RELEASE OF SPECTRIN-FREE VESICLES FROM HUMAN ERYTHROCYTES DURING ATP DEPLETION

I. Characterization of Spectrin-Free Vesicles

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ABSTRACT

Human erythrocytes incubated without glucose at 37°C (in vitro aging) release spectrin-free vesicles after 12 or more hours. The release of vesicles is dependent upon ATP depletion. If the endogenous level of ATP is maintained, vesicle release is completely inhibited up to 54 h. Vesicle release is independent of hemolysis because in vitro aged cells and cells that maintain their ATP levels lose identical amounts of hemoglobin up to 45 h.

93% of all the membrane particles released constitute a uniform population of spheres with a diameter of 185 ± 23 nm. These vesicles are of slightly varying densities due to varying contents of hemoglobin. Vesicles contain half the amount of membrane protein that is found in intact membranes when referred to the content of phospholipid phosphorus. This is primarily due to the absence of spectrin. However, their content of protein component III, glycophorin, and cholesterol remains the same as in intact membranes. Thus, the major integral membrane proteins are present in vesicles in similar quantities per surface area as in cells except for the enzyme acetylcholinesterase that is enriched up to twofold. The phospholipid composition of these vesicles is representative of the intact membrane except that the amount of phosphatidic acid is 10-fold higher and the amount of phosphatidylethanolamine is slightly lower than in erythrocytes.

These results suggest a selective release of membrane domains that lack peripheral membrane proteins and are enriched in acetylcholinesterase. This release of spectrin-free vesicles from cells aged in vitro could represent an acceleration of the physiological aging process.

Human blood contains a mixed population of old and young erythrocytes that can be separated into different age groups due to subtle changes in their properties (11, 41). Old cells are denser (33) but slightly smaller, have a decreased sialic acid content (21) and thereby a reduced surface charge (10, 48), have lost their deformability (46), and have become osmotically fragile (12, 44). One of the mechanisms responsible for the above changes in the senescent cell is a so-called "fragmentation"

that reduces the surface of the discocyte without hemolysis until a sphere is reached that has lost its deformability and is phagocytized (16).

Fragmentation was studied in vitro as early as the last century (for review see reference 45) and was first postulated to be a normal mode of erythrocyte destruction by Rous and Robertson (36). These fragments from in vitro aged cells were later morphologically described by Bessis (4) and Bessis and Mandon (5). Fragments have been found to be either microspheres or myelin forms or both. Their formation has been studied quantitatively by Ponder and Ponder (34). Several investigations have indicated that fragments are formed not only in vitro but also in vivo (7, 16, 47). It has been recognized further that erythrocytes incubated at 37°C under sterile conditions lose lipids (43). The remaining aged cells have roughly the same lipid composition as the fresh cells, suggesting a nonselective loss of lipids. Subsequent studies have clearly demonstrated not only a lipid loss but also a protein loss (37). Acetylcholinesterase has been found to be low in old cells (24). However, there is little evidence in the literature for the identification of any of these components in isolated fragments (45).

A different line of evidence starting with two independent and, at the onset, unrelated findings led us to the hypothesis that these so-called fragments might constitute not a representative fragment of the whole membrane but a very specific part of it. These findings are: sheep erythrocyte ghosts spontaneously release vesicles that do not contain spectrin but are enriched in intrinsic membrane proteins (29). Ghosts isolated from human erythrocytes aged in vitro are not only denser but also enriched in spectrin (31). These findings prompted us to investigate the fragmentation of ATP-depleted (aged) human erythrocytes. Our goal has been to characterize the released vesicles rather than the remaining cells. The following report demonstrates a very specific release of spectrin-free vesicles from human erythrocytes aged in vitro.

MATERIALS AND METHODS

Aging of Erythrocytes

Freshly drawn human blood was washed three times in 150 mM NaCl, 10 mM glycylglycine (pH 7.4). Washed cells were then incubated at 20% hematocrit under sterile conditions in a medium consisting of 50 mM glycylglycine (pH 7.4), 5 mM KCl and NaCl up to a total osmolarity of 285 mosM. In the control, the ATP level was maintained by including adenine (0.54 mM), inosine (12.7 mM), and glucose (2 g/liter). The total osmolarity was also kept at 285 mosM. In order to prevent bacterial growth, all incubation mixtures contained 0.2 mg/ml streptomycin and 200 U/ml penicillin G. These suspensions were placed in sterile Erlenmeyer flasks (Nalgene Labware Div. Nalge/Sybron Corp., Rochester, N. Y.) covered with parafilm and gently agitated in a water bath at 37°C. After 4–6 h of incubation, the cells were pelleted and the pH was readjusted in the supernate to pH 7.4. The pH usually dropped 0.1–0.3 U during the initial period of incubation, mainly in the glucose-containing control.

Isolation of Supernates Enriched in Vesicles

Incubations were stopped by cooling to 0°C. Aliquots were withdrawn for ATP determination (28). Within an hour, the suspensions were centrifuged in clear plastic tubes for 7.5 min at 1,500 rpm in an International centrifuge (International Equipment Co., Boston, Mass.), r_{bottom} = 20 cm. The supernate thus formed was carefully removed and either directly layered on dextran gradients or centrifuged to pellet the released particles.

