Journal of Supramolecular Structure 8:375–389 (1978)
Normal and Abnormal Red Cell Membranes 97–111

Vesicles Isolated From ATP-Depleted Erythrocytes and out of Thrombocyte-Rich Plasma

Hans U. Lutz

Laboratory for Biochemistry, Federal Institute of Technology, ETH, Universitätstrasse 16, CH 8092 Zürich, Switzerland

When human erythrocytes are depleted of endogenous ATP they release spectrin-free vesicles as a light vesicle fraction [Lutz et al: J Cell Biol 73: 548, 1977] and chains of rounded vesicles as well as flattened myelin forms in a heavy vesicle fraction. The heavy fraction retains some spectrin, and glycophorin is partially degraded. The release of both types of fragments is not dependent upon added Ca^{+2}, and 50 μM EGTA does not prevent the vesicle release. Concomitant with vesicle release, a large fraction of the major protein components of the cell is found in disulfide-bonded aggregates.

A protocol is outlined to recover erythrocyte-specific fragments from thrombocyte-rich plasma. It allows detection of spectrin-free vesicles in whole blood stored under blood bank conditions for 12 days. In freshly drawn blood no such vesicles are observed, but particles are obtained that are different from thrombocyte fragments and that show a prominent glycoprotein running slightly faster than glycophorin.

Key words: **thrombocyte-rich plasma, disulfide-bonded protein aggregates, erythrocyte fragmentation, human erythrocytes, in vitro aging, ATP-depletion, spectrin-free vesicles, fragments of erythrocytes.**

ATP-depleted human erythrocytes display a series of structural [1–3] and functional [3–6] alterations that are likely to help us understand the requirements to be fulfilled by a structurally intact, deformable, and functionally active erythrocyte membrane. If erythrocytes are depleted of ATP for more than 20 hours erythrocytes have become echinocytes [1–3], and some of these blebs are lost as spectrin-free vesicles [7]. Evidence has been presented which suggests that echinocytic blebs represent a membrane in which the lateral mobility of antigens is not restricted as in the rest of the membrane [8]. A similar behavior is expected for antigens in spectrin-free vesicles. Therefore echinocytes may contain part of their membrane in a state similar to that found in spectrin-free

Abbreviations: EGTA – ethyleneglycol bis (β-aminoethylether)-N,N'-tetraacetic acid; ATP – adenosine triphosphate; CPD – citrate-phosphate dextrose solution used to preserve whole blood; SDS – sodium dodecylsulphate; DDT – dithiothreitol.

0091-7419/78/0803-0375$02.90

vesicles, and thus their functional properties could well be an average of those found in vesicles and in ATP-loaded erythrocyte. However, the spectrin-free vesicles, named "microspheres" by morphologists [9], are not the only membrane fragment being released from ATP-depleted cells [9]. Thus a detailed study of functional properties of released fragments requires the myelin forms to be characterized as well. In this presentation some insight on both release of spectrin-free vesicles and myelin forms is given. The requirements for their release are analyzed and compared with those known to induce a release of spectrin-free vesicles when erythrocytes are incubated with Ca^{+2} and ionophore A23187 [10].

Spectrin-free vesicles are such a defined and easily derived structural entity from human erythrocytes that it would be interesting to know whether such vesicles have physiological significance. Some evidence along this line has been given for spectrin-free vesicles being released from erythrocytes stored under blood bank conditions [11]. In this study a protocol is worked out to search for erythrocyte-specific fragments in thrombocyte-rich plasma.

MATERIALS AND METHODS

Human blood collected in CPD was obtained from the Red Cross. If not otherwise indicated the donors had the following blood group: O, Rh^+, M/M.

ATP-Depletion Experiments

Whole blood was centrifuged for 7 min at 2,000 rpm in a Sorvall-SS34 rotor. The supernate was withdrawn as thrombocyte-rich plasma for the experiments described later. The buffy coat was removed and the cells made up to 50% hematocrit in 5 mM phosphate 150 mM NaCl (pH 7.4). This suspension was passed through a cottom column to remove most of the white cells and residual thrombocytes. The eluate was washed three times in the same buffer and was incubated at 20% hematocrit in a medium containing 60 mM glycylglycine (or 50 mM glycylglycine when 5 mM phosphate was added), 110 mM NaCl, 5 mM Kcl, 250 mg/liter penicillin G, and 300 mg/liter streptomycin. The pH was adjusted with 1 N NaOH to pH 7.5. The cell suspensions were placed in dialysis bags and these were immersed in 10 volumes of the same medium. The Erlenmeyer flasks containing the dialysis bags were incubated in a shaking water bath at 37° C. After 6, 12–15, and 18–24 hours, the pH was readjusted to 7.4 in the surrounding medium. At the given times the content of the dialysis bags was centrifuged for 7 min at 2,000 rpm in a Sorvall-SS34 rotor. The supernates collected were diluted up to 10-fold with isotonic buffered saline and centrifuged for 1–2 hours at 45,000g. The vesicle pellets were resuspended in the same buffer and analyzed; samples of these suspensions were layed on isotonic dextran step gradients consisting of two solutions with the densities $\rho = 1.05$ and $\rho = 1.085$ (not corrected for added salt). These gradients were centrifuged overnight in swing-out buckets at 100,000g. The following day the light fraction at the interface and the heavy one as the trailing fraction above the pellet were withdrawn from the top and washed once in a large volume of buffered saline. In some experiments the erythrocytes have been surface-labeled with ^{125}I using lactoperoxidase and glucose oxidase [12]. Under these conditions only the integral membrane proteins, glycophorin and protein component 3, are labeled.

