosts (10) age in vitro, they release vesicles that are depleted of spectrin and enriched in component III which is a glycoprotein. These vesicles are composed of headlike structures and protrusions. The intramembranous particles of the protoplasmic face are arranged in paired particle rows in vesicle protrusions.

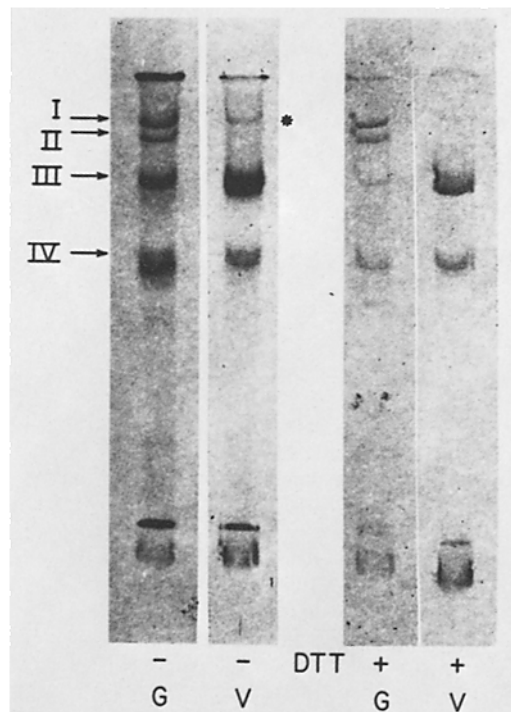


FIGURE 7 Autoradiographs of SDS-polyacrylamide gels from cross-linked ghosts and vesicles released from ghosts before and after reduction. Whole ghosts stored at 0°C for the same time as necessary to prepare vesicles and freshly prepared vesicles were cross-linked for 2 min with [^{35}S]DTSP at a final concentration of 2×10^{-4} M. The SDS-solubilized material was electrophoresed after incubation at 37°C for 30 min either in the presence or in the absence of DTT. All gels were run with 50 μg of total protein. G stands for whole ghosts and V for vesicles; the asterisk indicates location of component III-dimer. Roman numerals designate the major polypeptides.

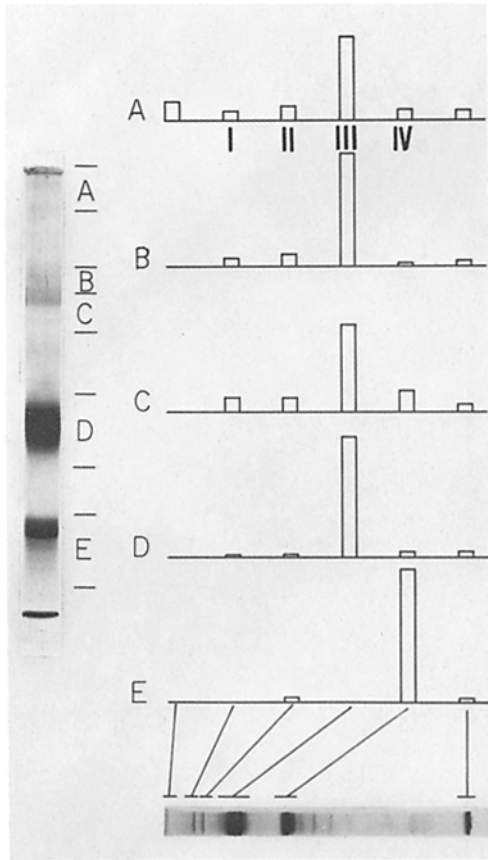


FIGURE 8 Quantitation of densitometric scans from autoradiographs of gels that were run with reduced material from fractions previously isolated from an unreduced gel of cross-linked vesicles. The 4% SDS-polyacrylamide gel shown on the left had been stained with Coomassie blue and shows the band pattern obtained from vesicles cross-linked for 6 min before reduction. Sections (A-E) were cut from unstained gels as indicated and treated as outlined in Materials and Methods. The five corresponding panels show the relative distribution of radioactivity in the polypeptide zones indicated below the panels for a 6% SDS-polyacrylamide gel and labeled with the corresponding Roman numeral on top of the graph.