Dextran Density Gradients

Linear dextran gradients (Dextran T70, Pharmacia, Inc., Piscataway, N. J.) were prepared with densities from ρ = 1.007 to ρ = 1.1 for hypotonic conditions and from ρ = 1.04 to ρ = 1.1 for isotonic conditions. The densities listed were not corrected for the salts added. The dextran solutions for hypotonic gradients contained 11 mM $NaKHPO_4$ and 1 mM EDTA (pH 7.4), and those for isotonic gradients were supplemented with 120 mM NaCl. The total volume of the gradients was 30–32 ml, to account for 6–8 ml of washed or unwashed particles that were layered on the gradient. The gradients were isopycnically centrifuged for a minimum of 8 h at 2–4°C in an SW 27 rotor at 25,000 rpm (81,000 g_{av}). After centrifugation, the content of the gradients was pumped from the bottom of the tube through a flow cuvette and was fractionated according to the absorbance trace at 280 nm. In the case of a gradient that was loaded with the supernate as collected from in vitro aged cells, the bands were visualized by their strong light scattering and were withdrawn from the top of the gradient.

All fractions collected from gradients were washed twice to remove dextran, using 11 mM $NaKHPO_4$ (pH 7.4) 150 mM NaCl for isotonic and 11 mM $NaKHPO_4$ (pH 7.4) for hypotonic washings.

Protein Characterization

Protein was determined by the method of Lowry et al. (27), unless the hemoglobin content was high. In these

samples, membrane protein was determined by integrating the amount of bound Coomassie Blue on sodium dodecyl sulfate (SDS) polyacrylamide gels, using a Helena Quick Scan Instrument (Helena Laboratories, Beaumont, Texas). The relative number was referred to that obtained from 50 μg of whole ghosts. The quantity "membrane protein" includes proteins that stain with periodic acid Schiff reagent (PAS) only to the extent that these components bind Coomassie Blue after a prestaining with PAS. Hemoglobin was measured by the method of Crosby and Furth (9). Polyacrylamide gel electrophoresis in SDS was carried out as described by Neville (30), using a stacking gel. The upper tray buffer had a pH of 8.64; the lower tray buffer and the gel were at pH 9.18. After removal of SDS, the gels were stained with PAS according to Fairbanks et al. (17). After extensive destaining in 0.1% metabisulfite, 0.1 N HCl and then in 10% acetic acid, the gels were stained with Coomassie Blue in 10% acetic acid and 10% isopropanol.

Lipid, Sialic Acid, and Enzyme Assays

Lipids were extracted and chromatographed by the methods of Broekhuyse (6). Lipids were characterized and phosphorus was measured as reported earlier (29). Cholesterol was measured in the lipid extract according to Courchaine (8). Sialic acid can be extracted completely by heating the sample for 1 h at 80°C in 0.1 N H_2SO_4 (42). If the sample has a high content of hemoglobin, however, the heat treatment seems to set free some porphyrins or their breakdown products that interfere with the reading at 549 nm. The least interference was found with the following protocol: the vesicles were incubated for 30 min at 37°C in 50 mM Tris acetate (pH 6.5) containing 0.02 U of neuraminidase per mg membrane protein (type VI, Sigma Chemical Co., St. Louis, Mo.). After cooling to 0°C, sulfuric acid was added to make a final concentration of 0.1 N. After 1 h in the cold, the precipitate (including most of the hemoglobin) was centrifuged for 20 min at 3,000 rpm in an International centrifuge. Sialic acid was determined from the supernates according to Warren (42), using *N*-acetylneuraminic acid as standard. Acetylcholinesterase was measured at room temperature following the protocol of Ellmann et al. (15). The initial rates of hydrolysis of the substrate acetylthiocholine were determined and the specific activities were expressed as micromoles acetylcholine per hour and milligrams membrane protein.

Electron Microscopy

The samples were diluted in the appropriate buffer to 100–500 $\mu g \times ml^{-1}$ membrane protein. The samples were fixed in 2.5% glutaraldehyde in the appropriate buffer. The grids were then stained for 30–60 s with 2% uranyl acetate. Electron micrographs were taken with a Zeiss EM 9 S electron microscope.

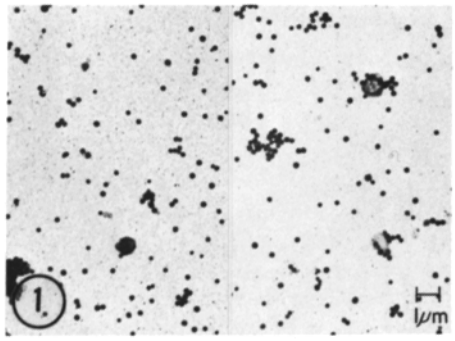

FIGURE 1 Electron micrograph of the particles present in the supernate from human erythrocytes aged in vitro for 54 h at 37°C. The particles adsorbed to the grid consist of electron-dense vesicles and larger cell fragments and some intact cells (not shown). × 3,400.

RESULTS

Vesicle Release During ATP Depletion

Human erythrocytes undergo ATP depletion during incubation at 37°C without glucose (in vitro aging). When the cellular ATP content has decreased to 15% of the preincubation level, the cells start to release vesicles. These vesicles are present in the overlying supernate when the cells are pelleted (Fig. 1). Besides the vesicles, the supernate contains free hemoglobin, fragments[1] of cells (as seen in Fig. 1) and, occasionally, some residual cells (not shown). The vesicles constitute >90% of the particles adsorbed to the grid and are a rather uniform population of electron-dense spheres of an average diameter of 185 ± 23 nm. Most of the vesicles are freely floating in the supernate, and some of them are linked together by small strands of membrane, the so-called myelin forms (5). Occasionally, myelin forms are found up to a length of 2 μm (not shown). It is important to note that the vesicles shown in Fig. 1 are present in the supernate as free vesicles and are not released by mechanical treatments (such as washing of the pelleted particles) from the few residual cells that are found in this supernate.