Gel Electrophoresis

SDS-polyacrylamide slab gels (8 × 2.7%) were performed in a system similar to that described by Neville [13], with a running gel in 0.38 M Tris and 0.1% SDS at pH 8.8. The stacking gel had a pH of 6.8, and the electrode buffer was 0.025 M Tris 0.3 M glycine and 0.1% SDS. The samples were incubated prior to electrophoresis for 30 min at 37° C in a medium containing 1% SDS, 40 mM DTT (if not otherwise indicated) 25% glycerol, 0.06 M Tris (pH 6.8) (final concentration).

Gels were stained for glycoproteins according to Eckhardt et al [14], and for proteins with Coomassie blue in 50% methanol, 10% acetic acid. Quantitation of protein components was carried out with a gel scanner Integraph (Bender and Hobein, Zürich) on Coomassie blue stained and dried gels. Quantitation of glycoproteins was performed on photographic negatives taken from gels stained with the fluorescent dansylhydrazine [14].

Isolation of Vesicles From Whole Blood

The protocol used is described in Figure 5. The filtration of thrombocyte-rich plasma was performed on a laminar flow Sartorius ultrafiltration system by using 3 filter units with a pore size of 0.8 μm. The filters were washed prior to use with water and isotonic saline. The completely liquid-filled system was connected to the flask containing the plasma to be filtered, and care was taken to avoid air from being trapped. The plasma was passed through the filter without artificially increasing the pressure.

The filtrate was centrifuged for 2 h at 45,000g. The pellet was resuspended in a small volume of buffered saline and 1 mg/ml aprotinin (a plasmin inhibitor, Novo, Copenhagen) was added. This suspension was layered on top of a dextran step gradient as outlined above.

A light and heavy fraction were collected from the gradient and washed once. The material thus obtained was resuspended in a small volume divided in three equal fractions and antisera added along with 1–2 mg aprotinin per ml. Commercially available anti-M and anti-N (Merz and Dade, Bern) have been used in a 1/1 dilution having agglutination titers of 1/8–1/12. Following the addition of sera, the samples were incubated for 30 min at 37° C and then pelleted in an Eppendorf centrifuge. These pellets were kept for several hours at 4° C and then they were resuspended and pelleted for 30 sec in a table centrifuge, the supernatant removed and the remaining pellets 3 times washed with buffered saline. The final pellets were made 1% in SDS and stored frozen.

Thrombocyte membranes were a gift from Dr Clemetson, Bern [15].

RESULTS

Light and Heavy Fractions of Vesicles Released From ATP-Depleted Human Erythrocytes

When human erythrocytes are depleted of endogenous ATP they release vesicular material that does not pellet upon centrifugation of the cell suspension for 7 min at 2,000 rpm in a Sorvall-SS34 rotor. The supernate thus obtained can be separated into a light and heavy fraction by a dextran step gradient (see Methods). With this step gradient the light fraction is found at the interface, and the heavy fraction is found as a trailing fraction above a tiny pellet containing some residual erythrocytes. The light fraction has the

properties of the previously described spectrin-free vesicles, with an average density of ρ = 1.062 [7] with no obvious signs of proteolytic damage (Fig 1B, compare background in the lower half of the gel with Fig 1A). However, the heavy fraction, which appears to have a similar protein composition, shows extended proteolysis evident from the high background in the trace of bound Coomassie blue in the lower half of the gel (Fig 1C). In addition, glycophorin from these vesicles is partially degraded (see arrows for glycophorin and its major breakdown product in Fig 1C). The number of vesicles found in the heavy fraction can be diminished by including phosphate in the incubation mixture, but their properties remain unchanged (Table I). Thus the diffuse Coomassie blue staining bands

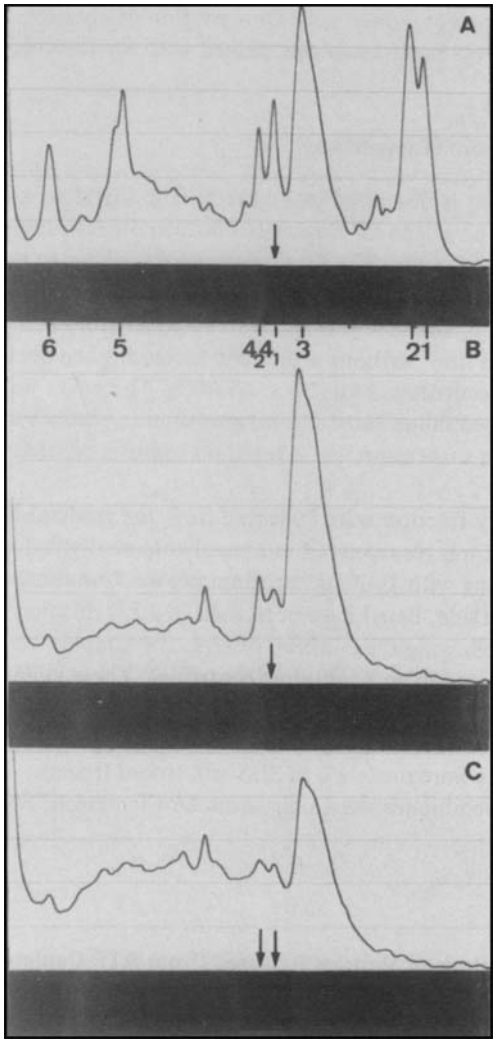

Fig 1. Protein compositions of light and heavy fractions of vesicles released from ATP-depleted human erythrocytes. Experimental details are given in Table I for assays with phosphate. Each graph consists of a scan from the dried Coomassie blue stained gel and of a photograph from the same gel previously stained for glycoproteins. A) fresh ghosts; B) light vesicle fraction; C) heavy vesicle fraction. The numbers listed below gel A refer to the major protein components of human erythrocytes. The arrows indicate the position of glycophorin and its major breakdown product where present.

