Biochimica et Biophysica Acta, 1065 (1991) 135–144

ADONIS 0005273691002323

BBAMEM 75252

Formation of large, membrane skeleton-free erythrocyte vesicles as a function of the intracellular pH and temperature

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(Received 11 October 1990)

Key words: Vesicle formation; Membrane skeleton; pH, intracellular; Temperature; Electron microscopy; (Human erythrocyte)

Vesiculation of intact erythrocytes can be induced by decreasing their intracellular pH and then heating the red cell suspension to a critical temperature value. While at intracellular pH 6 vesiculation begins at 45 °C, further decrease in the intracellular pH lowers the critical temperature. In addition, the critical temperature value can be modified by varying the length of the interval between titration and heating as well as by changing the temperature during this interval. The vesicles are large (1–3.5 μm in diameter), haemoglobin-containing and completely free of skeletal proteins. Pretreatment of the cells with diamide and 2,4-dinitrophenol had no substantial effect on vesiculation, while *N*-ethylmaleimide, chlorpromazine and wheat germ agglutinin proved to be inhibitory. Increasing the osmolarity of the incubation medium markedly decreased the critical temperature: red cells suspended in a solution of 600 mosM NaCl vesiculated at 42 °C instead of 45 °C when the intracellular pH was decreased to 6. We propose that the vesiculation is due to a purely physicochemical molecular mechanism which affects the state and dimension of the membrane skeleton. We also discuss the possible role of an altered haemoglobin–membrane interaction in preventing low pH-induced intramembrane particle aggregation in the membrane skeleton-free vesicles.

Introduction

Vesiculation of non-haemolyzed erythrocytes occurs under different experimental conditions, i.e., red cell ageing [1], treatment of the cells with Ca^{2+} and the ionophore A23187 [2,3], heating of the erythrocytes to 50 °C [4–7] at which temperature denaturation of spectrin occurs [8], treatment of the red blood cells with dimyristoylphosphatidylcholine vesicles [9] and titration of the erythrocytes with EDTA and $CaCl_2$ at 45 °C [10]. The main feature of these vesicles is that they contain haemoglobin and are either completely or partially free of skeletal proteins.

In this study we describe the formation of large (1–3.5 μm) membrane skeleton-free vesicles as a joint function of the intracellular pH and temperature as well as the effects of various agents on the vesiculation process. We suggest that the vesiculation is due to a purely physicochemical molecular mechanism which affects the properties of the membrane skeleton and that the formation of vesicles upon titration with EDTA and $CaCl_2$ at 45 °C [10] can also be explained by a similar mechanism.

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Materials and Methods

Formation and isolation of vesicles. Heparinized venous blood samples from healthy volunteers were washed several times in 0.15 M NaCl. After the last washing, the pellet was resuspended in unbuffered saline to get a 10% red cell suspension. This suspension containing 0.2 mM phenylmethylsulphonyl fluoride (PMSF) was then slowly titrated with 0.15 M HCl under constant stirring at room temperature in order to change the intracellular pH. Intracellular pH values were determined by measuring the pH of the haemolyzates of the titrated erythrocytes. The red cells were haemolyzed either osmotically or mechanically. Erythrocytes were pelleted, the supernatant was carefully removed and an equal volume of distilled water was added to the pellet. In other experiments the pellet was sonicated using an MSE type sonicator. In a given sample the same intracellular pH values were obtained with both haemolysing procedures.

Aliquots of titrated erythrocytes of different intracellular pH were heated to different temperature val-

ues for 40 min in order to determine those minimal temperature values which lead to significant and similar rates of vesiculation at a given intracellular pH. Vesiculation was checked by means of phase contrast microscopy or Nomarski optics. Vesicle release was not determined quantitatively by measuring a certain parameter of the shed membrane material, because the measured points did not overlap. For example at pH 6 significant vesiculation can be observed at 45 °C within 40 min, but vesicles are not formed at 44 °C during this time interval. On the other hand, at pH 5.8 many vesicles per field of view can be observed at 44 °C within a 40 min incubation period.

Vesicles were separated from the so-called mother cells as follows: the red cell suspensions were centrifuged in a Janetzki T 32c type table centrifuge at $400 \times g$ for 5 min and the supernatants containing numerous vesicles and fewer mother cells were filtered through Sartorius filters of 3 μm pore diameter. Filtering caused haemolysis of the mother cells, but the ghosts did not pass through the filters provided they were used only once. Ghosts could be clearly observed by using phase contrast or Nomarski optics even in the presence of extracellular haemoglobin. The pellets still containing many vesicles were resuspended in saline containing PMSF, then centrifuged and filtered as described above. This procedure was repeated several times to obtain a large number of purified vesicles. Filtering caused some fragmentation but no haemolysis of the vesicles. The vesicles were centrifuged at $660 \times g$ for 20 min at room temperature, washed once in a large volume of saline containing PMSF and used for morphological and biochemical investigations.

