

A possible physical mechanism of red blood cell vesiculation obtained by incubation at high pH

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Abstract

The membrane of human red blood cells is essentially composed of two parts, the lipid bilayer and the membrane skeleton that interacts with the lipid bilayer. The normal resting shape of the red blood cells at physiological pH 7.4 is the discocyte. However, at alkaline pH ≈ 11 the shape of red blood cells is composed of a spherical parent cell and large spherical daughter vesicles. The daughter vesicles may be free or connected to the parent cell by a narrow neck. In this paper we show that the shapes of red blood cells at pH ≈ 11 correspond to some of the calculated shapes of a closed lipid bilayer having an extreme area difference between the outer and the inner monolayer. Therefore, it is suggested that the observed shapes of the red blood cells at pH ≈ 11 are a consequence of the abolishment of the skeleton–bilayer interactions at this pH. © 1998 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The human red blood cell (RBC) has no internal structure. Therefore, its shape is determined solely by the physical properties of the membrane and the cell volume (Deuling and Helfrich, 1976; Waugh and Hochmuth, 1995; Zarda et al., 1977). The RBC membrane is essentially composed of two parts, the lipid bilayer and the membrane skeleton which is a continuous network of proteins. At normal conditions, the skeleton is attached to the inner side of the bilayer (Pasternack et al., 1985; Sikorski and Bialkowska, 1996).

The normal shape of the human RBC is a biconcave disc (Fig. 1a). Under certain conditions the discocytic RBC shape may be transformed into various other shapes such as the spiculated (echinocytic) shape or the cup (stomatocytic) shape (Bessis, 1973). These shape changes may occur due to a change in the difference between

the outer and the inner monolayer areas (ΔA) of the bilayer (Evans, 1974; Helfrich, 1974). It has been shown that a decrease in the area difference ΔA causes the discocytic RBC shape to change towards the stomatocytic shapes, while an increase in ΔA induces a transformation from the discocytic shape to the echinocytic shapes (Isomaa et al., 1987; Sheetz and Singer, 1974). These considerations can be applied to the shape changes occurring when manipulating the intracellular pH of RBCs. It has been shown that lowering or elevating intracellular pH gradually takes RBCs through stomatocytic or echinocytic shape transformations, respectively (Bessis, 1973; Ponder, 1971; Weed and Chailley, 1973). Therefore, it can be expected that lowering the intracellular pH decreases ΔA of the membrane bilayer while elevating the intracellular pH increases ΔA (Gedde et al., 1997).

When we equilibrated RBCs from neutral pH to pH ≈ 11 , the RBC shape were predominantly composed of a spherical parent cell and one or more spherical daughter vesicles of approximately similar size. The case with a parent cell and one large daughter vesicle is shown in Fig. 1b. This study was undertaken to analyze whether

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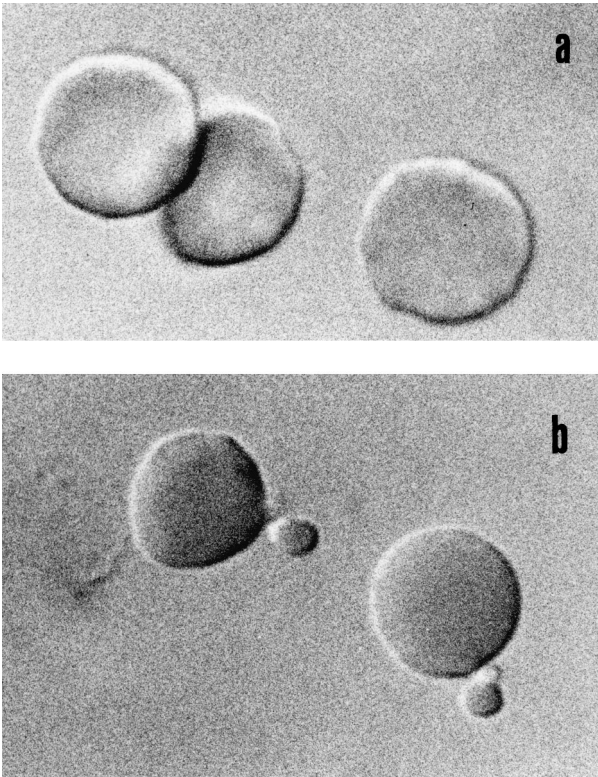