The kinetic of the vesicle release is shown in Fig. 2 for two parameters that are specific for

[1] Cell or ghost fragment here defined as those structures present in supernate collected from in vitro aged cells that are considerably smaller than whole cells but larger than vesicles and less electron dense than vesicles. The electron micrograph in Fig. 1 shows two of the smaller fragments observed.

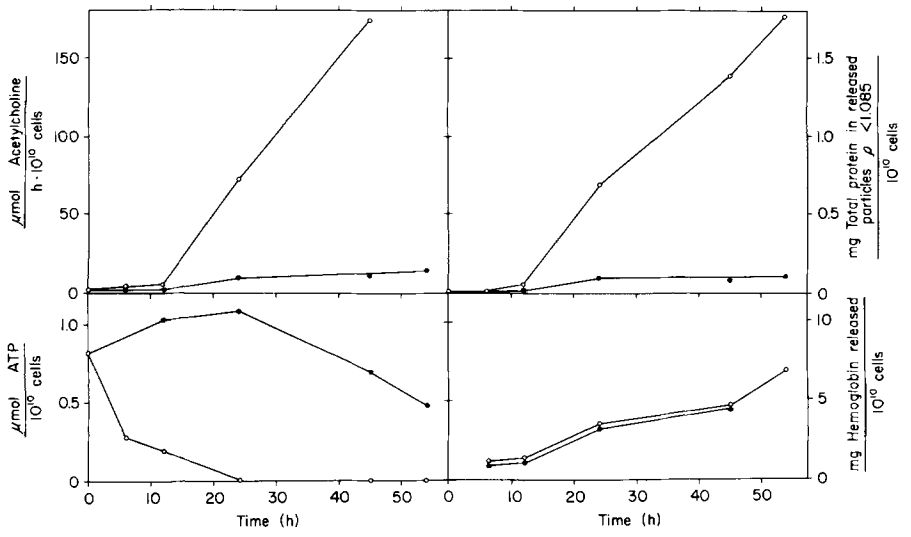

FIGURE 2 Time-course of vesicle release at 37°C monitored by two parameters and its correlation with ATP depletion but not with that of hemoglobin. All values are given per 10^{10} cells by referring the volume of the supernate to the number of cells (measured by Coulter Counter) from which it had been recovered. ATP was measured in duplicate in an aliquot of the suspension before removing the supernate. Total protein in particles with densities lower than $\rho = 1.085$ was determined by summing up all the protein recovered from hypotonic gradients that had been loaded with supernate as collected. Acetylcholinesterase activities and hemoglobin were measured in triplicate in the supernates. ○, in vitro aged erythrocytes; ●, control erythrocytes, ATP-maintained.

vesicles (see Fig. 4 and Table I). Up to 12 h, neither particles nor acetylcholinesterase (an enzyme bound to the vesicles) are released from in vitro aged cells. From this point on, the release of both particles and acetylcholinesterase increases linearly. Although the release of vesicles is dependent upon ATP depletion, it does not correlate with the loss of hemoglobin. In fact, both the in vitro aged cells and the control cells (ATP-maintained), up to 45 h, lose not significantly different amounts of hemoglobin to the supernate (Fig. 2). Control cells incubated in the presence of glucose (see Materials and Methods) maintain their ATP content up to 50 h. Correspondingly, vesicle release is completely inhibited.

Purification of Vesicles from Supernates

The supernates contain high amounts of hemoglobin that make impossible a direct analysis of the membrane proteins of vesicles. To further purify the vesicles, supernates were either directly layered on isotonic dextran gradients (*a*) or washed by centrifugation to recover the particles (*b*).

Method *a* provides a rapid technique for isolating highly purified vesicles but it does not allow quantitation of recoveries, because of the high level of hemoglobin compared to membrane protein found in the supernate as collected. Further, it is difficult to quantify the low buoyant density particles that sit on top of the dextran gradient because of the interfering free hemoglobin in the same region. However, the major vesicle fraction isolated by this method with an average density of $\rho = 1.062$ has been found to be the purest of all (as shown later in Fig. 6).

Method *b*, on the other hand, is applicable to large quantities. The particles within the supernate are pelleted, washed isotonically, and resuspended in a small volume (400 µg membrane protein per ml, at the most). This suspension contains vesicles that stay in suspension and some residual cells that pellet at 1 *g* after storage in the cold for several hours. The supernate thus formed is carefully removed and analyzed.

The SDS-polyacrylamide gels stained with Coomassie Blue of vesicles isolated by method *b* (Fig. 3, I) do not contain the spectrin bands I and II, but do contain components III and IV and, besides, a considerable amount of hemoglobin (intense band

