Low pH Induced Shape Changes and Vesiculation of Human Erythrocytes

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Abstract. Shape changes and vesiculation were induced in intact human erythrocytes by gradually decreasing pH in the cell suspension. A sequence of different shapes preceding vesiculation was documented, i.e. discocytes, stomatocytes, and stomatoacantocytes. The final state was characterized by spherical mother cells and vesicles released. Low pH-induced vesiculation was also studied in the presence of stomatocytogenic or echinocytogenic compounds. The action of stomatocytogenic compounds was inhibitory, and echinocytogenic compounds had no effect on low pH-induced vesiculation. Vesiculation induced by low pH was studied also in isotonic solutions of different sucrose/salt composition. It was concluded that (i) low intracellular pH is responsible for cell shape transformations as well as for release of vesicles, (ii) at temperature 37°C the intracellular pH value which induces the release of vesicles is 5.4, and (iii) the sequence of typical shape changes preceding vesiculation does not include echinocytes. The results are discussed on the basis of the layered membrane model of the shape formation and shape transformations of the human erythrocyte, and additionally considering the partial detachment of the membrane skeleton from the bilayer part of the membrane.

Key words: Human erythrocyte — pH — Cell shape changes — Vesiculation — Layered membranes

Introduction

Release of membrane vesicles from human erythrocytes can be observed *in vitro* under different experimental conditions. It occurs, for instance, in calcium (Allan et al. 1980; Allan and Thomas 1981) and in DMPC loaded erythrocytes (Ott et al. 1981; Bütikofer et al. 1987), in ATP depleted erythrocytes (Lutz et al. 1977), and

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it can be induced by various amphiphiles (Hägerstrand and Isomaa 1989, 1992). Further, vesiculation is observed in stored blood (Rumsby et al. 1977), heated erythrocytes (Coakley et al. 1978) and in erythrocytes incubated in urea solution (Fuhrmann 1968), and in low pH medium of about 5.4 (Leonards and Ohki 1983; Wagner et al. 1986; Brumen et al. 1987; Gros et al. 1987; Lelkes and Fodor 1991).

In various experiments it has been demonstrated that before releasing vesicles the erythrocyte first undergoes morphological changes. It is generally expected that evaginations of the membrane, as in echinocytes, lead to the formation of exovesicles, whereas invaginations, as in stomatocytes, lead to the formation of endovesicles. However, there are indications that the low pH induced vesiculation does not follow this pattern and therefore requires special attention. This is of particular interest because pH is well-known to determine the erythrocyte shape. The erythrocyte is a discocyte at the physiological value of pH, and it is a stomatocyte at sufficiently low pH values, whereas it is an echinocyte at high pH values (Deuticke 1968; Weed and Chailley 1972). The shape transformations are reversible with respect to the pH changes. It has been reported (Leonards and Ohki 1983) that upon incubation of erythrocytes at low pH budges were formed which were later pinched off as closed membrane vesicles. A scheme of morphological changes of erythrocytes preceding the vesiculation was proposed in which the shape transformation to echinocyte is to be considered as a prerequisite for the release of vesicles under conditions of low pH (Wagner et al. 1986). On the contrary, observations (Brumen et al. 1987; Lelkes and Fodor 1991) clearly showed the release of vesicles at low pH from stomatocytes and stomatoacantocytes. It is therefore of interest to study vesiculation of the erythrocyte under controlled experimental conditions of low pH with particular attention to observation of the cell shape transformations. These requirements were fulfilled in the report of Lelkes and Fodor (1991) where the induction of vesiculation was demonstrated to be a result of successive decreasing of intracellular pH and then of the heating of the system. Since pH by itself is a clear determinant of the reversible erythrocyte shape transformations (Deuticke 1968; Weed and Chailley 1972) it seemed appropriate to supplement the work of Lelkes and Fodor (1991) by experiments with a decrease of pH at constant temperature.

The present paper is a report of experiments in which vesiculation was induced under such conditions. The temperature was kept constant at 37 °C. Erythrocyte suspensions were gradually titrated from pH 7.2 to lower values until vesiculation occurred, and the concomitant cell shape transformations were documented. In order to ascertain the role of the cell shape changes, experiments were performed in which the pH induced cell shape transformations and vesiculation were studied in the presence of stomatocytogenic and echinocytogenic compounds. The initial cell shape in the experimental procedure was stomatocyte or echinocyte. Observations clearly showed that the echinocytic shape did not appear in the sequence of the typical shape changes as the final one preceding the release of vesicles. Finally,

experiments in isotonic salt solutions and in isotonic sucrose/salt solutions were done in order to confirm that the pH of the inner solution is the basic determinant of low pH induced vesiculation.

The mechanisms involved in the erythrocyte vesiculation are not yet completely understood. However, the variety of conditions under which vesiculation occurs indicates that the process arises from some general properties of the erythrocyte membrane. Lelkes and Fodor (1991) interpreted the formation of vesicles in conditions of low pH in terms of a molecular mechanism affecting the state and the dimensions of the erythrocyte spectrin network. We have continued their discussion by taking into consideration the general mechanical properties of a three layer membrane (Svetina et al. 1988). In this way the two processes, i.e. shape transformation and vesiculation, can be treated within the common concepts of the layered structure of cell membranes.

Materials, Methods and Theoretical Basis

Chemicals

Phenylmethylsulphonylfluoride (PMSF), dimyristoylphosphatidylcholine (DMPC), chlorpromazine, quinine, barbital, sodium salicylate, Tris (hydroxymethyl)-aminomethane, Tris (hydroxymethyl)-aminomethane hydrochloride, Triton X-100, sodium chloride, sodium lactate, lactic acid, sucrose, glutaraldehyde were all obtained from Sigma (St. Louis, MO. U.S.A.).