TABLE I. Properties of Light and Heavy Vesicle Fractions From ATP-Depleted Human Erythrocytes

Addition to incubation mixture	Type of fraction	Membrane protein (μgm/10 ml supernate)	Content of protein component 3 (%)	Extent of proteolytic damage to glycophorin (%)	Predominant structures observed on electron micrographs
None	Light	68	47	0	Rounded vesicles
	Heavy	152	21	10	Myelin forms and rounded vesicles in chains
5 mM phosphate	Light	80	49	2	Rounded vesicles
	Heavy	78	26	16	Myelin forms and rounded vesicles in chains

Erythrocytes were ATP-depleted for 45 hours as outlined in Methods, except that the cells were not passed through a cotton column but were washed 5 times in isotonic saline supplemented with phosphate only for those cells incubated in phosphate thereafter. Membrane protein was determined from Coomassie blue stained gels as outlined in ref 7. The content of protein component 3 was determined by scanning Coomassie blue stained and dried gels (see Methods). The extent of proteolytic damage to glycophorin was quantified by calculating the relative amount of stain found in the lower molecular weight form (see arrows in Fig 1) as a percentage of the total stain detected in both glycophorin and breakdown product for ghosts and vesicles. The observed percentage for ghosts was set at zero, and the difference between numbers determined for vesicles and for ghosts was listed.

in the lower half of the gel may originate from high-molecular-weight proteins that cause the high buoyant density of these vesicles.

On the other hand, when erythrocytes are washed thoroughly free of white cells and plasma (see Methods), the heavy vesicle fraction retains a small amount of spectrin (6% of the total membrane protein including bands 7 and 8) and does not show a diffuse Coomassie blue stained zone (gel 2b, Fig 2). Although no signs of extended proteolysis are seen on Coomassie blue stained gels, glycophorin is still partially degraded (data not shown). Addition of phenyl methane sulfonyl fluoride (10–20 mg/liter) does not prevent glycophorin from being degraded in the heavy vesicle fraction under optimum conditions (not shown). Further evidence for some proteolysis to persist even under optimum conditions for ATP-depletion is clearly obtained from cells that have been surface-labeled with

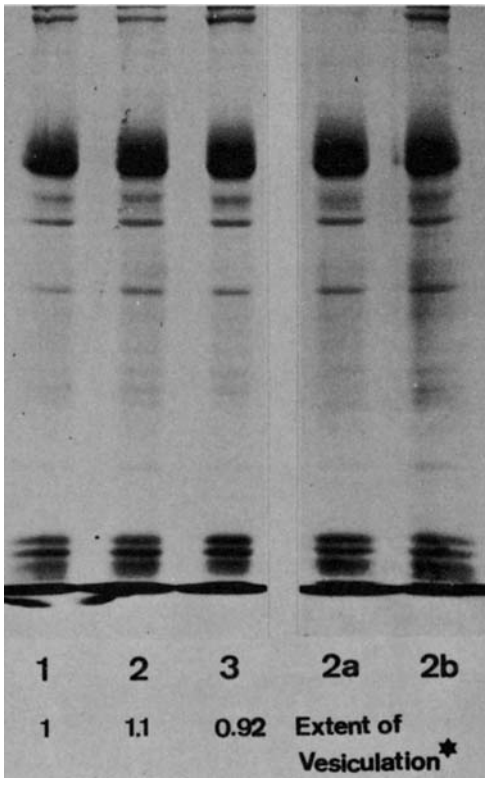

Fig 2. Extent of vesiculation and protein composition of vesicles released from ATP-depleted human erythrocytes in the presence of Ca^{+2} or EGTA. Erythrocytes were washed as outlined in Methods and surface-labeled with ^{125}I [12] prior to incubation. ATP-depletions were carried out for 38 hours in the medium given in Methods supplemented with 5 mM phosphate and with no further addition in experiment 1, with 50 μM $CaCl_2$ in experiment 2, and with 50 μM EGTA in experiment 3. 2a refers to the light vesicle fraction; 2b, to the heavy vesicle fractions.

*Extents of vesiculation are listed as relative numbers based on the number of cpm from ^{125}I recovered in the vesicle pellet originating from 10 ml of supernate from ATP-depleted cells. Extent 1, listed for the control (experiment 1), corresponds to 16,712 cpm recovered in the vesicle pellet. Extent data shown for experiments 1 and 2 are averages from two separate experiments. Radioactivity and protein were determined in triplicate.

^{125}I. Whereas ghosts from fresh cells contained 91,396 cpm per mg protein, those of ATP-depleted cells retained only 59,670 cpm per mg protein. The vesicles recovered from ATP-depleted cells account for an estimated fraction of 12% of the loss.

Most interesting is the morphology of the two fractions. The light one displays rounded vesicles, as previously described, and the heavy one shows rounded vesicles mostly in chains, and some vesicles appear to carry with them fragments of erythrocytes (Fig 3A). Among these structures elongated strands or flattened myelin forms are detected (Fig 3B) that are abundant in preparations revealing extended proteolysis (not shown).