Experiments with different reagents. The following reagents were used: *N*-ethylmaleimide (Serva), diazenedicarboxylic acid bis(*N*,*N*-dimethylamide) (Serva), chlorpromazine hydrochloride (Aldrich), 2,4-dinitrophenol (Fluka), wheat germ agglutinin (Sigma) and 600 mosM NaCl. Red cells were washed in isotonic saline and diluted to 5% in phosphate-buffered saline (pH 7.4) containing the following reagents: 2 mM *N*-ethylmaleimide (NEM), 2 mM diazenedicarboxylic acid bis(*N*,*N*-dimethylamide) (diamide), 60 μM chlorpromazine and 4 mM 2,4-dinitrophenol (DNP), respectively. After 60 min incubation at 37 °C, the cells were centrifuged, diluted to 5% with unbuffered saline, titrated with 0.15 M HCl to different pH values at room temperature and heated. Since NEM- and diamide-treatment decrease the heat denaturation temperature of spectrin and the fragmentation temperature of erythrocytes without titration [13–15] — from 49 to 45 °C under the conditions employed by us — in experiments concerning these reagents, both control and pretreated cells were titrated to pH values where vesiculation occurs below 45 °C (see Results). In the case of chlorpromazine and DNP, the reagents were also included in the unbuffered suspension at the indicated concentrations and the titrated cells (pH_{ic} 6) were heated to 45 °C. Since in the presence of WGA strong red cell agglutination occurs after centrifugation, the following procedure was used: washed red cells were diluted to 1% with unbuffered saline containing 5 μg/ml WGA. After a 60 min incubation at room temperature the suspension was titrated to pH 6 in the presence of WGA and was then heated to 45 °C. The pH of the unbuffered red cell suspension was 7.2.

Washed red cells were diluted to 5% with 600 mosM NaCl. After 60 min at room temperature, the suspension was titrated to pH 6 followed by determination of the critical temperature.

Electron microscopy. After titration and heat treatment the red cells were centrifuged at $660 \times g$ for 20 min and the pellets containing both mother cells and vesicles were fixed with 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7) at room temperature. Fixed cells and vesicles were pelleted as described above and embedded in agar. The samples were then postfixed with 1% osmium tetroxide for 1 h, stained en bloc with 0.5% aqueous uranyl acetate for 30 min, dehydrated in graded ethanol and embedded in Durcupan ACM. Thin sections were made with an LKB Ultrotome III type ultramicrotome and were stained with uranyl acetate and lead citrate.

For freeze-fracture electron microscopy vesicles were purified as described above, fixed with 1.5% glutaraldehyde for 30 min, washed in cacodylate buffer then glycerinated. The samples were freeze-fractured and replicated in a Balzers 510 type freeze-etch apparatus as described previously [11]. In some cases aliquots of the titrated and heated red cell suspension containing both mother cells and vesicles were added to 1.5% glutaraldehyde then pelleted, washed, glycerinated and freeze-fractured. Both replicas and thin sections were investigated in a Philips EM 300 electron microscope at 60 kV accelerating voltage.

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. Washed, control red cells and mother cells were lysed in 7.5 mM Tris-HCl buffer (pH 7.6) containing 0.2 mM PMSF at 4 °C, the ghosts were pelleted at $10000 \times g$ for 30 min, washed twice in the same buffer and processed for SDS-gel electrophoresis. Purified red cell vesicles were lysed in the same buffer, centrifuged at $100000 \times g$ for 1 h and the pellet was processed for gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli [12] and the gel slabs were stained with Coomassie blue.

Results

When erythrocytes of decreased intracellular pH were incubated at different temperature values, vesicu-

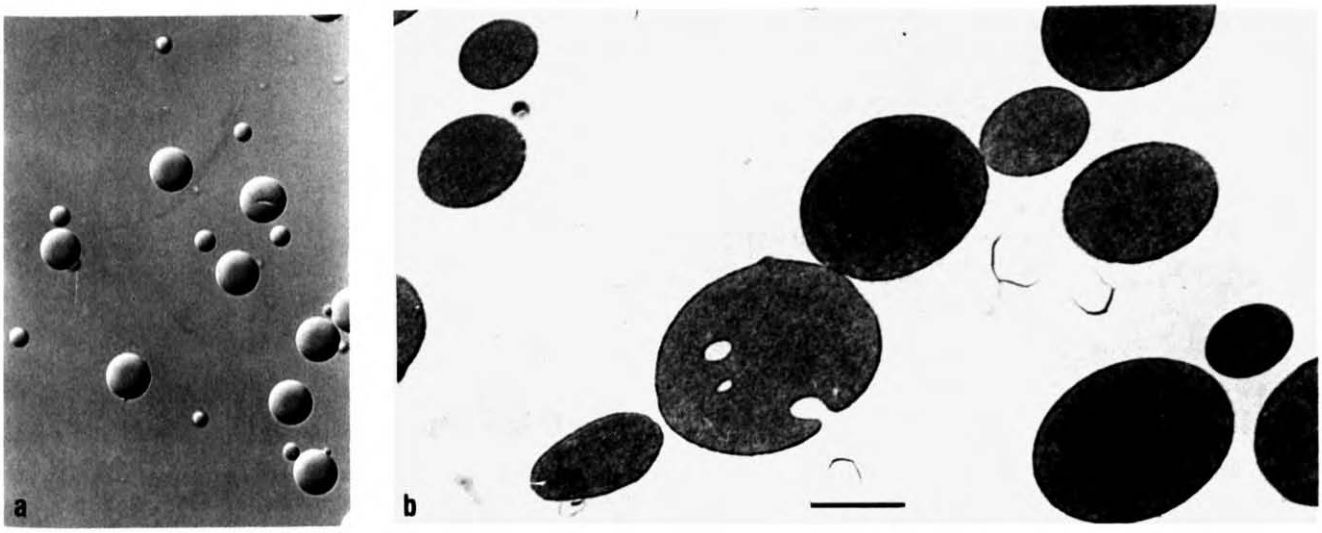


Fig. 1. Nomarski micrograph (a) and low magnification electron micrograph (b) of red cells showing vesiculation as a result of decreased intracellular pH and elevated temperature. The vesicles are large and no haemolysis accompanies vesicle formation. Magnification: (a) 800×; (b) 6400×. Bar, 2 μm.

lation of the red cells could be detected (Fig. 1a). The vesicles are very large (1–3.5 μm in diameter), they are of ovoid and spherical shape and contain haemoglobin (Fig. 1b). In addition to vesicle release, titration and heating of the erythrocytes also caused membrane internalization (Fig. 1b) the intensity of which was much less than that of exovesiculation. Vesiculation is a joint function of the intracellular pH and temperature. The curve in Fig. 2 shows the minimal temperature values causing vesiculation as a function of the intracellular pH. At an intracellular pH 6, vesiculation begins at 45 °C, but further decrease in the pH lowers the critical temperature in the way shown by the solid line. The vesiculation was found to be a sensitive process, i.e., the curve could be modified by varying the length of the interval between titration and the beginning of heating and by changing the temperature during this interval (Fig. 2). The vesiculation temperature was progressively decreased with increasing time and increasing temperature.