Preparation of red blood cells

Freshly drawn blood was used. The plasma was removed and the red blood cells were washed three times in an isotonic solution of NaCl-Tris (NaCl 150 mmol/l, Tris/Tris-HCl 10 mmol/l, pH 7.2) and then in an isotonic solution of NaCl-Tris-PMSF (NaCl 150 mmol/l, Tris/Tris-HCl 10 mmol/l, PMSF 0.2 mmol/l, pH 7.2). Finally, the red blood cells were resuspended in the latter solution at 0.1 hematocrit. Six milliliters of this suspension were used in each experiment.

Procedures for inducing vesiculation

The lactate buffer solution pH 4.5 was prepared from sodium lactate solution 1 mol/l, and the pH was adjusted with lactic acid.

A. Gradual lowering of pH. The suspension of red blood cells was first incubated at 37 °C for 10 minutes. In order to lower the pH gradually, 50 μl of sodium lactate buffer solution (pH 4.5) was then added every 5 minutes until pH 5.4 was achieved. Then, the suspension was incubated at 37 °C for 30 minutes. The concentration of free hemoglobin in the supernatant was below 7 mg/l, and the final hematocrit changed less than by 3% of the initial value. Due to the addition of lactate solution

the final osmolarity was about 20% higher in this and in all further experiments. The cell suspension was titrated (i) by EDTA and $CaCl_2$ as reported by Leonards and Okhi (1983), (ii) by calcium, magnesium or sodium acetate (Brumen et al. 1987), and (iii) by lithium or potassium lactate (Gros et al. 1987).

B. Gradual lowering of pH in the presence of membrane active compounds. 6 ml of the cell suspension was centrifuged at 1500 g, the supernatant solution was replaced by isotonic solution of NaCl-Tris-PMSF (NaCl 150 mmol/l, Tris/Tris-HCl 10 mmol/l, PMSF 0.2 mmol/l, pH 7.2) with membrane active compounds added (quinine 2 mmol/l, chlorpromazine 0.25 mmol/l, sodium salicylate 20 mmol/l, barbital 40 mmol/l). The hematocrit was 0.1. This cell suspension was incubated at 37 °C for 1 hour before gradual lowering of pH.

C. Gradual lowering of pH in the isotonic sucrose/salt solution. Two isoosmotic solutions of initial osmolarity 300 mmol/l were prepared with respect to different fractions of NaCl and sucrose (150 mmol/l NaCl, 0 mmol/l sucrose, and 30 mmol/l NaCl and 240 mmol/l sucrose). The PCV values of both cell suspensions were 0.1. The lowering of pH was achieved by the addition of sodium lactate buffer solution, pH 4.5. A portion of the suspension was treated for further examination of cell shapes by light and electron microscopy. Another portion was analyzed for values of inner pH, external pH and external osmolarity.

Observations of erythrocyte shapes

During the titration, the cell shapes were observed under light microscope (immersion, ×1000). Selected samples were further prepared for scanning electron microscopy. One hundred μl of the suspension were added to 100 μl solution of glutaraldehyde, 0.25 mol/l in isotonic solution of NaCl-Tris-PMSF. The glutaraldehyde solution was always adjusted with sodium lactate buffer to the pH value of the cell suspension. For scanning electron microscopy samples were further prepared according to Bessis (1960). The shapes observed were classified according to the nomenclature of erythrocyte shapes of Bessis (1972).

Determination of pH

pH changes in cell suspensions were monitored using a thermostatic Ultra-Micro pH electrode and an IL 213-03 pH-meter (Instrumentation Laboratory Inc., Lexington, USA). For measurements of intracellular pH, the cells were separated from the suspension by centrifugation (Beckman Microfuge 11, Beckman, USA; 12,000 rpm). The pocket cells were hemolysed by addition of a detergent, Triton X-100. pH of the hemolysate was measured by the same apparatus as above.

Vesicles

Free vesicles were separated from the cells by centrifugation (Beckman Microfuge 11, Beckman, USA; 12,000 rpm). The orientation of the vesicle membrane was determined from measurements of the catalytic activity of acetylcholinesterase (Ellman et al. 1961) in suspension of vesicles in the presence or absence of the detergent Triton X-100.

Theoretical basis – the concept of layered membranes

The erythrocyte membrane can in general be considered as being composed of three layers: the two layers of the bilayer part of the membrane and the membrane skeleton network. The behavior of this system can in first approximation be described by assuming that these three layers, taken to be laterally homogeneous, are in tight contact and can slide one over another. The shape of a cell is thus determined by its volume and the elastic properties of the individual layers (Svetina et al. 1988). The classical bilayer couple model (Sheetz and Singer 1974) is a simplified version of the above more general model as it considers only the two layers of the bilayer part of the membrane which are also assumed to be incompressible. Then, the parameter which essentially determines the cell shape at a given cell volume and membrane area is the difference between the areas of the outer and the inner layer, i.e. $\Delta A = A_{\text{out}} - A_{\text{in}}$. In the quantitative treatment of this model (Svetina and Žekš 1989) the cell shape corresponds to the minimum value of the membrane bending energy at constant values of the cell volume, cell membrane area, and the difference between the areas of the two membrane layers. A result of this theory relevant to our analysis is that consecutive cell shape transformations as a consequence of the monotonously changing parameters ΔA and/or the cell volume (at constant membrane area) can only occur within limited regions in the volume/area difference phase diagram. Some boundaries of these regions are characterized by specific limiting shapes. The limiting shapes of interest in the vesiculation phenomenon are composites of linked spheres where the radii of the spheres can only have two values (Svetina and Žekš 1989). Some examples of such limiting shapes are shown in Fig. 1. It can be seen that at higher values of ΔA the limiting shapes involve exovesicles, whereas at lower values of ΔA they involve endovesicles.