Effect of Calcium on the Release of Spectrin-Free Vesicles

In view of the finding [10] that spectrin-depleted vesicles are released from human erythrocytes within 90 min following the addition of calcium and ionophore A23187, the question arose whether calcium that is known slowly to enter ATP-depleted cells [16] is necessary for the release of spectrin-free vesicles during ATP-depletion. Thus erythrocytes were depleted of ATP with varying concentration of calcium chloride in media not containing phosphate. All Ca^{+2} concentrations exceeding 50 μM resulted in enhanced hemolysis and proteolytic damage to vesicles and cells, but up to this concentration the activity of acetylcholinesterase found in the supernates after pelleting the cells was comparable to that found in the absence of Ca^{+2}, reaching 32% higher levels at 50 μM Ca^{+2}. Since the addition of 50 μM Ca^{+2} did not significantly enhance the release of vesicles, only traces of Ca^{+2} might be needed to induce the release. Such low concentrations of Ca^{+2} could have been introduced as Ca^{+2} adsorbed to washed cells or as contaminant in sodium chloride. Therefore the release of spectrin-free vesicles was studied in the presence of EGTA as well. In these experiments cells were surface labeled with ^{125}I (see Methods) to quantify integral membrane proteins. The extent of vesiculation was almost identical

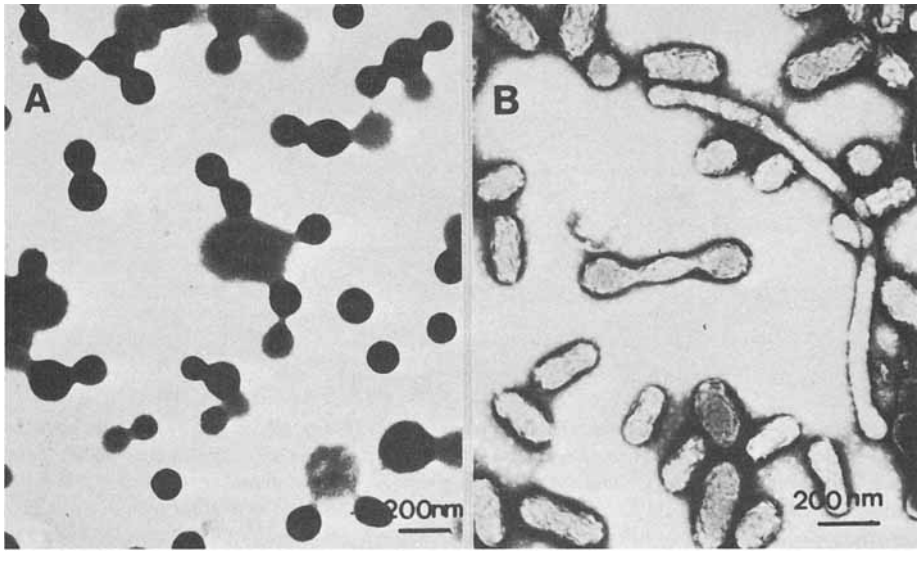

Fig 3. Electron micrographs from heavy fractions obtained following staining with uranylacetate. A) 15-sec staining revealing the electron-dense material trapped within vesicles; magnification ×33,000. B) 60-sec staining, focused on the surface of these structures; magnification ×40,000.

in preparations with or without Ca^{+2}, as well as with 50 μM EGTA when the amount of ^{125}I was measured in washed vesicle pellets from supernates of ATP-depleted cells (including both light and heavy fractions) (Fig 2, gels 1–3). The protein compositions of all three vesicle pellets are identical as are the compositions of all light and heavy fractions respectively demonstrated in Figure 2 for the assay with Ca^{+2} (gel 2a and 2b). Consequently the release of spectrin-free vesicles is not dependent upon added calcium and thus occurs by a different series of phenomena than that observed with calcium and ionophore.

Formation of Disulfide-Bonded Protein Aggregates in ATP-Depleted Erythrocytes That Have Released Spectrin-Free Vesicles

Introduction of calcium with ionophores causes the endogenous transglutaminase to be activated [17, 18] and ATP to be rapidly depleted [19]. Thus ATP-depletion and transglutaminase-dependent protein cross-linking precede the release of spectrin-free vesicles when calicum is introduced with ionophore. A similar pair of events occurs during in vitro aging: ATP is depleted [7] and disulfide-bonded protein aggregates are formed, irrespective of added Ca^{+2} or EGTA. This is shown in Figure 4 in erythrocytes that have released spectrin-free vesicles. (A thorough analysis of these aggregates has been published by Liu and Palek [20]). Since disulfide formation is dependent on oxidative conditions, the release of spectrin-free vesicles, as found in the light fraction, is partially inhibited by

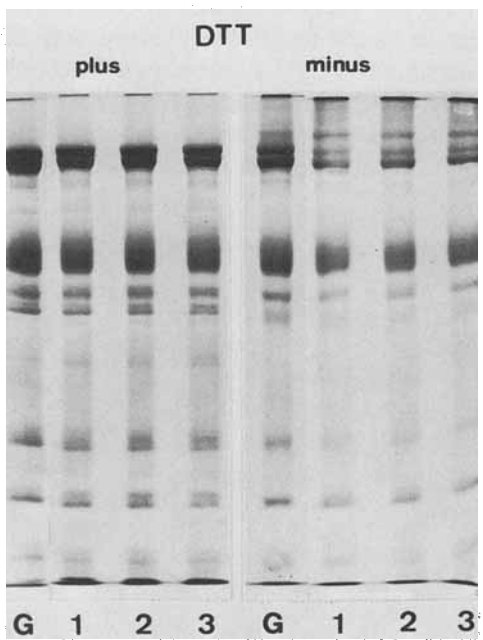

Fig 4. Formation of disulfide-bonded protein aggregates in ATP-depleted erythrocytes that have released vesicles. The conditions were as outlined in Figure 2. After having removed the vesicles from ATP-depleted cells, the cells were washed once and ghosts were isolated and frozen in 1% SDS without addition of NEM, which would have eliminated the slight formation of aggregates seen in ghost proteins electrophoresed without prior reduction. Each sample applied to the gel contained 15 μg of protein and was electrophoresed with or without prior reduction with DTT. 1) ghosts from ATP-depleted cells; 2) ghosts from ATP-depleted cells with 50 μM $CaCl_2$; 3) ghosts from ATP-depleted cells with 50 μM EGTA. G, fresh ghosts.

nitrogen, but the release of vesicles found in the heavy fraction is not affected (Lutz and Müller, unpublished results, December 1977).