We did not try to induce vesiculation at intracellular pH values higher than 6 or lower than 5. The reason for this is that at intracellular pH values lower than 5 the red cells haemolyse while at intracellular pH values higher than 6 only those temperature values cause vesiculation which are very near 49 °C at which temperature heat-denaturation of spectrin and an other kind of vesiculation occurs.

Isolation of the vesicles by repeated centrifugation and filtering caused some fragmentation, but no haemolysis of the vesicles (Fig. 3).

Coomassie blue-stained SDS-polyacrylamide gel profiles of membranes from control erythrocytes, mother cells and isolated vesicles can be seen in Fig. 4. The gel patterns clearly demonstrate the complete absence of skeletal proteins from the vesicle membrane as compared to the control and mother cells. Although Fig. 4 shows only the gel of vesicles derived from cells of intracellular pH 6 heated to 45 °C, vesicles released at other pH and temperature values (e.g. pH 5.4 and 41 °C) also showed the same profile (not shown).

The effects of various agents on the vesiculation are summarised in Table I. Since the SH-reagents diamide and NEM decrease the heat denaturation temperature of spectrin and consequently can cause red cell fragmentation without titration [13–15], the experiments concerning these reagents were carried out on erythrocytes which were titrated to pH 5.8 and 5.4, and which were then heated to 44 and 41 °C, respectively. At

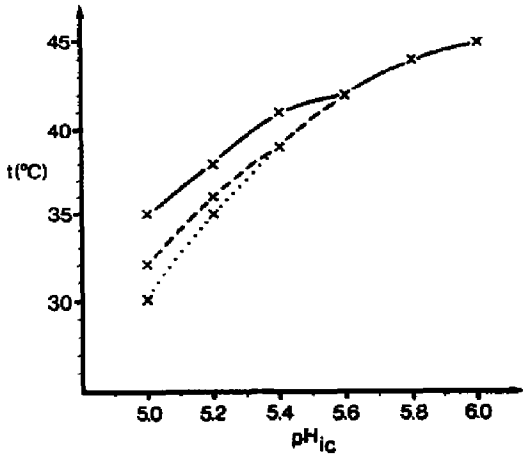


Fig. 2. Minimal temperature values causing vesiculation as a function of the intracellular pH. Time interval between titration and heating: ———, 0 min; — — —, 6 h at 25 °C; ·····, 6 h at 30 °C

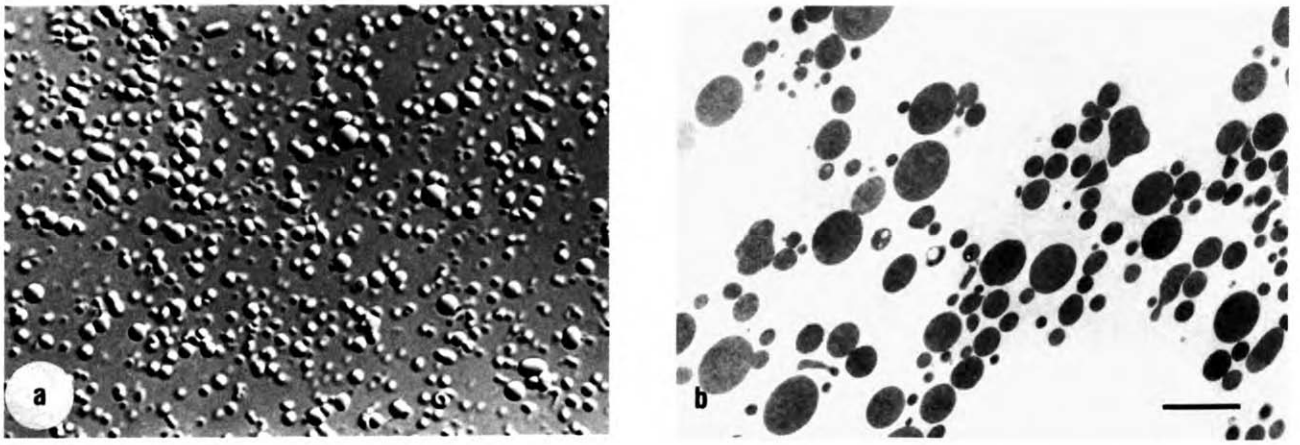


Fig. 3. Nomarski (a) and transmission electron micrograph (b) of vesicles which were separated from the mother cells. Note that the vesicles are slightly fragmented but are not haemolyzed. Magnification: (a) 1000×; (b) 5300×. Bar, 2 μm.

these temperature values neither diamide- nor NEM-pretreated, non-titrated cells showed vesiculation within 40 min. While diamide had no effect on vesiculation, NEM proved to be inhibitory. In contrast to control cells, NEM-pretreated erythrocytes were in the process of budding, but only very few free vesicles could be observed (Fig. 5a). While the crenator DNP had no substantial effect on vesiculation, the cup-former chlorpromazine was strongly inhibitory (not shown). WGA showed about as pronounced an inhibitory effect as chlorpromazine: it not only prevented vesicle release, but the number of budding forms were also severely reduced (Fig. 5b). Preincubation of the erythrocytes in hyperosmotic NaCl solution markedly promoted vesicle formation: a medium of 600 mosM decreased the critical temperature from 45 °C to 42 °C when the intracellular pH was decreased to 6 (not shown).