The analysis of low pH induced vesiculation requires a generalization of the bilayer couple model related to the presence of the membrane skeleton. A trilayer membrane parameter ΔA_{eff} can be introduced representing the effective difference in the layer areas (Svetina et al. 1988). The meaning of the qualitative effect of the parameter ΔA_{eff} on the erythrocyte shape transformations (as indicated in the legend to Fig. 1) is the same as of the parameter ΔA of the bilayer couple model, however, its value is determined by the elastic constants and equilibrium areas of all three layers, and the distances between them (Svetina et al. 1988). It can be shown that ΔA_{eff} increases when either the area of the outer layer increases or

when the area of the inner layer decreases whereas reverse changes in areas of these two layers result in a decrease of ΔA_{eff}. The effect of the middle layer is complex, strongly depending on the interplay of all parameters which determine ΔA_{eff}. Recent data on elastic properties of the erythrocyte membrane layers show that values of the expansivity moduli of the two leaflets of the bilayer are significantly larger than the value of the skeleton expansivity modulus (Mohandas and Evans 1994). Consequently, for changes in layer areas of the same order of magnitude the trilayer parameter ΔA_{eff} is nearly equal to the bilayer parameter ΔA.

The possibility of a partial detachment of the membrane skeleton from the bilayer part of the membrane can also be considered. It was shown (Iglič et al. 1995) that when the skeleton is stretched, its partial detachment can occur when the decrease of the elastic energy of the skeleton upon detachment is larger than the increase of the energy due to the diminished skeleton/bilayer interaction. The process is more favorable when cells have shapes involving narrow necks such as in the exovesicular limiting shapes shown in Fig. 1.

Results

Changes of erythrocytes shapes were continuously monitored using light microscopy in an experiment in which the cells were first suspended in isotonic salt solution

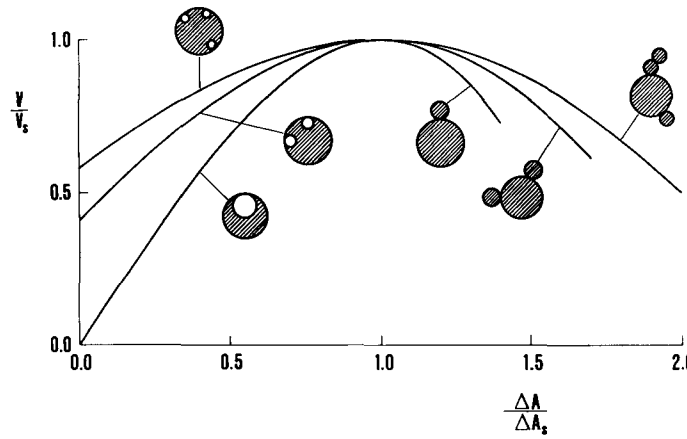


Figure 1. The dependence of relative cell volume (cell volume, V, divided by the volume of the sphere with the same membrane area, V_s) on the relative area difference (area difference, ΔA, divided by the area difference corresponding to a sphere with the same membrane area, ΔA_s) for some examples of the limiting shapes composed of connected spheres. Spheres may only have two different radii (Svetina and Žekš 1989). The shaded areas mark the cell interior. At relative area differences larger than one daughter vesicles are exovesicles, and at relative area differences smaller than one they are endovesicles. The same predictions apply if ΔA is replaced by ΔA_{eff}.

at 37°C and then the pH of the suspension was gradually lowered. In this experiment discocytes changed to stomatocytes, and further, stomatocytes transformed to stomatoacantocytes. Finally, when pH was close to 5.4, vesicles were released as an abrupt event, and the erythrocytes became spherical. Since in a cell population cells are distributed with respect to their shape (Weed and Chailley 1972) the above sequence of cell shape transformations refers to the majority of cells. Electron micrographs in Fig. 2 are representative for shapes of cells in suspension at pH values indicated. In the case shown titration was performed with sodium lactate buffer solution. In additional experiments different chemicals were used, nevertheless, the morphological changes preceding vesiculation as well as the release of vesicles were observed to be the same in all cases. Thus, the important result in the context of the present paper has been that the echinocytic shape is not observed as the final one in the sequence of shapes preceding vesiculation.

Vesicles are of a micro-size as it can be seen from Fig. 2D. The vesicle membrane is oriented like the membrane of a normal cell. This could be concluded from determination of the catalytic activity of acetylcholinesterase in the absence and presence of Triton X-100 in the suspension of vesicles. Within the accuracy of the measurements there was no difference between the two values. This orientation of the vesicle membrane has already been determined by Leonards and Ohki (1983) whose procedure of inducing vesiculation by lowering pH was essentially the same as in our experiments. They also reported that the vesicles were free of the cytoskeleton.

Further experiments were designed to assess the possible role of intracellular pH in vesiculation. Two isotonic incubating solutions were prepared differing with respect to their sodium chloride/sucrose ratio: one contained 30 mmol/l sodium chloride and 240 mmol/l sucrose, and the other one was an isotonic sodium chloride, sucrose free solution. According to the theoretical treatment of osmotic and acid-base equilibrium across the red cell membrane the difference between the intra and the extracellular pH of about 0.5 can be calculated in the first case whereas in the case of pure sodium chloride solution the difference in pH is negligible (Freedman and Hoffman 1979; Brumen et al. 1979). Fig. 3 gives the calculated lines of constant intracellular pH in the phase diagram with respect to the extracellular pH and sodium chloride/sucrose ratio at 37°C. The calculations were performed using the mathematical model from Brumen et al. (1979). The measurements of intracellular and extracellular pH at which vesiculation occurred are in accord with these predictions (see Table 1). In sodium chloride/sucrose solution vesiculation occurred at the suspension pH of 4.9 and intracellular pH of 5.4, whereas in the sodium chloride solution at pH 5.4 (for experimental conditions see Fig. 3, dots I and II, respectively). Observations of erythrocyte shapes in sodium chloride/sucrose solution at the suspension pH of 6.1 and in the pure sodium chloride solution at the suspension pH of 6.2 (for experimental conditions see Fig. 3, dots III