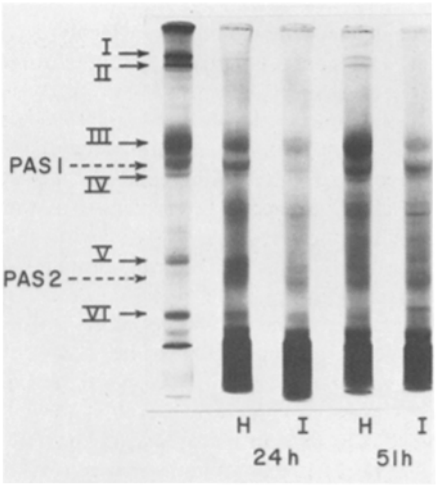

FIGURE 3 SDS-polyacrylamide gels from hypotonically or isotonically washed particles recovered from the supernate of human erythrocytes aged in vitro for 24 h at 37°C. Due to the high content of hemoglobin in these washed particles, the gels were overloaded with respect to hemoglobin, giving rise to the densely stained region at the lower end of the gel. The membrane protein in these fractions was determined by the amount of bound Coomassie Blue. 24 H, 50 μg membrane protein of hypotonically washed particles aged for 24 h; 24 I, 27.9 μg membrane protein of isotonically washed particles aged for 24 h; 51 H, 18 μg membrane protein of hypotonically washed particles aged for 51 h; 51 I, 37 μg of isotonically washed particles aged for 51 h. A Coomassie Blue band pattern from human ghosts is shown on the left as a reference to determine the components present in the particles. This ghost preparation had been frozen before electrophoresis and therefore displays spectrin aggregates on top of the gel. The location of PAS 1 and PAS 2 will be demonstrated in Fig. 7.

at and below tracking dye) and some other low molecular weight components.

Methods *a* and *b* outlined above are the ones that we recommend for isolating these vesicles. In order to study the properties of these vesicles in more detail and to clarify the ambiguous results obtained upon PAS staining of SDS-polyacrylamide gels from isotonically washed vesicles (see later Fig. 7), method *b* has also been applied by using a hypotonic buffer instead of an isotonic one. When particles are washed and stored in a hypotonic buffer, the residual cells lyse. SDS-polyacrylamide gels from these hypotonically washed particles then also reveal, besides the components seen after isotonic washing, spectrins I and II (Fig. 3, H).

The vesicle preparations obtained by method *b* from ATP-depleted cells can further be separated into different density classes (Fig. 4). On an isotonic linear dextran gradient from $\rho = 1.04$ to $\rho = 1.1$ (gradient I, Fig. 4), three fractions can be distinguished. The low buoyant density fraction *1* contains some ghosts and ghost fragments (judged from electron micrographs) that must be sealed to ions because they otherwise would be seen in their normal density range from $\rho = 1.05$ to $\rho = 1.065$. The vesicles found in fraction *1* are similar to those shown in Fig. 1, except that they are less electron dense. Fraction *2* is the trailing edge of the major fraction *3*. Both fractions consist of a really uniform population of vesicles as shown in Fig. 5 for fraction *3*. These observations are summarized in Table I.

From a hypotonic gradient from $\rho = 1.007$ to $\rho = 1.1$, three fractions can be isolated, of which the minor fraction *1* contains faintly stained vesicles and a few ghost fragments. On the other hand, fraction *2* contains a rather pure population of vesicles that appear faint on electron micrographs without uranyl acetate, suggesting that these structures are low in hemoglobin. The major fraction *3*

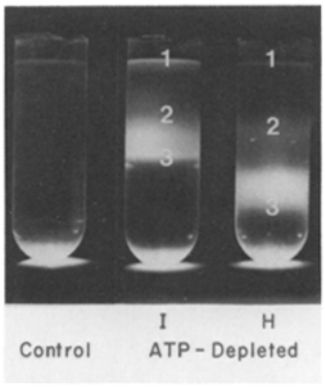

FIGURE 4 Isolation of pure vesicles from washed particles recovered from the supernate of human erythrocytes aged in vitro for 51 h by isopycnic dextran gradient centrifugation. The gradient on the left shows the band pattern obtained from the isotonically washed particles of control erythrocytes that were able to maintain their ATP level (virtually an empty gradient). The gradient in the center shows the band pattern of isotonically washed particles from in vitro aged erythrocytes on an isotonic gradient ($\rho = 1.04$ to $\rho = 1.1$). The gradient on the right shows the band pattern of hypotonically washed particles from the same erythrocytes on a hypotonic gradient ($\rho = 1.01$ to $\rho = 1.1$). The numbers refer to the fractions isolated. These numbers will be used subsequently (*1–3*).

contains electron-dense vesicles (even in the absence of uranyl acetate), some with protrusions (similar to myelin forms), and a few ghosts and ghost fragments (see also Table I). The control gradient in Fig. 4 has been loaded with isotonically washed particles derived from cells that maintained their ATP level. Except for a tiny band on top of the gradient, virtually no vesicles can be found.

The properties of each of these fractions are given in Table I. The density differences seen in Fig. 4 between the fractions are partially due to the hemoglobin content. One of the most striking properties is the high hemoglobin content in hypotonically washed particles (fractions *2* and *3*). Not only have the particles been washed, but each fraction has undergone two additional washes in hypotonic buffer before analysis. It has further been found that hemoglobin is not highly aggregated or endogenously crosslinked by disulfide bridges, because electrophoresis of samples of unreduced, isolated vesicles did not reveal elevated amounts of hemoglobin dimers (not shown).