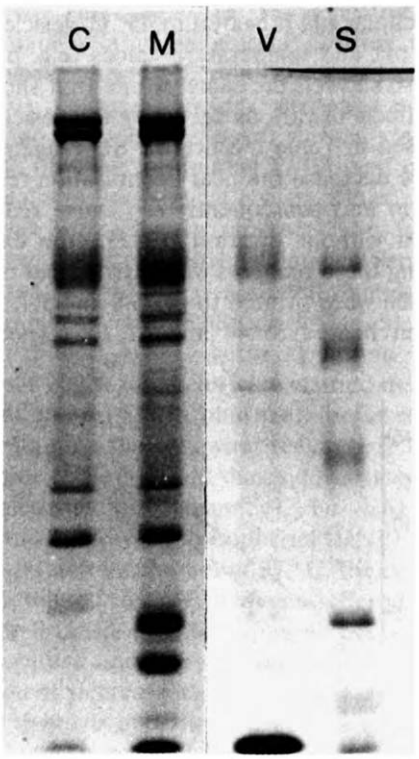


Fig. 4. Coomassie blue-stained SDS-polyacrylamide gels of membranes of control cells (C), mother cells (M) and vesicles (V). Note the complete absence of cytoskeletal proteins from the vesicle membrane. (S) Molecular weight standards: phosphorylase *b*, 94 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; trypsin inhibitor, 20, 1 kDa; α-lactalbumin, 14,2 kDa.

At higher magnification, thin section electron micrographs revealed a dense layer beneath the membranes of the mother cells, but not in the control erythrocytes. This layer, in the case of cross-sectioned membranes, occasionally showed a filamentous substructure (Fig. 6a) very similar to that observed by Tsukita et al. [16] in glutaraldehyde/tannic acid-fixed ghosts, but not in spectrin-depleted vesicles. That this submembrane layer does in fact have a reticular appearance could be demonstrated by investigating tangentially sectioned membranes (Fig. 6b). Such dense layers frequently showing discontinuities beneath the membranes of the mother cells could never be detected in the forming or detached vesicles (Fig. 6c). The empty endocytotic vesi-

TABLE I

Effects of various agents on the rate of vesiculation

Agent	Concn.	Effect
Diamide	2 mM	none
N-Ethylmaleimide	2 mM	significant decrease
2,4-Dinitrophenol	4 mM	none
Chlorpromazine	60 μM	almost complete inhibition
Wheat germ agglutinin	5 μg/ml	almost complete inhibition
Hyperosmolarity	600 mosM NaCl	significant increase

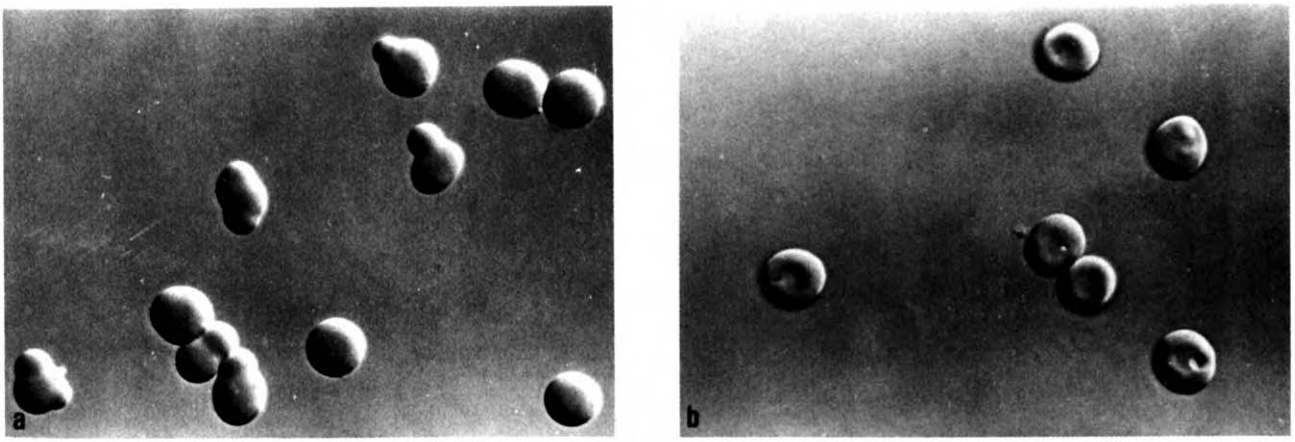


Fig. 5. Nomarski micrographs of titrated and heated erythrocytes pretreated with NEM (a) and WGA (b). Note that NEM-pretreatment slows down, while WGA-pretreatment almost completely prevents vesiculation. For further details see text. Magnification: 1000×.

cles were also found to lack the dense submembrane layer described above (not shown). Pretreatment of the cells with agents listed in Table I had a different effect on the appearance of the submembrane layer. NEM could prevent neither the formation of this layer nor that of the discontinuities seen in Fig. 6c. WGA strongly hindered the condensation of the submembrane material. In WGA-pretreated cells only a poorly visible layer could be observed with no or minimal discontinuities. Chlorpromazine-pretreated cells showed a clearly visible discontinuous submembrane layer as well as many endovesicles after titration and heating. These vesicles could also be seen in chlorpromazine-pretreated, titrated cells without the submembrane layer (not shown).

When titrated and heated erythrocytes were fixed with glutaraldehyde 30 min after vesiculation had occurred, no aggregation of the intramembrane particles could be detected in either the mother cells or the vesicles even when the intracellular pH of the erythrocytes was previously adjusted to 5.4 (Fig. 7).