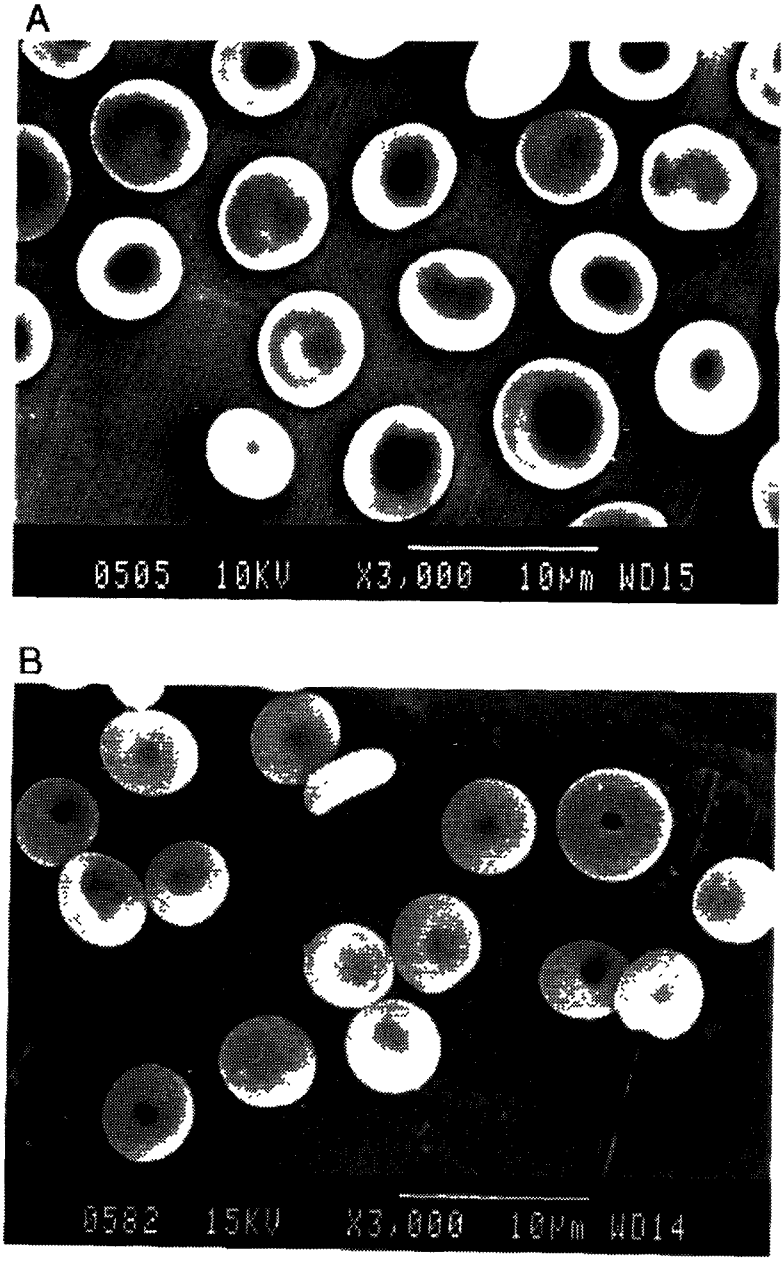
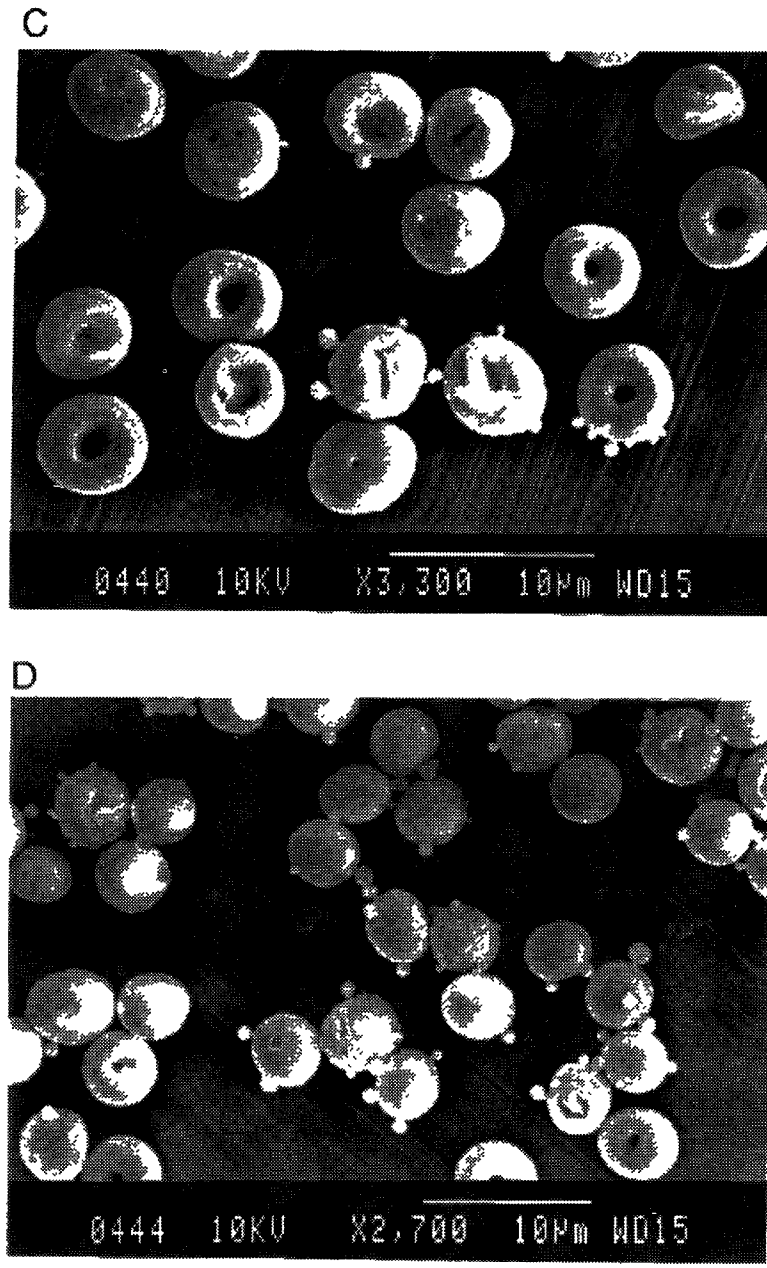


Figure 2. Shape transformations of human erythrocytes in the procedure of gradual lowering of pH in suspension of cells by adding sodium lactate (see Materials, Methods and Theoretical Basis). Electron micrographs are representative of cell morphology at



pH values indicated in the continuous process of shape transformations. Bar: 10 μm. (*A*) pH 7.2, mostly discocytes, (*B*) pH 6.2, mostly stomatocytes, (*C*) pH 5.8, mostly stomatoacantocytes, (*D*) pH 5.4, spherocytes and vesicles. Temperature: 37 °C.