Isolation of Vesicles From Whole Blood

Whole blood stored for more than 12 days in CPD contains spectrin-free vesicles that can be isolated from thrombocyte-rich plasma following the protocol outlined in Figure 5. The light and heavy fractions (Fig 6) retain proteins reminiscent of thrombocyte membranes (compare with gel T Fig 7A) and of spectrin-free vesicles. After agglutination of the particles from these fractions with rabbit anti-M and anti-N sera and repeated washing of the agglutinated and pelleted material, the protein composition of these pellets is similar to that of spectrin-free vesicles isolated from in vitro aged human erythrocytes (Fig 6), except that they retain actin (component 5) but very little spectrin. In spite of using blood from a donor with a homozygous genotype M/M, anti-N agglutinates these vesicles to some degree and anti-M reacts weakly with vesicles. These data suggest the vesicles to have lost some of the antigens, although they contain glycophorin (evident from gels stained for glycoproteins, not shown). When thrombocyte-rich plasma from whole blood stored under blood bank conditions for 4 weeks is processed the same way, light and heavy fractions following the step gradient reveal a protein composition

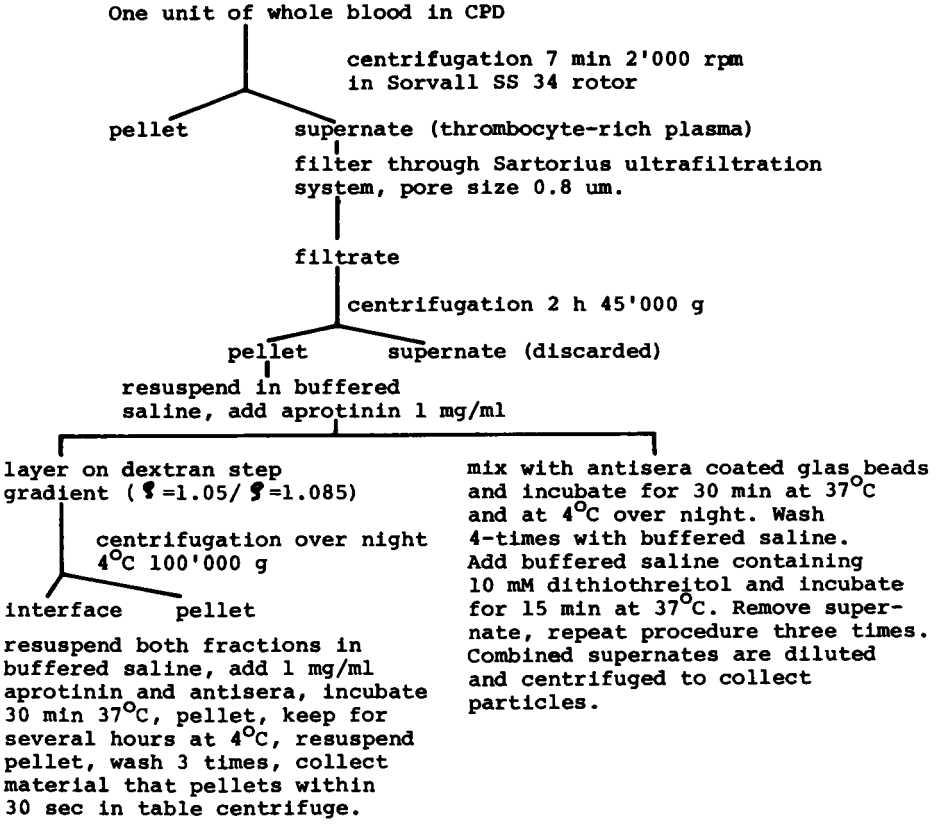

Fig 5. Isolation of erythrocyte-specific fragments.

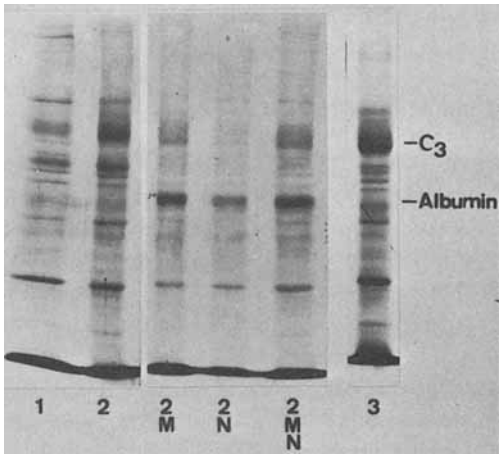

Fig 6. Spectrin-free vesicles from whole blood stored under blood bank conditions. Whole blood stored for 13 days was treated as outlined in Figure 5. Protein compositions are shown in gel 1 for the light vesicle fraction; in 2, for the heavy vesicle fraction of the dextran step gradient. Equal amounts of the heavy fractions were agglutinated with M/N blood group antisera and the protein composition of the agglutinated and pelleted material shown in 2_M, 2_N, 2_{MN}. M refers to added anti-M;N, to anti-N. C_3 denotes protein component 3.

similar to spectrin-free vesicles without further purification by agglutination (shown for heavy fraction in Fig 6).