The second striking finding concerns the high specific activity of acetylcholinesterase in these vesicles. We determined an average activity of 383 ± 59 μmol per h and mg membrane protein from eight different major vesicle fractions from three independent experiments. White ghosts have an average specific enzyme activity of 100 μmol per h and mg membrane protein. The specific enzyme activity of isolated vesicles is therefore three- to fourfold higher than in ghosts and is completely accessible because the addition of Triton X-100 (0.25–0.5%) does not increase the activities (not shown). A similar enrichment of acetylcholines-

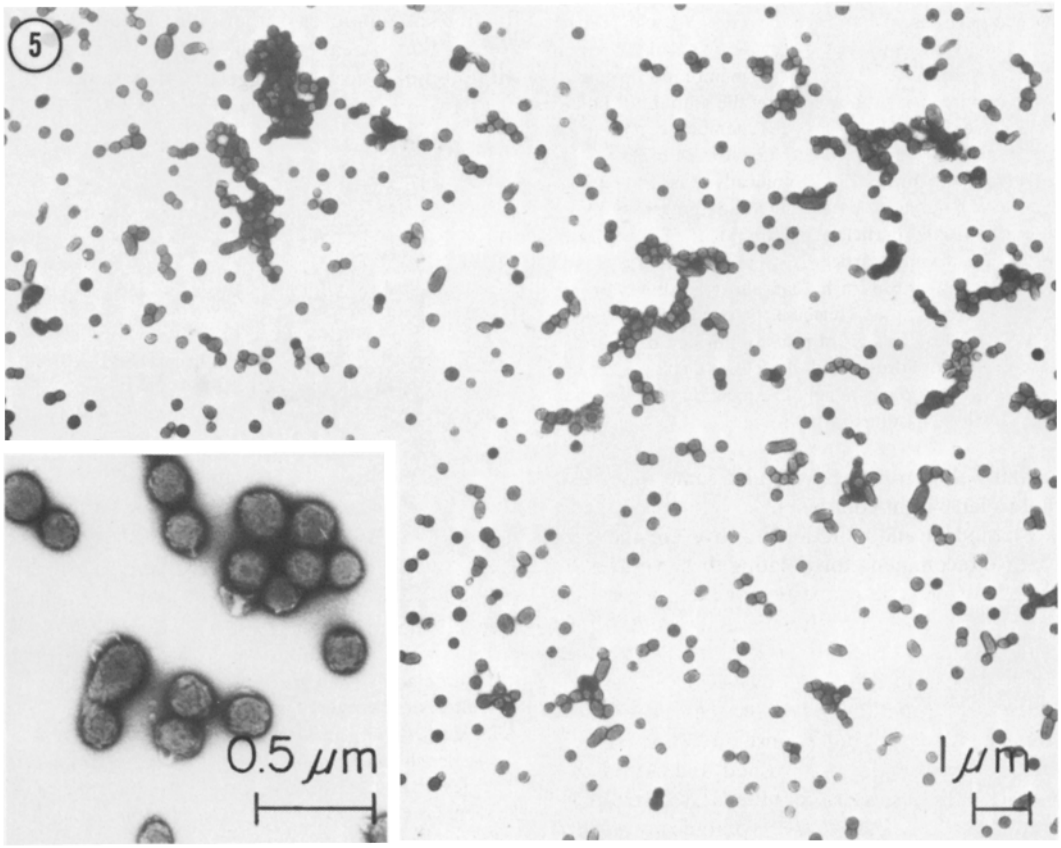

FIGURE 5 Electron micrograph showing the major vesicle fraction isolated under isotonic conditions from in vitro aged erythrocytes. The fraction (*3*, washed) was isolated from an isotonic gradient loaded with the isotonically washed particles that were recovered from the supernate from erythrocytes aged for 51 h. × 7,800; inset, × 31,300.

TABLE I

Properties of Vesicles Released from In Vitro Aged Human Erythrocytes Isolated from Dextran Density Gradients Loaded with Washed Particles

Fraction	Isotonic conditions				Hypotonic conditions			
	Total membrane protein	Ratio of hemoglobin/membrane protein*	Acetylcholinesterase	Structures‡ found	Total membrane protein	Ratio of hemoglobin/membrane protein*	Acetylcholinesterase	Structures‡ found
	μg		μmol Acetylcholine · h^{-1} · mg^{-1} membrane protein*		μg		μmol Acetylcholine · h^{-1} · mg^{-1} membrane protein*	
Washed particles from supernate		4.5	406	V, GF, G		3.3	311	V, GF, G
Fraction *1*	10	1.4	249	V, GF, G	33	n.d.	394	V
Fraction *2*	47	4.5	356	V	53	3.9	377	V
Fraction *3*	83	4.8	301	V	127	3.9	228	V, GF, G
Pellet	30	4.5	101	G	26	1.4	97	G
Recoveries in %	72		56		88		75	

* Membrane protein was determined by measuring bound Coomassie Blue and referring these values to that found for 50 μg hemoglobin-free ghost protein determined as given in reference 27.

‡ Certain structures observed in electron micrographs are expressed for each fraction by the following symbols: V, vesicles, GF, ghost fragments (see footnote 1 for characterization), G refers to both ghosts and cells since many of these structures are found to be more ghost-like after washing the fractions than they do in the original supernate (especially for hypotonic washes). These data do not imply a quantitation; except for the pellets, all the fractions contain vesicles.

terase can be calculated on the basis of recoveries. After 54 h, 23% of the total acetylcholinesterase activity[2] is recovered in the vesicles, while only 7.3% of the membrane protein is found. In the experiment shown in Table I, the acetylcholinesterase is high in fraction *2* of both types of gradients and considerably lower in the third fraction of the hypotonically washed preparation. This result agrees with the above-mentioned content of leaky ghosts and ghost fragments and will further be substantiated by the amount of spectrin (see Fig. 6).