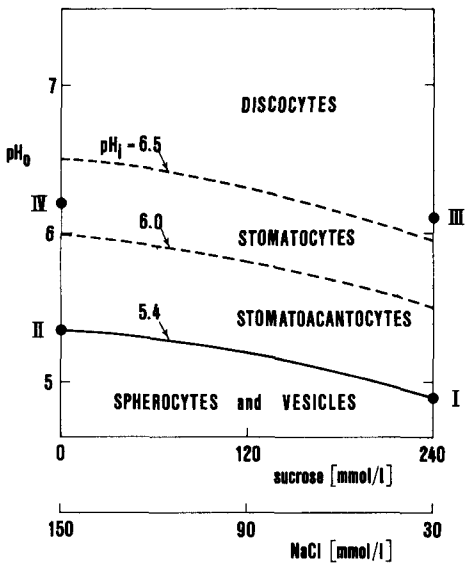


Figure 3. Theoretically predicted regions of typical erythrocyte shapes in the phase diagram with respect to extracellular pH (pH_o) and the sucrose/salt ratio of the isotonic medium. The lines of constant intracellular pH (pH_i) which separate these regions were calculated by the mathematical model of osmotic conditions of human erythrocytes (Brumen et al. 1979). The full line represents the conditions in which vesiculation occurs, and the broken lines separate the regions where cells have predominantly the indicated shapes. Dots denote the experimental conditions in which erythrocytes were incubated, their shape was observed and intracellular pH was determined (see Table 1).

Table 1. Intracellular pH (pH_i), suspension pH (pH_o), and erythrocyte morphology in solution of sodium chloride and sucrose; temperature 37 °C

Experiment[1)]	NaCl (mmol/l)	Sucrose (mmol/l)	pH_o	pH_i	Cell morphology
I	30	240	4.9	5.3	spherocytes and vesicles
II	150	0	5.35	5.4	spherocytes and vesicles
III	30	240	6.1	6.5	discocytes
IV	150	0	6.2	6.25	stomatocytes

[1)] see Fig. 3, experimental conditions indicated by dots

and IV, respectively) revealed that in sodium chloride/sucrose solution a significant portion of cells were still discocytes whereas in chloride solution the erythrocytes were stomatocytes. Observation of erythrocytes under the light microsope under the four experimental conditions indicated are shown in Fig. 4. The correspondence between the intracellular and the extracellular pH on one and the shape and the vesiculated state of erythrocytes on the other hand suggests that intracellular pH is a critical parameter determining the shape as well as the vesiculation of these cells.

In order to relate the process of pH induced vesiculation to the predictions of the bilayer couple model, experiments were performed also in the presence of the stomatocytogenic compounds, chlorpromazine or quinine (Svetina et al. 1992) or the echinocytogenic compounds, Na-salicylate or barbital. These compounds were used in concentrations which induce shape changes but no vesiculation by themselves (Fujii et al. 1979). The presence of the stomatocytogenic compounds caused cell transformation to stomatocytes already in the initial solution. During the further procedure of lowering of pH, the cell shapes did not undergo significant changes. The stomatocytes became wrinkled at pH about 6.2. The cells became spherocytes when pH dropped to 5.4, and almost no release of vesicles could be observed. In the presence of echinocytogenic compounds the cell shape was echinocytes at pH 7.4, discocytes around pH 6.8, stomatocytes at pH about 6.0, and stomatoacantocytes at pH about 5.6. Finally, at pH 5.4 vesiculation occurred and the cell shape was spherocytes. Vesiculation reached the same extent as observed in the absence of echinocytogenic agents. The entire sequence of shape transformations from echinocytes to vesiculated spherocytes was the same as in the absence of agents. Figs. 5A and 5B show the final state of the red cells achieved at pH 5.4 in the presence of stomatocytogenic compounds and echinocytogenic compounds, respectively. It thus could be concluded that the tested stomatocytogenic compounds inhibited pH induced vesiculation, whereas the echinocytogenic compounds tested had no effect on vesiculation.

Discussion

Based on the observations, a general scheme of cell shape transformations in conditions of low pH as well as under the influence of membrane active compounds can be set up. The essential characteristic of it is the generality of the sequence of cell shapes from discocytes over stomatocytes to spherocytes, induced by changes of intracellular pH. At 37°C, vesiculation occurred at the intracellular pH value of 5.4. The vesicles released had their membranes oriented like it is the case in the normal cell, however, there were no preceding echinocytic shapes. Although in the presence of stomatocytogenic agents the general pattern of shape changes remained preserved, vesiculation was inhibited. Below a consistent explanation of all these observations is presented.