Thrombocyte-rich plasma from fresh blood collected in CPD and processed in the same day according to the protocol in Figure 5 does not contain spectrin-free vesicles, but both light and heavy fractions show many protein bands originated from thrombocytes. However, when the heavy fraction from the dextran step gradient is agglutinated with antisera M or N, a prominent protein band (see arrow in Fig 7A) is enriched in the pellet that has no counterpart in thrombocyte membrane proteins. Both M and N antisera seem to have the same effect. The prominent band appears to be a glycoprotein running slightly faster than glycophorin (Fig 7B). The prominent band does not originate from added sera, nor is it a degradation product of thrombocyte glycoprotein because the thrombocyte protein pattern remains unchanged when thrombocyte membranes are incubated with antisera (T_M Fig 7A and B). Identical results are obtained when thrombocyte-rich plasma from an N/N homozygous donor is used (data not shown).

In order to avoid agglutination and copelleting of thrombocyte fragments, preliminary experiments have been performed using immunadsorption to antisera-coated glass beads. Anti-M and anti-N sera from MRC Blood Group Reference Lab in London (titer 1/124 and 1/64, respectively) were covalently coupled by dithiobis succinimidyl propionate (Pierce) to aminoalkyl glass beads (Pierce) following the protocol from Lomant and Fairbanks [21]. Since the antibodies were reversibly coupled, the washed beads could be treated with reducing agent to release antisera and bound material. The supernate after this reduction was diluted and centrifuged to collect particles. In two preliminary experiments the pellets thus obtained reveal the prominent glycoprotein and serum albumin but decreased amounts of protein bands originating from thrombocytes (Fig 8). However, the binding capacity of the glass beads was low, such that the supernate still contained a large fraction of this protein. The amount of protein that can be agglutinated

or adsorbed to immobilized antisera is very low and does not exceed 30 μgm per unit of blood. Although this material behaves like a vesicle, it may be of other origin than erythrocytes. Experiments are in progress to check whether this glycoprotein is partially identical to glycophorin.

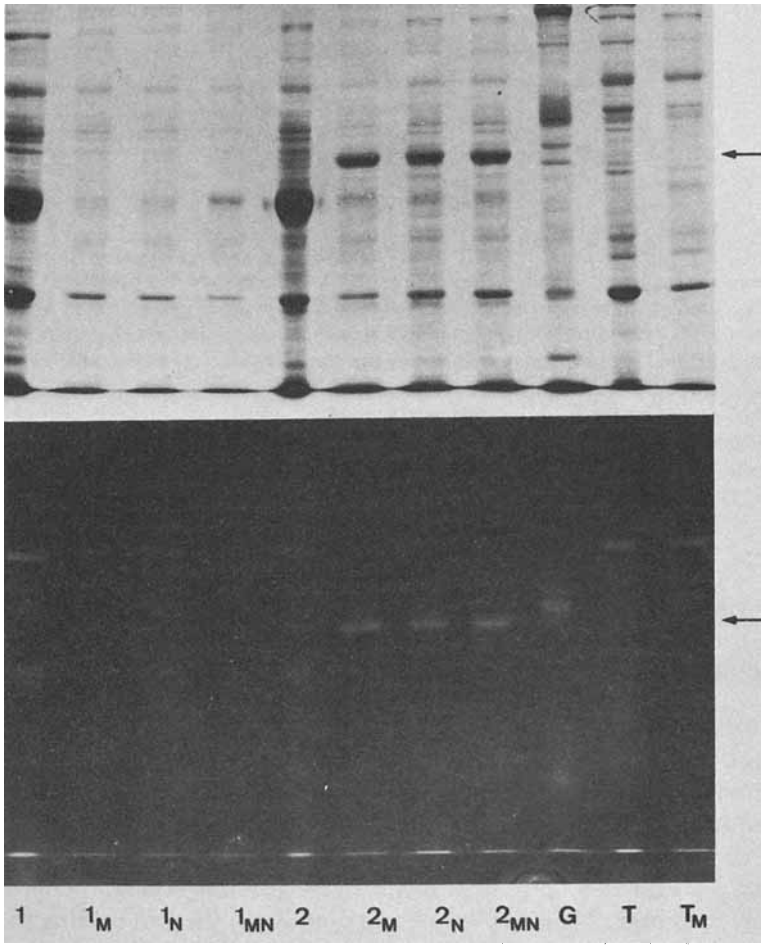

Fig 7. A) Protein composition of light and heavy particle fractions isolated from fresh thrombocyte-rich plasma. Plasma was processed as outlined in Figure 5 except that 20 mg/liter PMSF was added to the plasma prior to filtration. The light and heavy fractions following the dextran step gradient were divided in three equal parts, and anti-sera were added as indicated by subscript M or N (anti-M or anti-N). 1, supernate collected from all three agglutination assays from the light fraction; 2, supernate collected from all three agglutination assays from heavy fraction; 1_M, 1_N, 1_{MN}, agglutinated and pelleted material from the light fraction; 2_M, 2_N, 2_{MN}, agglutinated and pelleted material from the heavy fraction; G, 15 μgm protein of fresh human erythrocyte membranes; T, 15 μgm protein of thrombocyte membranes; T_M, thrombocyte membranes incubated with anti-M and pelleted.

B) Glycoprotein composition of light and heavy particle fraction isolated from the fresh thrombocyte-rich plasma.