Discussion

In this study we describe the formation of large membrane skeleton-free erythrocyte vesicles as a function of intracellular pH and temperature. The question arises, what type of mechanism is responsible for the phenomenon? Johnson et al. [17] reported that the volume of isolated human erythrocyte ghosts is reduced by decreasing the pH, and a further volume reduction can be induced by adding salts even at pH 4.8, where pH-induced shrinkage is maximal. From these results, the authors concluded that the observed volume changes depend on both intermolecular and intramolecular forces. Johnson et al. [17] also described a decrease in the volume of isolated red cell skeletons by raising the temperature from 0°C to 37°C. In keeping with this observation Stokke et al. [18] demonstrated that the intrinsic viscosity of isolated human erythrocyte spectrin dimers decreases when the temperature is increased from 4°C to 38°C. This means that the end-to-end distance of spectrin dimers decreases with increasing temperature [18].

Based on these data and our results, the mechanism of vesiculation can, in our opinion, be explained as follows: the extension of a meshwork depends on both the intermolecular distances and on the lengths of the individual molecules that form the meshwork. In the human erythrocyte membrane skeleton intermolecular distances decrease by lowering the pH and reach a minimum at pH 4.8, the reported isoelectric point of spectrin [19]. As mentioned above, elevated temperature induces a decrease in the length of spectrin molecules [18] thereby causing some shrinkage of the isolated skeletons [17]. The curve in Fig. 2 clearly demonstrates that the vesiculation depends on both the intracellular pH and the temperature. At higher intracellular pH higher temperature values lead to vesiculation. This means in our opinion that the lower the rate of reduction in the intermolecular distances the higher the rate of decrease needed in the length of spectrin molecules to induce that rate of skeletal condensation which causes vesiculation and vice versa. The contraction may form large skeleton-free areas below the lipid bilayer due to disruption of the connections between the skeletal proteins themselves and between these proteins and the bilayer. Further shrinkage of the skeleton then causes these areas to bud off from the erythrocytes and to a lesser extent to be internalized. This is supported by the observation that unlike in mother cells no dense reticular layer could be observed beneath the membranes of the forming or detached skeleton-free vesicles (Fig. 6c). It is not quite clear why

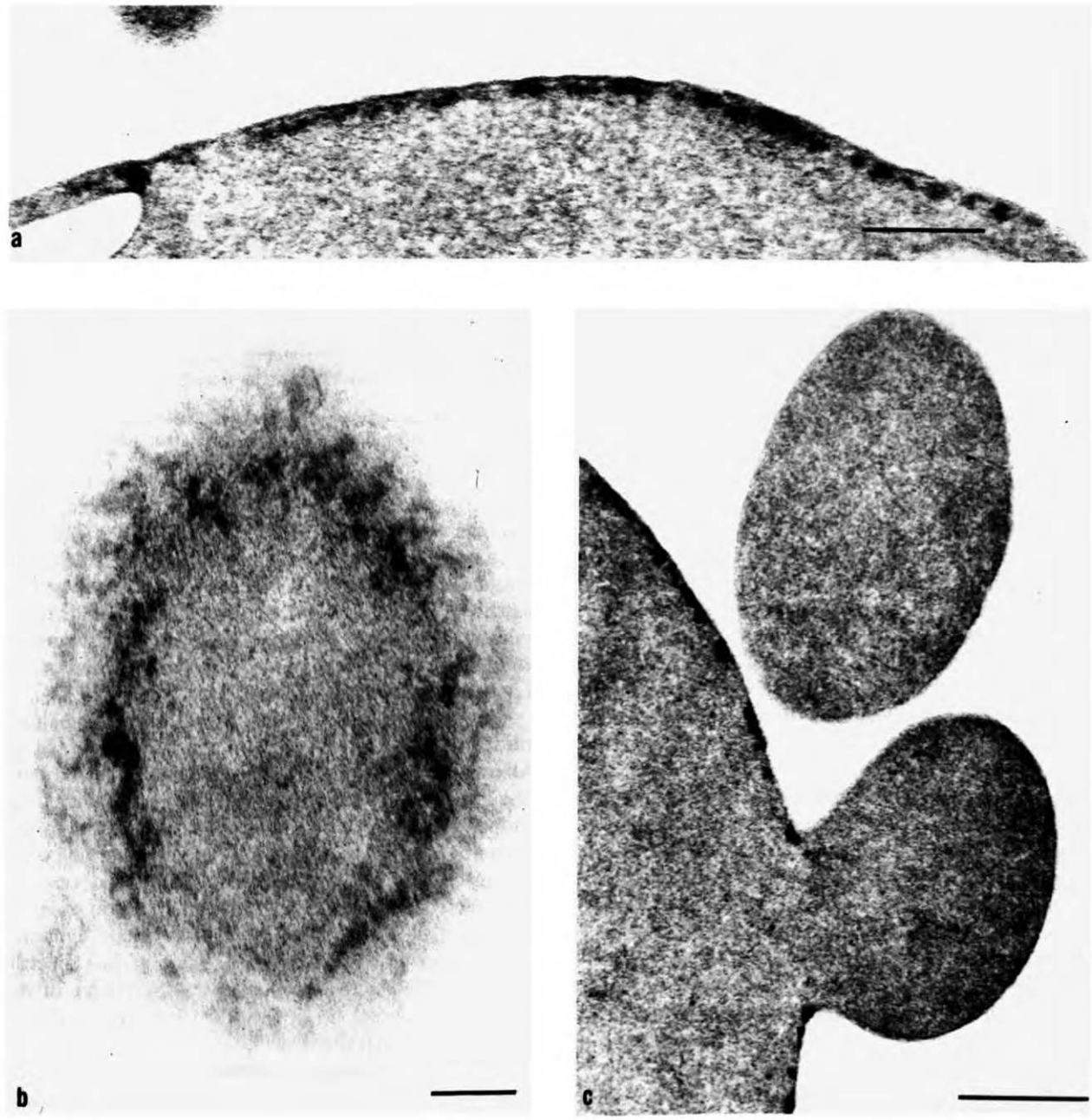
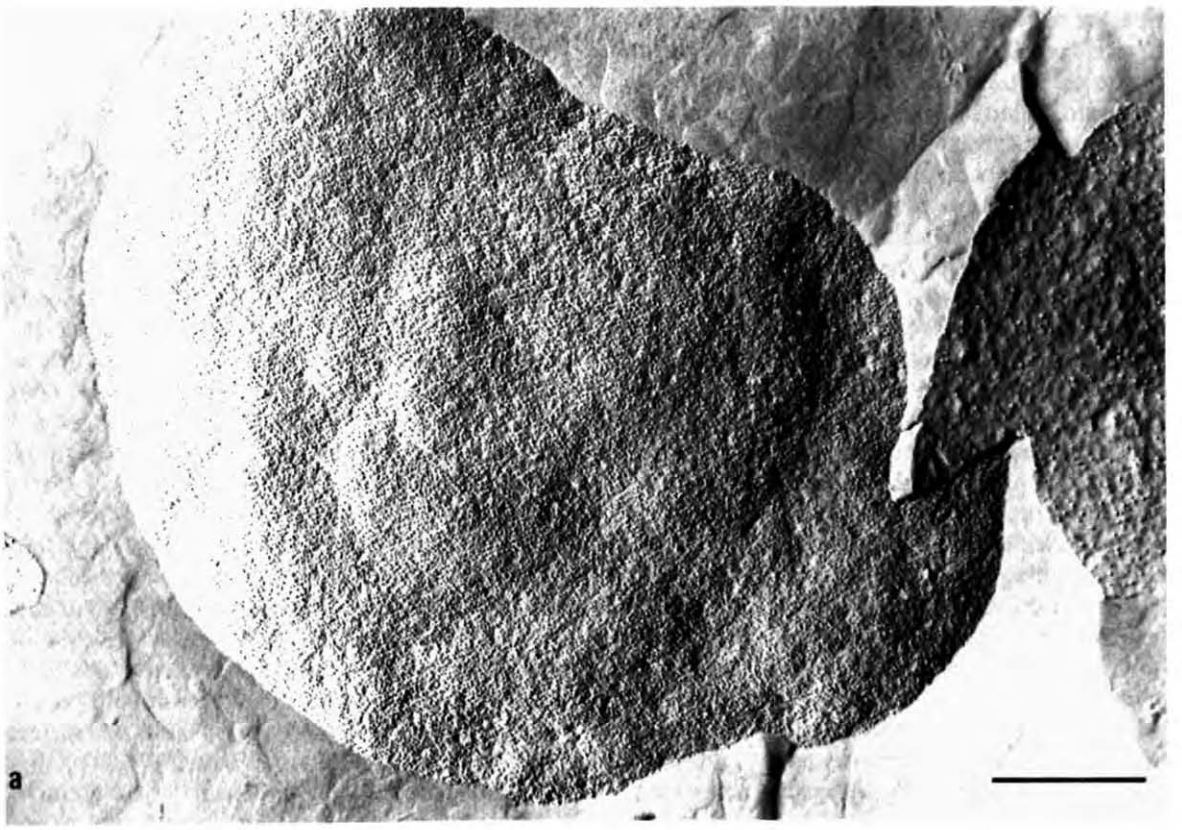