It is generally believed that the process of the release of membrane vesicles from erythrocytes is related to structural changes of the cell membrane. The broad variety of cases in which vesiculation can be observed (cf. Introduction) indicates that the same macroscopic behavior can be the result of different structural modifications of the membrane. The discussion of the phenomenon of vesiculation induced by low pH of the incubating medium which follows acknowledges this fact by stating that vesiculation represents the final stage of the process of the erythrocyte

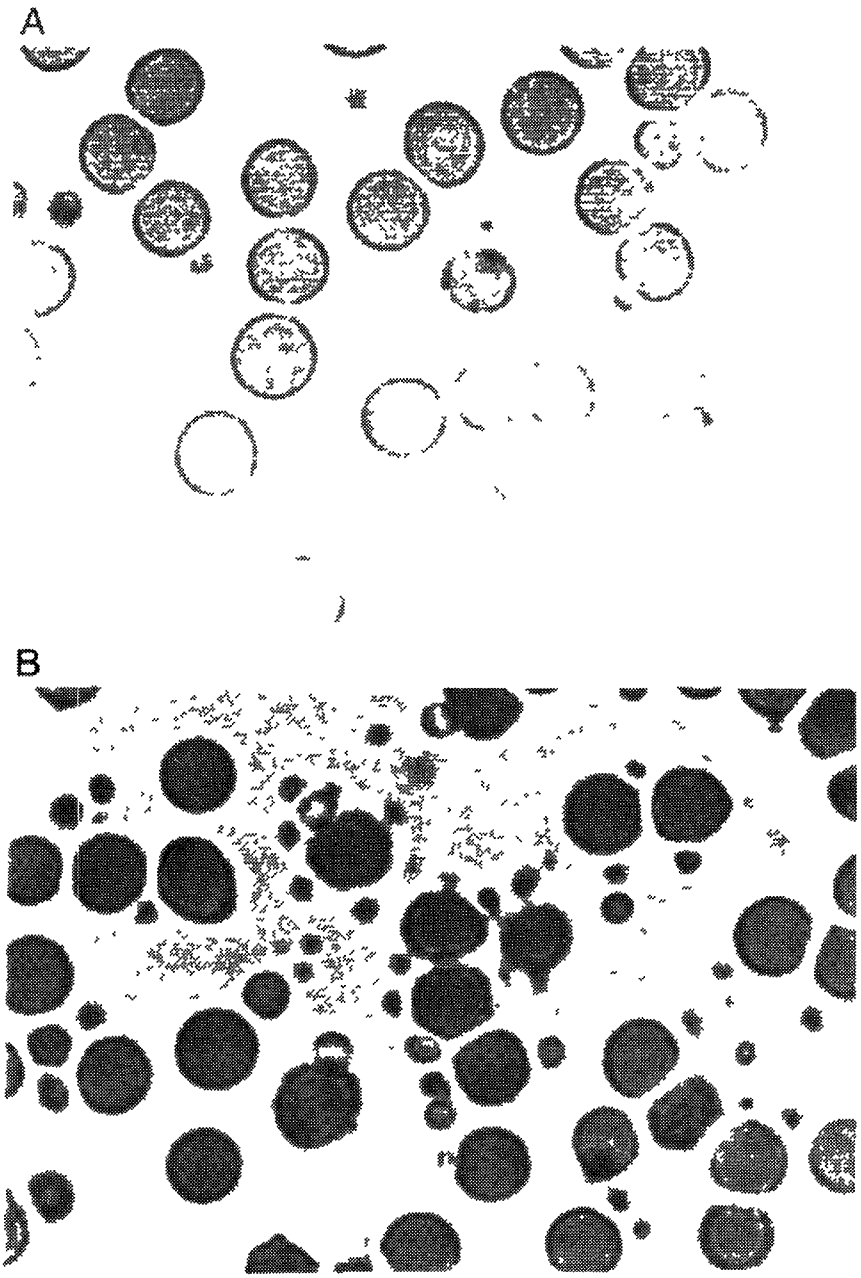
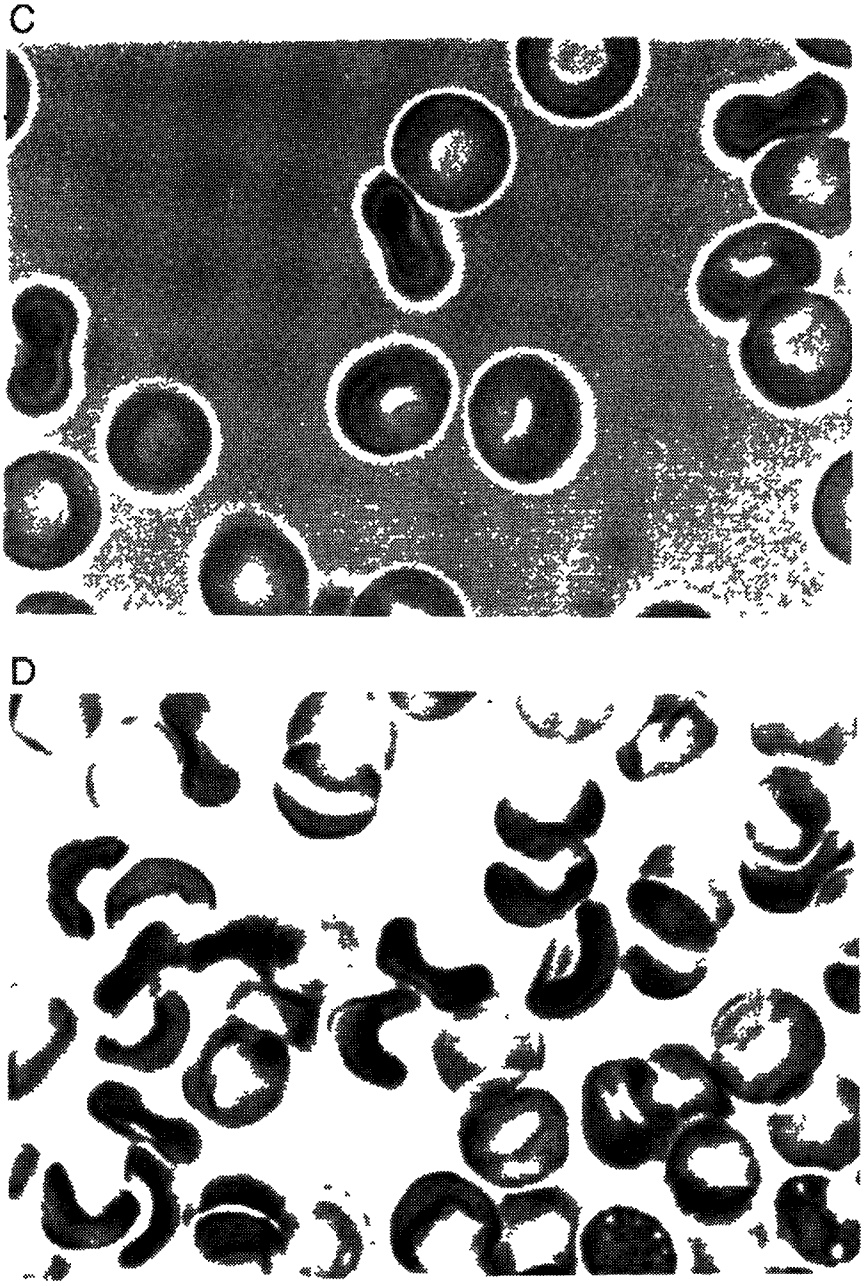