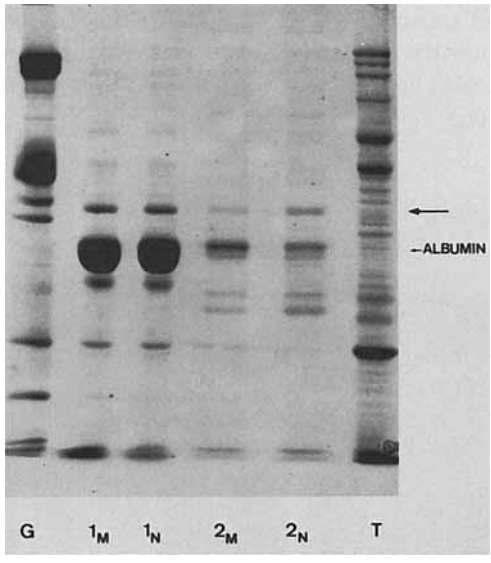

Fig 8. Protein composition of particles derived from fresh thrombocyte-rich plasma adsorbed to anti-M- or anti-N-coated glass beads. Fresh whole blood M/M was processed as outlined in Figure 5 and given in Methods. 1_M shows the protein composition of the material not bound to glass beads coated with anti-M; 2_M shows the protein composition of the particles that were bound and released by adding reducing agent; 1_N and 2_N show the equivalent results obtained with anti-N-coated glass beads (when antisera-coated glass beads were processed without adding the particles obtained after filtration and centrifugation of the filtrate, no protein could be found pelleted after reduction of the glass beads); G, 20 μgm protein of human erythrocyte membranes; T, 20 μgm protein of thrombocyte membranes. The arrow points to the prominent glycoprotein found in these particles (see also Fig 7).

DISCUSSION

Light and Heavy Vesicles

ATP-depleted erythrocytes release two types of fragments, a light fraction containing essentially spectrin-free vesicles, or microspheres, and a heavy fraction that contains chains of rounded vesicles and flattened myelin forms. The heavy fraction retains a small portion of spectrin and shows several indications of proteolytic damage, even under optimum conditions. Since myelin forms are abundant in preparations with extended proteolysis, it is likely that these structures have evolved from chains of rounded vesicles by proteolytic damage. Whereas spectrin-free vesicles from the light fraction do not show obvious signs of proteolysis, the heavy vesicle fraction and the residual cells do. These results are compatible with earlier findings that ATP-depleted cells release low-molecular-weight glycopeptides [22, 23]. However, the question remains unsettled why the light vesicle fraction hardly undergoes proteolysis and the heavy one is extensively proteolyzed. Whether this difference is due to potential proteases being trapped along with spectrin in the heavy vesicle fraction is not known. It is still not even clear whether the residual spectrin found in heavy vesicles is located in the chains of rounded vesicles or in the scarcely seen fragments of erythrocytes.

Requirements For Vesicle Release From ATP-Depleted Human Erythrocytes

During in vitro aging of human erythrocytes 50 μM Ca^{+2} does not enhance vesicle release and 50 μM EGTA has no inhibitory effect. Thus the well-documented endogenous transglutaminase that requires at least 0.5 mM Ca^{+2} to be active [17, 18] remains inactive during in vitro aging. Instead the proteins undergo disulfide-bonded aggregation that prevents spectrin from being trapped in vesicles. Although these changes are occurring concomitantly with vesicle release, they are not absolute requirements for vesicle release; under nitrogen, disulfide formation and release of spectrin-free vesicles are partially inhibited, but heavy vesicles are still formed (Lutz and Müller, unpublished results). We have previously shown that ATP has to be depleted for spectrin-free vesicles to be released. We then hypothesized that during ATP-depletion more and more of the yet unknown connections between integral membrane components and spectrin are destroyed. Such connections could well involve a phosphorylated site of spectrin that has recently been shown to play an important role in maintaining erythrocyte shape [6].

Although a loss of interaction between integral and peripheral membrane proteins is obviously necessary and theoretically conceivable, we lack similarly simple perceptions concerning the other major alteration that seems to be required: the formation of phosphatidate. We found phosphatidate to be enriched in spectrin-free vesicles from in vitro, aged human erythrocytes when its relative content was compared to that found in fresh erythrocytes. Allan et al [10] did not detect phosphatidate in spectrin-depleted vesicles induced by Ca^{+2}-ionophore A23187, but they report a stimulated phosphatidate labeling capacity due to diacylglycerol formed [24]. Phosphatidate found in spectrin-free vesicles from in vitro, aged erythrocytes can hardly be formed from diacylglycerol because ATP is depleted before vesicles are released.

Although the protein composition of the two types of spectrin-free vesicles is very similar, the lipid composition as well as the events favoring release differ. However, ATP-depletion is common to both types.

Isolation of Erythrocyte-Specific Fragment From Whole Blood

Since microspheres have been observed in blood stored under blood bank conditions [25] (and even in situ during erythrocyte phagocytosis [26]) and in fresh blood from certain patients [25], it is obvious to analyze such microspheres for their protein composition. On the other hand, it is unlikely that in vivo aged erythrocytes release spectrin-free vesicles to a large extent in circulation because their cellular ATP is not known to drop sufficiently to induce vesicle release [27]. Nevertheless a sensitive method has been worked out to test this possibility, taking advantage of the M/N bloodgroup antigens that occur only on erythrocytes and involve glycosylated side-chains as well as the N-terminus of glycophorin [28]. Using the protocol outlined in Figure 5, spectrin-free vesicles can be purified to some degree from thrombocyte-rich plasma of blood stored under blood bank conditions. The weak agglutinability observed with anti-M when M/M cells are used and the partial agglutination found with anti-N are not unexpected because it is known that blood stored under these conditions loses antigenic material to the supernate.