Protein Components of Isolated Vesicles

As mentioned above, the major protein component of isolated vesicles is hemoglobin (Fig. 6). The membrane proteins found in vesicles are components III, IV, PAS 1, PAS 2, PAS 3 (for PAS components, see Fig. 7), and some minor, low molecular weight Coomassie Blue components. In vesicles isolated under isotonic conditions (method *b*), spectrin is almost completely absent; only traces are found in the major fraction *3*. Exactly the same protein composition, with no spectrin at all, is found in vesicles isolated from an isotonic gradient that has been loaded with the supernate as collected after pelleting the cells (method *a*) (see D, Fig. 6). Protein component III that has been reported to contain acetylcholinesterase (3) represents 44 ± 3% of the total membrane protein (measured by Coomassie Blue) found in three independent preparations of spectrin-free vesicles in which the residual amount of Coomassie Blue stain in the spectrin I and II region was <2% of the total. While the specific activity of acetylcholinesterase is three- to fourfold higher, the amount of component III is only 1.5–1.8 times higher in these vesicles than in ghosts.

As expected, spectrin is present in some fraction derived from hypotonically washed particles. It is completely absent in fraction *2* that does not contain ghost fragments and has the highest specific activity of acetylcholinesterase.

Unlike the gel system used by Fairbanks et al. (17), the Neville system (30) allows the separation of component III and PAS 1. The faint band just underneath component III contains PAS 1 (Fig. 6) as demonstrated in Fig. 7. Most of the PAS stain of isotonically isolated vesicles is found in PAS 2 instead of PAS 1 which is the major PAS component in ghosts as shown for the same conditions in Fig. 7. Since the same vesicles isolated under hypotonic conditions still have PAS 1 as the major PAS component (Fig. 7), we assume that the PAS stain that is found comigrating with PAS 2 stems from PAS 1. A quantitation and a comparison of

[2] The total activity of acetylcholinesterase has been calculated by using the established value of 28.9 IU/g hemoglobin at 25°C and the amount of hemoglobin measured in the cells. The total membrane protein per 10^{10} erythrocyte ghosts has been determined to be 5.2 mg as measured in three ghost preparations.

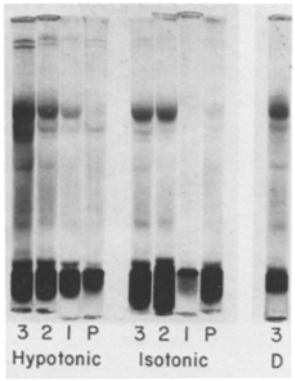

FIGURE 6 Protein composition of vesicles isolated by density gradient centrifugation from washed and unwashed particles recovered from supernates of erythrocytes aged in vitro for 51 h. The SDS-polyacrylamide gels (6.5%) were stained with Coomassie Blue. The numbers refer to the fractions isolated from dextran gradients (see Fig. 4), and each of these fractions shown here has been characterized in Table I. D refers to the major vesicle fraction (*3*) that was isolated from an isotonic gradient loaded with supernate as collected from cells aged in vitro for 45 h. The amount of membrane protein on each gel was determined by measuring bound Coomassie Blue, and the values are expressed in μg: 51 h hypotonically washed, 1:32.4; 2:14.5; 3:8.1; pellet: 5; 51 h isotonically washed, 1:15.3; 2:12.3; 3:2.8; pellet: 7.5; D: 20.

PAS stain in both types of vesicles with that from ghosts should therefore include PAS 1 and PAS 2. A ratio of the relative amount of PAS stain in both PAS 1 and PAS 2 over total protein has been determined for each gel shown in Fig. 6 before staining with Coomassie Blue (Table II). These data indicate a 1.4- to 1.7-fold enrichment of PAS stain in the isolated vesicles, a value very similar to that found for component III. The enrichments are identical for vesicles of the same purity whether isotonically or hypotonically isolated. Purified vesicles thus contain intrinsic membrane proteins but no extrinsic ones, except for hemoglobin and some minor cytoplasmic components. The relative amounts of component III and PAS 1 and PAS 2 are 1.5- to 1.8-fold higher and the specific activity of acetylcholinesterase 3- to 4-fold higher than in ghosts.

Lipid Composition and Sialic Acid Content of Isolated Vesicles

We have already shown that purified vesicles have virtually no spectrin; thus their buoyant density is reduced by a 1.9-fold decrease in the amount of membrane protein per phospholipid phosphorus. This is shown for the isotonically washed particles that constitute almost pure vesicles (Table III). The decrease of this ratio in vesicles is again dependent on the purity of the fraction; the higher the content of ghost fragments (spectrin as indicator), the higher the ratio.

When the amounts of integral membrane proteins are referred to the content of phospholipid

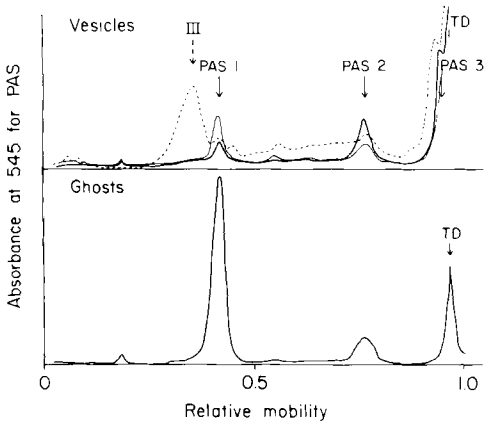

FIGURE 7 PAS absorption profiles from erythrocyte ghosts and isolated vesicles released from in vitro aged erythrocytes. The upper panel shows PAS absorbance profiles (solid lines) of SDS-polyacrylamide gels from vesicles of erythrocytes aged for 51 h isolated under isotonic conditions (heavy solid line) and isolated under hypotonic conditions (light solid line). The dashed line shows the Coomassie Blue trace corresponding to the isotonically isolated vesicles with its major component III. PAS 3 comigrates with the tracking dye (*TD*). The lower panel shows a PAS profile of an SDS-polyacrylamide gel from 50 μg membrane protein of whole ghosts.