Figure 4. Shapes of erythrocytes as observed under light microscope in four different experimental conditions indicated in Fig 3 and Table 1 (*A*) spherocytes and vesicles, case I NaCl 30 mmol/l, sucrose 240 mmol/l, pH_o 4 9, pH_i 5 3, (*B*) spherocytes and



vesicles, case II NaCl 150 mmol/l, pH_o 5 35, pH_i 5 4, (*C*) mostly discocytes, case III NaCl 30 mmol/l, sucrose 240 mmol/l, pH_o 6 1, pH_i 6 5, (*D*) mostly stomatocytes, case IV NaCl 150 mmol/l pH_o 6 2, pH_i 6 25

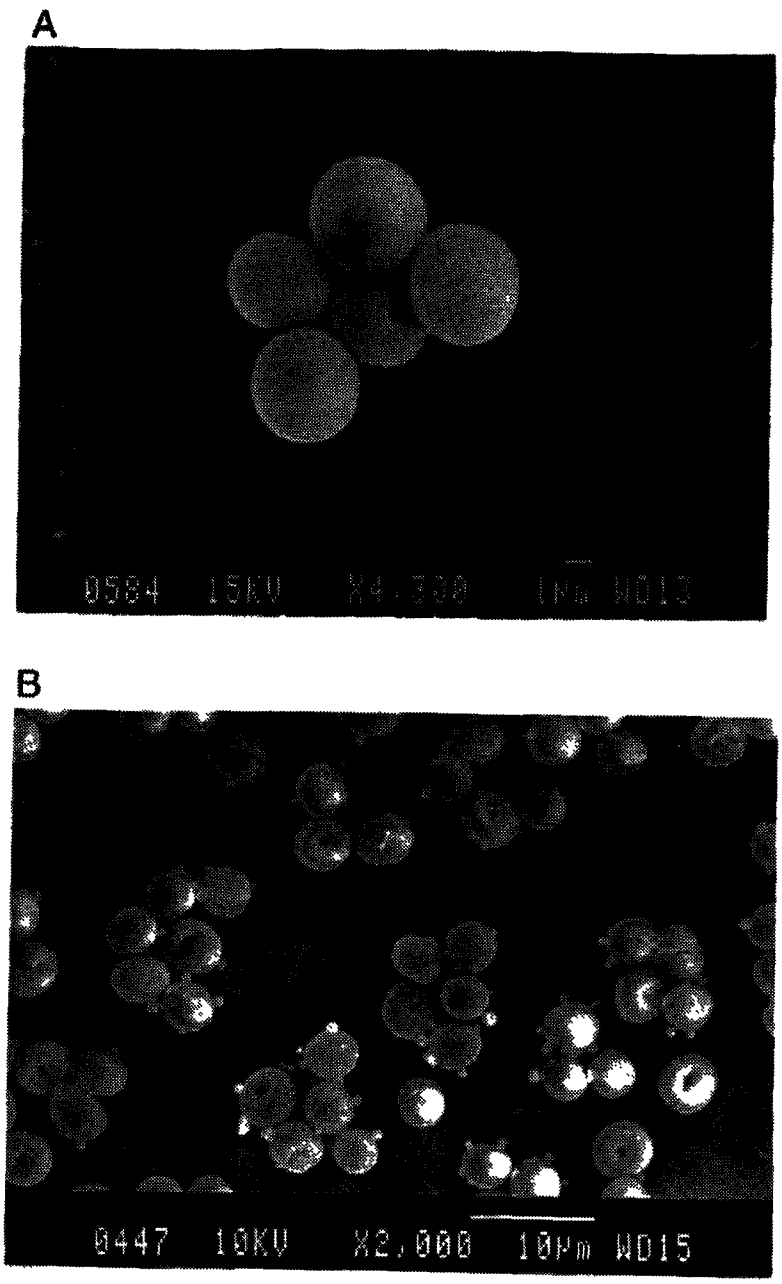


Figure 5. Low pH-induced vesiculation (pH 5.4 and temperature 37°C) in the presence of stomatocytogenic compound chlorpromazine (*A*) and echinocytogenic compound Na-salicylate (*B*). Bars: 1 μm and 10 μm.

shape transformations. In relation to the latter process it has been shown that its interpretation can be at two levels: at the microscopic level, the elasticity parameters are determined from the specific structural and compositional details of the membrane layers, and at the macroscopic level the shapes are determined from the values of the elasticity parameters irrespective of their structural background (cf. Materials, Methods and Theoretical Basis).

Previous and also our present experimental data on low pH induced vesiculation point to the intracellular pH as the main factor determining the process. Lelkes and Fodor (1991) proposed that the vesiculation is due to a purely physicochemical mechanism which affects the state and the dimensions of the membrane skeleton in the sense that spectrin aggregation causes shrinkage of the skeleton. This hypothesis is supported by the experimental findings that spectrin as the major constituent of the erythrocyte membrane skeleton aggregates at 37 °C and pH 5.4 (Liu et al. 1977; Smith and La Celle 1979), i.e. under the same conditions as vesiculation is triggered. It can be concluded that the aggregation of spectrin is the basic structural reason for the low pH-induced vesiculation of human erythrocytes.