Fig. 1. Micrographs showing red blood cells (a) incubated at $\text{pH} \approx 7.4$ and (b) equilibrated at $\text{pH} \approx 11$ (37°C , 60 min). For photography (Nomarski interference contrast microscopy, Olympus BH-2) RBCs were fixed with 2% glutaraldehyde and applied between object glass and cover glass as previously reported (Isomaa et al., 1987; Hägerstrand and Isomaa, 1989).

the prelytic formation of these large RBC vesicles may be explained by some simple physical mechanism.

2. Theory

Recent studies show that the interactions between the skeleton and the lipid bilayer in RBCs may be seriously disturbed at a pH above ca 9.5 (Low et al., 1991; Paulitschke et al., 1995) so that the physical properties of the RBC membrane at this high pH are determined mainly by the lipid bilayer. Therefore, it is not surprising that RBCs at $\text{pH} \approx 11$ (Fig. 1b) may attain shapes similar to that of the giant lipid vesicles (Sackmann, 1991; Svetina and Žekš, 1996), whose membrane consist only of the lipid bilayer. Because an elevation of intracellular pH is thought to increase the value of ΔA as indicated above we propose that the RBC shapes at extreme $\text{pH} \approx 11$ correspond to the shapes of a closed bilayer with an extreme area difference of the two monolayers ΔA at a given membrane area and a given volume of the cell.

The problem of finding the shapes of a closed bilayer with an extreme ΔA at a given membrane area (A_0) and

a given cell volume (V_0) can be mathematically formulated by stating a variational problem. Within the Euler–Lagrange theory (Deuling and Helfrich, 1976; Svetina and Žekš, 1996) the variation of the functional

$$Q = \Delta A - \lambda_A (A - A_0) - \lambda_V (V - V_0), \quad (1)$$

$\delta Q = 0$ is sought. The Lagrange multipliers λ_A and λ_V are determined from the constraints for the membrane area and the enclosed cell volume

$$\int dA = A_0, \quad \int dV = V_0, \quad (2)$$

where dA and dV are infinitesimal cell area and volume elements, respectively. Since the distance between the neutral surfaces of the bilayer leaflets (h) is much smaller than the dimensions of the RBC, the area difference ΔA can be approximately written as

$$\Delta A = h \int (c_1 + c_2) dA, \quad (3)$$

where c_1 and c_2 are the two principal curvatures defined so that they are positive for a sphere. It was shown that $\Delta A/A$ changes up to 0.02 for different RBC shape transformations (Beck, 1978; Iglič, 1997; Sheetz and Singer, 1974) proving that already very small changes in ΔA can cause significant changes of the RBC shape (Svetina and Žekš, 1996). The change in the thickness of both leaflets of the bilayer during RBC shape transformation is very small and the value of h can be considered as a constant (Berndl et al., 1990; Svetina and Žekš, 1996).

Due to simplicity the analysis is restricted to axisymmetric cell shapes. The Cartesian coordinate system is chosen in which the symmetry axis points in the x direction while the shape is described by the function $y(x)$. In this system the principal curvatures are

$$c_1 = + (1 + y'^2)^{-1/2} y^{-1}, \quad (4)$$

$$c_2 = - y'' (1 + y'^2)^{-3/2}, \quad (5)$$

where $y' = dy/dx$ and $y'' = d^2y/dx^2$ while the area and volume elements are

$$dA = 2\pi (1 + y'^2)^{1/2} y dx, \quad dV = \pi y^2 dx. \quad (6)$$

The variation $\delta Q = 0$ is performed by solving the Euler–Poisson equation

$$\frac{\partial q}{\partial y} - \frac{d}{dx} \left(\frac{\partial q}{\partial y'} \right) + \frac{d^2}{dx^2} \left(\frac{\partial q}{\partial y''} \right) = 0, \quad (7)$$