Fig. 6. Higher magnification electron micrographs of vesiculating erythrocytes. (a) Observe the dense, granulofibrillar layer beneath the plasma membrane of a mother cell; (b) Tangentially sectioned membranes show reticular appearance of the submembrane layer; (c) No submembrane layer can be detected in the forming or detached vesicles. Observe the patchy, discontinuous appearance of the submembrane layer in the mother cell. Magnification: (a) 100000×; (b) 67500×; (c) 41500×. Bars, (a) 0.2 μm; (b) 0.2 μm; (c) 0.5 μm.

this layer, which in all probability represents the membrane skeleton, can be revealed in the mother cells, but not in control erythrocytes. One possibility is that contraction of the membrane skeleton and subsequent release of the skeleton-free membrane areas lead to an increased density of the skeletal proteins beneath the

Fig. 7. Freeze-fracture electron micrographs of a titrated and heated erythrocyte sample. Red cells were titrated to pH 5.4 and vesiculation was induced at 41°C. After vesiculation had occurred the sample was kept at room temperature for 30 min, then fixed. Note the random distribution of the intramembrane particles in both the mother cells (a) and the vesicles (b). Magnification: (a) 42000×; (b) 57000×. Bars, (a) and (b) 0.5 μm.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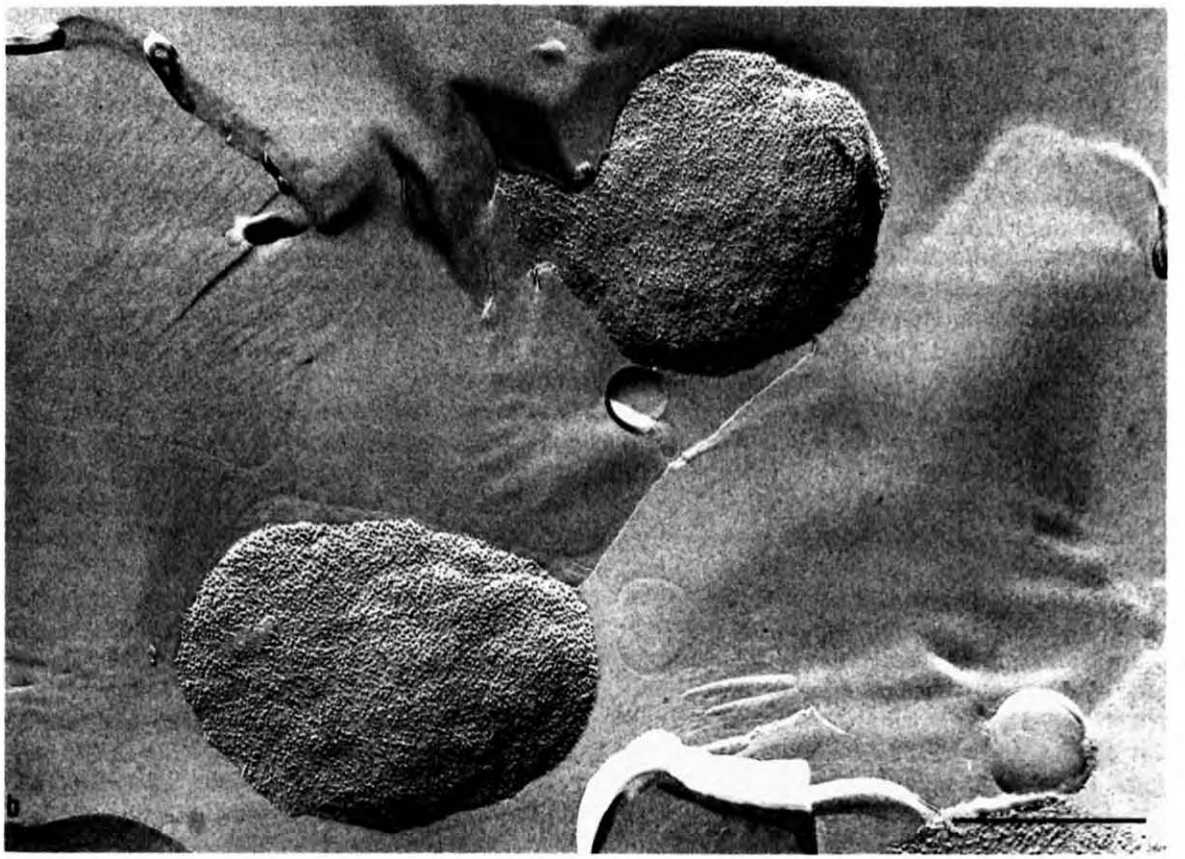
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membranes of the mother cells. This denser layer can then be visualized by means of conventional electron microscopy even in the presence of haemoglobin.

According to Crandall et al. [20] the surface elasticity modulus of acid-titrated erythrocytes increases with time becoming more and more substantial with decreasing pH. This fits in very well with our observation demonstrating a decrease in the vesiculation temperature as a result of increasing the time between titration and heating at pH values lower than 5.6 (Fig. 2). Together, these results further support the role of skeletal contraction in the vesiculation. Therefore, we believe that it is reasonable to assume that the vesicle formation described here can be explained by a purely physicochemical molecular mechanism which affects the state and dimension of the membrane skeleton.

One might say that the vesiculation described by us is due primarily to the heat denaturation of spectrin occurring normally at about 49 °C, but as the pH is shifted toward the isoelectric point it takes place at lower temperature values. Two observations are inconsistent with this hypothesis. First of all, using differential scanning calorimetry, Brandts et al. [21] demonstrated that the transition temperature for the A transition of ghosts, which is due to the thermal unfolding of spectrin in the membrane, is largely insensitive to pH-changes in both moderate and high salt media. Secondly, the results of our experiments with NEM- and diamide-pretreated red blood cells also seem to contradict the hypothesis mentioned above. Since both NEM- and diamide-pretreatment are known to decrease the denaturation temperature of spectrin and the fragmentation temperature of erythrocytes [13–15], one might assume that these reagents should promote vesiculation if it were also a result of heat denaturation of spectrin at lower temperature values. However, at temperature values where these reagents did not cause red cell fragmentation by themselves, diamide did not affect vesiculation, while NEM proved to be significantly inhibitory (Fig. 5a). Therefore, although some irreversible change in the molecular structure of skeletal proteins occurs most likely simultaneously with vesiculation, this alteration as the result of a simple thermal unfolding of spectrin and as the major cause of vesiculation can in our opinion be excluded.

The inhibitory effect of NEM can be explained by the fact that this reagent, unlike tetrathionate and diamide [15], not only decreases the denaturation temperature of spectrin, but also profoundly affects spectrin self-association in the membrane [14,15] causing a decreased association between dimers and consequently a looser skeletal organization. Although pretreatment of the cells with NEM cannot prevent the formation of skeleton-free membrane areas in the mother cells, the weaker contraction of the looser skeleton leads to slower vesiculation (Fig. 5a). Since the supramolecular organization and mechanical characteristics of membrane skeletons of erythrocytes from patients with hereditary pyropoikilocytosis and elliptocytosis can be mimicked by NEM pretreatment [14], we think that the procedure described by us may be in principle a simple and useful supplementary method to study the skeletal defects in different haemolytic anaemias.

It is known that the cytoplasmic segment of glycophorin A can bind to the membrane skeleton upon addition of glycophorin-specific ligands such as monoclonal antibodies and WGA, and that this binding significantly affects the mechanical properties of the erythrocytes [22–25]. In the WGA-treated cells, the increased binding of the skeleton to the membrane strongly hinders both the condensation of the skeletal material and the formation of skeleton-free membrane areas upon titration and heating. Since the formation of these areas is the prerequisite for vesiculation, it is not surprising that the binding of WGA to the outer surface almost completely prevented vesiculation (Fig. 5b). It is important to emphasize that in our case WGA acts via a transmembrane mechanism and not by forming a cell wall-like structure on the outer surface. Our experiments using radioiodinated WGA showed that under the conditions employed by us about 600000 lectin molecules were bound specifically to one erythrocyte (data not shown). This number, which approximates that of the glycophorin monomers in one red blood cell, is too low for the formation of an exoskeleton.