It remains then to connect the vesiculation event with the preceding cell shape transformations. This can be done within the concept of layered membranes (cf. Materials, Methods and Theoretical Basis). The general scheme of the pH effect on shape transformations of erythrocytes is represented as discocyte – stomatocyte transformation under conditions of lowering pH, and discocyte – echinocyte transformation under conditions of increasing pH, in both cases with respect to the physiological value of pH (Weed and Chailley 1972). This means that low pH induces transformation of discocytes into an invaginated shape, and high pH induces transformation into an evaginated shape. Qualitatively, this scheme is the same as that of shape transformations predicted in dependence on the difference between the areas of the bilayer leaflets (ΔA) in the bilayer couple model or on the corresponding area difference parameter (ΔA_{eff}) of the trilayer model. Thus, the basic point of the present discussion is the postulation of the relation between the intracellular pH and the area difference: low pH is assumed to correspond to a decreased value of the area difference, and high pH to an increased value of the area difference.

From the described correspondence between the area difference and pH it can be expected that the low pH induced vesicles are endovesicles (see Fig. 1) which have their membranes oriented oppositely to the normal cell. However, we could confirm that membranes of vesicles induced by low pH are oriented normally. On the other hand, it can be expected that if the final stage of the shape transformations are exovesicles, the intermediate shapes of the shape sequence should also include echinocytes. In this respect the experimental evidence is not very clear either. Wagner et al. (1986) claimed that vesicles were released in low pH conditions after the cells assumed the shape of echinocytes, whereas no echinocytes were observed

in our experiments or in those of Lelkes and Fodor (1991). Some other experimental evidence (Lutz et al. 1977; Allan et al. 1980; Allan and Thomas 1981; Ott et al. 1981; Bütikofer et al. 1987; Hägerstrand and Isomaa 1989, 1992) which can support the view of the necessity of the cell transformation to echinocytes before vesicles are released may not be relevant for the present discussion since the experimental procedures were different and the sizes of the vesicles were significantly smaller than those induced by low pH.

A consistent interpretation of the low pH vesiculation observed in the present work, and of preceding shape transformations can be based on different pH responses of different membrane layers, i.e. of the two layers of the bilayer part of the membrane and of the membrane skeleton. The simplifying assumption of this system is that the area expansivity moduli of the two layers of the bilayer are much larger than the area expansivity modulus of the skeleton (Mohandas and Evans 1994). Consequently, a change in the area of either of the two leaflets of the bilayer affects the value of ΔA_{eff} much more than the same change in the area of the skeleton. In view of this it is plausible to assume that initial lowering of pH below its normal value induces a continuous shape transformations from the discocyte to the stomatocyte essentially due to the continuous decrease of ΔA, i.e. of the difference between the areas of the two bilayer leaflets. Our data (Fig. 3) obtained from NaCl/sucrose solution on the involvement of intracellular pH indicate that the decrease of ΔA must come from an increase of the equilibrium area of the inner leaflet.

As the vesiculation is caused by the aggregation of spectrin it cannot be considered as the final stage of the process governing the pH dependent cell shape changes. An immediate consequence of the aggregation of spectrin occurring at pH 5.4 is a drastic decrease of the equilibrium area of the skeleton. Therefore, the skeleton if underlying the bilayer over its whole area is highly stretched. The corresponding stretching energy can be released by a partial detachment of the skeleton from the bilayer part of the membrane (Iglič et al. 1995). It is plausible to assume that this actually happens, in particular because vesicles obtained in the process of the low pH induced vesiculation were found to be skeleton depleted (Leonards and Ohki 1983). It has been shown (Iglič et al. 1995) that partial skeleton detachment is a shape-dependent process, and that it is particularly well pronounced in shapes with narrow necks such as in the limiting shapes involving exovesicles (see Fig. 1) where the skeleton may remain underlying the bilayer of the large parent cell and is not present in small vesicles. Consequently, the partial detachment of the skeleton occurs concomitantly with the discontinuous cell shape change from the initial shape which is in the stomatocytic region of the volume/area difference phase diagram presented in Fig. 1, e.g. in the region of the limiting shapes including endovesicles, to a limiting shape including exovesicles. The occurrence of such a discontinuous shape transition explains why echinocytes as intermediate shapes

are not observed. It has to be noted that the described shape transition is opposed by an increase of the elastic energy of the bilayer part of the membrane. Namely, in the initial shape the bilayer leaflets have their equilibrium areas such that their difference corresponds to the ΔA value of the stomatocyte shape which means that they are not stressed. In order to attain the larger ΔA value of the exovesicular limiting shape, the outer leaflet must become correspondingly dilated, and the inner leaflet must become correspondingly compressed, both processes requiring energy. The discontinuous shape transition can thus occur only if the absolute value of the energy decrease due to the partial detachment of the skeleton is larger than the increase of the elastic energy of the bilayer (Iglič et al. 1996).

The presented view about the system behavior is supported by the data obtained concerning the low pH-induced vesiculation in the presence of echinocytogenic and stomatocytogenic compounds. The effect of echinocytogenic compounds (Na-salicylate, barbital) can easily be understood by assuming that the target of their action is the outer leaflet of the membrane bilayer (Sheetz and Singer 1974). The absence of influence on the sequence of the cell shape transformations or on vesiculation can be explained by the absence of the interaction between these compounds and the inner bilayer leaflet as well as the membrane skeleton. On the other hand, the stomatocytogenic compounds (chlorpromazine, quinine) affect the sequence of shape transformations and inhibit the release of vesicles. In terms of the trilayer membrane model and considering area elasticity moduli of the constituting layers, it can be deduced also for the stomatocytogenic compounds that their site of action is the inner layer of the bilayer (Svetina et al. 1988; Sheetz and Singer 1974). When vesiculation occurs at pH 5.4 in the presence of stomatocytogenic compounds, the value of the bilayer area difference ΔA is lower than in their absence. The inhibitory effect of the stomatocytogenic compounds can thus be understood on the basis of the fact that the increase of the bilayer elastic energy to reach the limiting exovesicular shape is larger than is the energy gain due to the skeleton detachment, and that therefore, the corresponding discontinuous shape transition can not occur.

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