sions. The vesicles are depleted of the intramembranous particles seen on the exoplasmic face of ghosts and thus contain almost exclusively intramembranous particles on the protoplasmic face. Based on the assumption that most of the integral membrane proteins are located within intramembranous particles (16, 19), the paired particles in the protoplasmic face should contain components III and IV (the molecular weight of component IV

corresponds to that of the major integral membrane protein designated component 3 in human erythrocytes). Although component III is only 1.3 times more prevalent in vesicles than in ghosts when its amount is referred to that of phospholipid phosphorus (10), short-time modifications with DTSP is found to yield dimers and multimers of this polypeptide only in vesicles. Therefore, the paired particle rows are composed of a subpopulation of particles containing those molecules of component III that readily form dimers upon DTSP modification. The tendency to form component III dimers and multimers could then serve as the basis for the preferential uptake of paired particles into vesicles. The polymerization of paired particles into rows that form most probably a helix or simple rings is presumed to occur while the protrusions are budding. It seems most likely

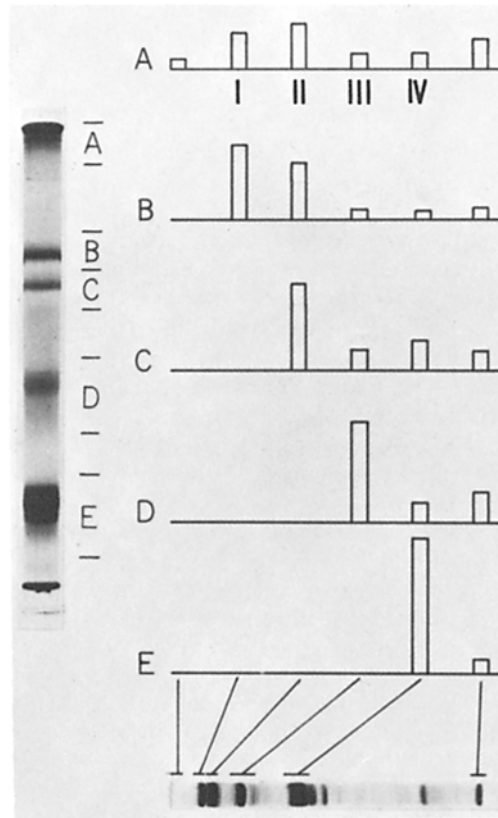


FIGURE 9 Quantitation of densitometric scans from autoradiographs of gels that were run with reduced material from fractions previously isolated from an unreduced gel of cross-linked ghosts. For experimental details, see Fig. 8.

that the formation of this highly regular structure is responsible for the spontaneity of the specific vesiculation process in aging sheep erythrocyte membranes. The initiation of this type of vesiculation is probably controlled by a loss of yet unspecified interactions between spectrin and component III (for review, see reference 15).

Enhanced cross-linkage of protein component III most likely reflects an altered structural arrangement of this protein in vesicle protrusions rather than an enhanced lateral mobility of component III. This interpretation is strengthened by the fact that similar numbers of accessible amino groups are present in component III of vesicles and ghosts (see Materials and Methods). An increased lateral mobility of component III would result in enhanced cross-linkage of component III to amino lipids rather than another component III because a rapidly diffusing protein would encounter more frequently free amino groups of lipids than of other component III proteins. Thus, the cross-linking data can be understood as the result of an altered arrangement, as a correlate to the structural rearrangement seen on a supramolecular level in the paired particle rows.

The results presented here have been explained as being due to major rearrangements of the membrane components during in vitro aging of sheep erythrocyte membranes. Rearrangements of integral membrane components occur by diffusion in the plane of the membrane. Translational diffusion of the major integral membrane protein in fresh human erythrocytes is limited due to the interaction with spectrin (5, 13). Rearrangements of integral membrane proteins (for example, aggregation) occur in spectrin-depleted ghosts (5) more readily than in intact cells. In intact erythrocytes, however, rearrangements of integral membrane proteins can occur as a consequence of ATP-depletion that leads to dephosphorylation of integral and peripheral membrane proteins (6). A gradual loss of negatively charged phosphoryl groups from the cytoplasmic side of the membrane may therefore be one explanation for the rearrangement of intramembranous components in in vitro aging of sheep erythrocyte membranes. Another explanation would involve alterations of the properties of membrane lipids. However, the phospholipid composition of vesicles differs only in minor components from that of ghosts (10).

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