The vesicles isolated from stored blood differ from those obtained from washed cells upon ATP-depletion in their retention of protein component 5 and the appearance

of a new protein band with an apparent molecular weight of 140,000. Overall their composition appears to be comparable to that reported for microvesicles from stored blood as reported by Rumsby et al [11] in a drawing of a gel. Thus it can be concluded that blood stored in CPD undergoes vesiculation leading to spectrin-free vesicles. This vesiculation may be reduced by supplementing CPD with adenine, which has been shown to maintain high cellular ATP levels and erythrocyte survival rates after transfusion [29, 30]. It may well be that storage of blood under nitrogen further improves the survival, because under these conditions at least the release of spectrin-free vesicles is partially inhibited.

When the protocol outlined in Figure 5 is applied to thrombocyte-rich plasma from freshly drawn blood, particles are agglutinated that differ in protein composition from thrombocyte membranes and spectrin-free vesicles. They contain a prominent glycoprotein that has a slightly lower apparent molecular weight than glycophorin. Although the total amount of protein found in this particle fraction from one unit of blood was very low and thus did not allow chemical characterizations exceeding gel electrophoresis, the same results were obtained repeatedly for both M/M and N/N erythrocytes. Immunoadsorption using antisera-coated glass beads resulted in retention of the prominent glycoprotein, whereas some other proteins seen in agglutinated particles that clearly originated from thrombocytes were reduced. However, the reversible immunoadsorption technique has to be improved, because only a fraction of the prominent protein found in the particle suspension was adsorbed. Thus an erythrocyte-specific antiserum must be used that is not identical to M/N antisera.

In preliminary experiments using immunreplica techniques with antibodies against glycophorin, Claude Bron (Lausanne) and I have obtained some evidence that the particles isolated from thrombocyte-rich plasma contain protein components that form precipitations with anti-glycophorin. These studies will show whether particles containing a glycophorin breakdown product are released from erythrocytes in circulation presumably as a mode to correct for suffered damage.

ACKNOWLEDGMENTS

The author thanks Mrs N. v. Fischer for technical assistance, Dr T. Bächi, Institute for Medical Microbiology, University of Zürich for the electron micrographs, and Drs M. Metaxas and M. N. Metaxas from the Red Cross in Zürich for helpful discussions concerning the use of antibodies and for providing typed blood units. This work has been supported by SNSF grant 3.022-0.76, Berne.

REFERENCES

1. Nakao M, Nakao T, Yamazoe S: Nature 187:945, 1961.
2. Féo CJ, Leblond PF: Blood 44:639, 1974.
3. Weed R, Lacelle P, Merrill E: J Clin Invest 48:795, 1969.
4. Tadano K, Hellums JD, Lynch EC, Peck EJ, Alfrey CP: Blood Cells 3:163, 1977.
5. Sheetz M, Singer SJ: J Cell Biol 73:638, 1977.
6. Birchmeier W, Singer SJ: Cell Biol 73:647, 1977.
7. Lutz HU, Liu SC, Palek J: J Cell Biol 73:548, 1977.
8. Gordon JA, Marquardt MD: Nature 258:346, 1975.
9. Bessis M, Mandon P: N Rev Fr Hematol 12:443, 1972.

10. Allan D, Billah MM, Finean JB, Michell RH: Nature 261:58, 1976.
11. Rumsby RH, Trotter J, Allan D, Michell RH: Biochem Soc Trans 5:126, 1977.
12. Reichstein E, Blostein R: J Biol Chem 250:6256, 1975.
13. Neville D: J Biol Chem 246:6328, 1971.
14. Eckhardt AE, Hayes CE, Goldstein IJ: Anal Biochem 73:192, 1976.
15. Clemetson KJ, Pfueller SL, Luscher EF, Jenkins CSP: Biochim Biophys Acta 464:493, 1977.
16. Palek J, Church A, Fairbanks G: In Bolis L, Hoffmann JF, Leaf A (eds): "Membranes and Disease." New York: Raven Press, 1976, pp 41.
17. Lorand L, Weissmann LB, Epel DL, Brunner-Lorand J: Proc Natl Acad Sci USA 73:4479, 1976.
18. Anderson DR, Davis JL, Carraway KL: J Biol Chem 252:6617, 1977.
19. Taylor D, Baker R, Hochstein P: Biochem Biophys Res Commun 76:205, 1977.
20. Palek J, Liu SC, Snyder LM: Blood 51:385, 1978.
21. Lomant AJ, Fairbanks G: J Mol Biol 104:243, 1976.
22. Brovelli A, Suhail M, Pallavicini G, Sinigaglia F, Balduini C: Biochem J 164:469, 1977.
23. Brovelli A, Pallavicini G, Sinigaglia F, Balduini CL, Balduini C: Biochem J 158:497, 1976.
24. Allan D, Michell RH: Biochem J 166:495, 1977.
25. Weed RI, Reed CF: Am J Med 41:681, 1966.
26. Essner E: J Biophys Biochem Cytol 7:329, 1960.
27. Boron ER: J Lab Clin Med 62:263, 1963.
28. Wasniowska K, Drzeniek Z, Lisowska E: Biochem Biophys Res Commun 76:385, 1977.
29. Dawson RB: Transfusion 17:242, 1977.
30. Zuck TF, Bensinger TA, Peck CC, Chillar RK, Beutler E, Button LN, McCurdy PR, Josephson AM, Greenwalt TJ: Transfusion 17:374, 1977.