Considering the effects of NEM and WGA it is concluded that vesiculation can be affected by changing protein–protein interactions between the skeletal proteins themselves and between the skeletal proteins and the bilayer: decreased interactions between the spectrin molecules and increased interactions between the skeleton and the bilayer hinder vesicle formation.

The inhibitory effect of chlorpromazine must have resulted from the intense endovesiculation that occurred mainly after titration but before heating of the erythrocytes. Chlorpromazine which incorporates into the inner leaflet of the erythrocyte membrane [26] causes not only stomatocytosis but also endovesiculation. Although this latter phenomenon normally occurs at a concentration of the drug about one order of magnitude higher than that used by us [27], in our case the lower concentration of chlorpromazine together with the additive effect of intracellular acidification caused significant membrane internalization. This led to a greatly decreased surface to volume ratio, which prevented exovesiculation after heating even if the condensation of the skeleton was not inhibited.

The only agent which significantly enhanced vesiculation was the increased osmolarity of the incubation medium. One may be tempted to say that it is the

crenation and the resulting spicule-formation of the erythrocytes that is responsible for the increased vesiculation observed. However, DNP which is also a crenator did not cause an increase in vesicle formation at all, and the echinocytes formed upon treatment with increased osmolarity as well as DNP changed their shape into stomatocytes during acidification. Therefore, vesicles were released from stomatocytes upon heating, even though the erythrocytes were pretreated with crenators. On the other hand, it is known that neutral salts can decrease the Stokes radius of spectrin implying the contraction of the individual molecules [28]. Consistent with this observation Johnson et al. [17] could demonstrate a contraction of the isolated spectrin-actin lattices upon treatment with increasing concentrations of NaCl and KCl. Since incubation in hyperosmotic solutions leads to an increase in the intracellular concentration of monovalent cations, we think that this increase can cause, like elevated temperature values, some contraction of the individual spectrin molecules and can thereby promote vesiculation.

Leonards and Ohki [10] reported on the formation of large membrane skeleton-free vesicles as a result of titration of the red cells with EDTA and $CaCl_2$ at 45 °C. Although the vesicles obtained by this procedure have been used to study membrane aggregation and fusion as well as different transport processes [29–31], the mechanism of vesiculation remained obscure. We believe that the formation of vesicles described by these authors can be explained by a mechanism similar to the one proposed by us. Although neither EDTA nor Ca^{2+} can penetrate the red cell membrane, hydrogen ions are released as a consequence of EDTA and $CaCl_2$ addition. Thus, the authors must have decreased the intracellular pH inadvertantly, which induced vesiculation at 45 °C. Indeed, we found that the intracellular pH of erythrocytes showing vesiculation as a result of EDTA and $CaCl_2$ treatment at 45 °C is close to 6. Although the authors described that in control experiments no vesiculation was found in a solution of pH 5.5 at 45 °C the erythrocytes may have been incubated in a solution of low buffering capacity which was unable to decrease the intracellular pH sufficiently. Therefore, it seems likely that the method of Leonards and Ohki [10] is a particular form of the more general method described by us.

When the intracellular pH of erythrocytes was adjusted to 5.4 and then vesiculation was induced at 41 °C, no aggregation of the intramembrane particles could be detected in either the mother cells or the vesicles (Fig. 7). Both observations are unexpected considering some previously published studies. Whereas in ghosts the low pH-induced particle clustering has been ascribed to the isoelectric precipitation of spectrin on the inner surface of the plasma membrane [19], our results indicate that a low pH-induced skeletal contraction does not necessarily cause particle aggregation in intact red cells. Moreover, heating the erythrocytes to 50 °C or treating them with diamide — both agents are known to induce clustering of spectrin [32,33] — also failed to cause particle aggregation in nonhaemolyzed red cells [34]. Together, these data call for a reappraisal regarding the role of the membrane skeleton in the redistribution of the red cells integral membrane proteins.

The lack of particle aggregation in the membrane skeleton-free vesicles is not less surprising if we consider the strong low pH-induced particle aggregation in the skeleton-free, stripped ghosts described by Gerritsen et al. [35]. The reason for the lack of pH-induced particle aggregation in the skeleton-free vesicles is not quite clear, but we believe that an altered haemoglobin–membrane interaction plays a role in it. The erythrocyte membrane can bind haemoglobin and the cytoplasmic segment of Band 3 molecules was reported to be the binding site [36]. The interaction of haemoglobin with Band 3 seems to be electrostatic in nature. In addition to Band 3, inner layer phospholipids may also be involved in the haemoglobin–membrane interaction [37–39], especially in the skeleton-free vesicles, where the inner membrane surface is completely unmasked. Since the isoelectric point of haemoglobin is 6.8, at pH 5.4 haemoglobin is definitely positively charged, but the net charge of phosphatidylserine is still negative at this pH value. Therefore, haemoglobin may interact with phosphatidylserine electrostatically upon intracellular acidification, and this interaction may hinder particle aggregation by preventing phosphatidylserine from undergoing low pH-induced lateral phase separation [35] even in the complete absence of spectrin. Although this idea still requires verification, the example demonstrates well that data obtained on haemoglobin-free ghosts produced by hypotonic lysis cannot be directly extrapolated to haemoglobin-containing red cells and vesicles.

Acknowledgements

We thank Dr. Ágnes Enyedi for her help in SDS-polyacrylamide gel electrophoresis. The excellent technical assistance of Ms. Gabriella Papp and Ms. Mária Berényi is also acknowledged.

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