TABLE II

*Relative Content of PAS 1 Plus PAS 2 in Isolated Fractions from Dextran Gradients**

Fraction	Isotonic gradient	Hypotonic gradient
Ghosts	–	1.0
Fraction *1*	1.4	1.5
Fraction *2*	1.7	1.5
Fraction *3*	1.4	1.1
Pellet	0.6	1.1

* SDS-polyacrylamide gels (6.5%) stained with PAS were scanned at 545 nm. The areas under the densitometric scan of PAS 1 and PAS 2 were added. The ratio of this value over that of the total content of membrane protein was set to 1 for ghosts.

phosphorus, it becomes evident that this ratio remains the same in vesicles and ghosts (Table III). In addition, the ratio of cholesterol to phospholipid content as well as the ratio of sialic acid to phospholipid content are preserved in vesicles. We thus conclude that the whole bilayer with integral membrane proteins is budding to form vesicles and leaves behind spectrin. To further substantiate this conclusion, we analyzed the phospholipid composition of the isolated vesicles. The four major phospholipids of erythrocytes are present in vesicles to such amounts that it can be concluded that the intact, asymmetrically organized bilayer (49) is taken up into vesicles (Table IV). However, all major components are slightly lower than in erythrocytes, and one of them, phos-

TABLE III

*Protein/Phospholipid Ratios, Lipid, and Sialic Acid Content of Vesicles Released from In Vitro Aged Erythrocytes**

Preparation	Conditions	Membrane protein / Lipid phosphorus	Protein comp. III / Lipid phosphorus	Sialic acid / Lipid phosphorus	Cholesterol (total) / Lipid phosphorus
		μg protein/μg P	*μg protein/μg P*	*μg sialic acid/μg P*	*μg cholesterol/μg P*
Washed particles from supernate	Isotonic 51 h	22	9	1.02	11.4
Major vesicle fraction	Isotonic 51 h	–	–	1.1‡	–
Washed particles from supernate	Hypotonic 51 h	24	–	–	11.1
Major vesicle fraction	Isotonic 45 h	19‡	9	–	–
	Hypotonic 54 h	–	–	1.01‡	–
	Hypotonic 45 h	29‡	7	1.13‡	–
Fresh ghosts	Hypotonic	42	10	1.25§	12.1

* All values for phosphorus (P), cholesterol, and sialic acid were determined in triplicate assays as given in Materials and Methods if not otherwise indicated. Membrane protein and component III were measured according to bound Coomassie Blue as outlined in Material and Methods.

‡ In these cases there was not enough material to permit assay three times.

§ Established value from earlier measurements after digesting the ghosts in 0.1 N H_2SO_4 for 1 h at 80°C. If the content of sialic acid of ghosts was determined by the same method as that applied to vesicles, the readings were 20–30% lower than the one listed.

TABLE IV

*Phospholipid Composition of Vesicles from Erythrocytes**

Phospholipid	Erythrocytes‡	Vesicles average	1.	2.
Phosphatidylcholine	29.4 ± 0.9	27.2	26.3	28
Phosphatidylethanolamine	29.5 ± 0.3	24.9	25.6	24.2
Sphingomyelin	27.8 ± 0.7	25.9	25.2	26.5
Phosphatidylserine + phosphatidylinositol	9.7 ± 0.3	8.8	9.0	8.5
Phosphatidic acid + lysophosphatidic acid	0.6 ± 0.3§	7.7‖	8.9	6.6
Lysophosphatidylethanolamine	0.4	2.1	2.0	2.2
Lysophosphatidylcholine	0.4	1.8	2.3	1.4
Start + unidentified	2.2	1.5		

* The values represent phospholipid phosphorus in percent of total phosphorus recovered from the plates. Recoveries were 92% or more.

‡ The values and the standard deviations are derived from three measurements of two different lipid extracts from fresh erythrocytes.

§ Phosphatidic acid in lipid extracts from fresh ghosts has been found to be 2–3% of the total phosphorus.

‖ Lysophosphatidic acid was <2% of total phosphorus.

phatidylethanolamine, is significantly lower. This loss is compensated by slightly higher amounts of lysocompounds (lysophosphatidylethanolamine and lysophosphatidylcholine) and significantly higher amounts of phosphatidic acid. The content of phosphatidic acid is 10-fold higher than in whole cells but only 3–4 times higher if compared to lipid extracts from ghosts.

DISCUSSION

Human erythrocytes aged in vitro release spectrin-free vesicles containing lipids, integral membrane proteins, and hemoglobin. The release of this uniform population of vesicles is dependent upon ATP depletion and can be prevented from occurring by maintaining the endogenous ATP concentration. Vesicle release is not related to hemolysis. We thus have established that the so-called fragmentation of erythrocytes undergoing ATP depletion (45) is a very specific exocytosis of the bilayer with most of its integral membrane proteins. Since the proportion of spectrin is higher in ATP-depleted erythrocytes than in fresh cells (31), spectrin remains with the cells during budding of these vesicles. It is further known that old erythrocytes isolated from circulating blood are similarly enriched in nonhemoglobin protein (26). Thus it is likely that the process studied here could mimic in vivo aging of erythrocytes. Whether the microspheres existing in blood plasma (16, 45) have a composition similar to that of those released from in vitro aged cells awaits further investigation.

Besides protein component III and some minor Coomassie Blue components, these vesicles contain glycophorin (38). However, glycophorin is present in isotonically washed vesicles in both the high and low molecular weight form (PAS 1 and PAS 2). This finding is not due to boiling of the samples before electrophoresis, as has been reported by Tuech and Morrison (40). It occurs in all isotonic but not in hypotonic preparations. Since the sum of the PAS stain measured in PAS 1 and PAS 2 is similar in both isotonic and hypotonic preparations of comparable purity, we conclude that PAS stain in PAS 2 stems from PAS 1 (glycophorin). Thus the relatively high amount of stain in PAS 2 does not represent a preferential uptake of PAS 2 that has been shown to exist as a separate component despite comigrating with the low molecular weight form of PAS 1 (19, 20).

Spectrin is virtually absent in pure vesicle preparations and has been found to be increased in aged cells (31). As will be demonstrated in a subsequent paper[3] and has been reported in an abstract (32), spectrin undergoes aggregation during in vitro aging of the cells and is most likely involved in budding of these vesicles. Recently, some evidence has shown that intramembranous particles are linked to spectrin in fresh ghosts (13). Under conditions that cause aggregation of spectrin, no particle aggregation could be demonstrated in fresh ghosts, but almost particle-free vesicles were budding off from the ghosts (14). Occasionally, those authors could find some protein in the isolated vesicles, and this protein happens to be component III. We think that, with in vitro aging of cells, more and more of these connections between intramembranous particles and spectrin are destroyed, thereby enabling these particles to be taken up into budding vesicles. Since protein component III and glycophorin are present in vesicles in amounts comparable to those found in ghosts when referred to phospholipid phosphorus content, the density of intramembranous particles in the vesicles is expected to be similar to that found in ghosts. On the contrary, the enzyme acetylcholinesterase is enriched up to twofold when normalized to the content of phospholipid phosphorus. This result was not unexpected, since a very similar enrichment has been found in spontaneously released vesicles from sheep erythrocyte ghosts (29). As has been found for sheep erythrocyte ghosts, the enzyme recoveries here never exceed the protein recoveries, suggesting a preferential uptake of this enzyme rather than an enzyme activation. This result would imply that acetylcholinesterase-rich domains exist and/or that rearrangements take place during in vitro aging.

The loss of acetylcholinesterase has long been recognized as being one of the major changes that occur during aging of erythrocytes (35). Here it has been shown that the enzyme is not just lost or inactivated but is recovered in the released vesicles.

We have demonstrated that these vesicles have a high content of hemoglobin and release only 20% of it upon hypotonic washing. Although no highly aggregated forms of hemoglobin have been found, a gel-like precipitation of hemoglobin onto the inside of the membrane, similar to Heinz bodies (25), cannot yet be excluded.

The low density of these vesicles is due to almost a twofold decrease in membrane protein

[3] Palek, J., S. Liu, and H. U. Lutz. Manuscript in preparation.

when membrane protein is referred to the content of phospholipid phosphorus. Their rich lipid content is not due to a preferential uptake of a particular lipid since the ratio of the cholesterol to phospholipid content remains the same as in ghosts. In addition, the phospholipid composition of these vesicles suggests that the lipid is released from the cell in the form of the intact bilayer rather than in the form of the outer monolayer. These results are in good agreement with the earlier studies on lipid loss from old erythrocytes (43). The atypical phospholipid, phosphatidic acid, is, however, 10-fold higher in the isolated vesicles than in erythrocytes and threefold higher than in ghosts. The only phospholipid that is significantly decreased even after adding the amount in its lysocompound is phosphatidylethanolamine. Yet, the decrease in phosphatidylethanolamine could, in turn, be due to an enhanced production of 1,2-diacylglycerol (2) that has not been measured here.

An explanation for phosphatidic acid formation has been suggested by Allan et al. (1): when erythrocytes are stimulated by a Ca^{2+} ionophore, 1,2-diacylglycerol is phosphorylated to phosphatidic acid by endogenous ATP during the first 15 min of incubation. A related report by the same group (2) shows that erythrocytes treated with this ionophore released vesicles within 90 min that have a protein composition similar to that of the vesicles that we have described here, except that their spectrin content is higher. Although those authors reported a synthesis of phosphatidic acid from 1,2-diacylglycerol during the incubation with the ionophore, neither the isolated microvesicles nor the residual cells have been shown to contain phosphatidic acid. In contrast, we clearly can demonstrate phosphatidic acid in vesicles released from in vitro aged cells.

Whether phosphatidic acid is enzymatically produced by a phospholipase D, whether it is formed as outlined by Allan et al. (1), or whether it is produced by a chemical hydrolysis of a phospholipid in the outer layer of the membrane upon structural rearrangements during aging, awaits further investigation. However, phosphatidic acid could be formed from phosphatidylethanolamine that has been shown to become accessible to nitrophenylation and phospholipases upon ATP depletion (22, 23). If it were produced asymmetrically within the membrane, it could well be a trigger for expansion of the outer monolayer and, thus, for budding of the bilayer by a mechanism similar to that suggested for amphipathic ions that interact with the membrane (18, 39).

H. U. Lutz as a visiting scientist to the Worcester Foundation gratefully acknowledges the financial support received for this research from the institutional funds of the Worcester Foundation. In particular, he thanks Dr. F. Welsch and Dr. M. Hoagland for the opportunity to stay at the Worcester Foundation. This investigation was further supported in part by the Boston Sickle Cell Center.

Received for publication 25 June 1976, and in revised form 24 January 1977.

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