where q is defined by $Q = \int q(x, y, y', y'') dx$. Taking into account the Eqs. (3)–(6) the above Euler–Poisson Eq. (7)

can be solved by the ansatz for the sphere of an origin $(x_0, 0)$ and a radius R , $y = (R^2 - (x - x_0)^2)^{1/2}$, where

$$R = 2/(\lambda_A \pm (\lambda_A^2 - 2\lambda_V)^{1/2}), \tag{8}$$

$\lambda_A \rightarrow \lambda_A/h$ and $\lambda_V \rightarrow \lambda_V/h$. Since there are, in general, two radii subject to Eq. (8) it can be concluded that the shapes of a closed bilayer corresponding to the extreme area difference of the two monolayers (ΔA_ℓ) at a given cell volume and a given membrane area can be composed of spheres having two different radii.

In the simplest case the shapes of the maximal monolayer area difference ΔA_ℓ are composed of a spherical parent cell with the radius R_p and N spherical daughter vesicles with the radius R_d , where $N \geq 1$. Such shapes adequately correspond to the observed RBC shapes at $\text{pH} \approx 11$, as presented in this work (Fig. 1b). At a given membrane area A_0 and cell volume V_0 the radius of the parent cell R_p and the radius of the daughter vesicles R_d of the described cell shapes of the extreme ΔA can be determined from the constraints (2).

In the following analysis, dimensionless quantities are introduced. For the unit length, the radius of a sphere R_s with the membrane area A_0 is chosen. In accordance with the definition of the radius R_s , the relative area $A_0/4\pi R_s^2$ is equal to one.

At a given relative cell volume $v_0 = 3V_0/4\pi R_s^3$ and a given number of daughter vesicles N , the relative radius of the parent cell $r_p = R_p/R_s$ and the relative radius of the daughter vesicles $r_d = R_d/R_s$ of the described cell shapes of the maximal area difference ΔA_ℓ are determined from the constraints for the relative membrane area and relative cell volume:

$$r_p^2 + Nr_d^2 = 1, \quad r_p^3 + Nr_d^3 = v_0. \tag{9}$$

The corresponding relative area difference $\Delta a_\ell = \Delta A_\ell/8\pi h R_s$ is

$$\Delta a_\ell = r_p + Nr_d. \tag{10}$$

The system of Eq. (9) can be solved analytically only for $N = 1$, describing the RBC shape with a single daughter vesicle (Igljč et al., 1995). In the case of $N = 1$ the system of Eq. (9) can be transformed into the equations

$$v_0 = \Delta a_\ell (1 - \beta), \quad 1 = \Delta a_\ell^2 - 2\beta, \tag{11}$$

where we have introduced a new variable $\beta = r_p r_d$ and where now $\Delta a_\ell = r_p + r_d$. The system of Eq. (11) can be re-expressed as a single equation for Δa_ℓ :

$$\Delta a_\ell^3 - 3\Delta a_\ell + 2v_0 = 0. \tag{12}$$

Eq. (12) has, in general, three real solutions. However, only one of them is positive

$$N = 1: \quad \Delta a_\ell = 2 \cos [(\pi - \arccos(v_0))/3] \tag{13}$$

The second part in Eq. (11) can be re-expressed as

$$r_p r_d = (\Delta a_\ell^2 - 1)/2. \tag{14}$$

By taking into account that $\Delta a_\ell = r_p + r_d$ it follows from the Eq. (14) that

$$N = 1: \quad r_p = \frac{1}{2} (\Delta a_\ell + (2 - \Delta a_\ell^2)^{1/2}), \tag{15}$$

$$N = 1: \quad r_d = \frac{1}{2} (\Delta a_\ell - (2 - \Delta a_\ell^2)^{1/2}), \tag{16}$$

where the relative difference between the areas of the two monolayers Δa_ℓ is given by the Eq. (13).

In general, for $N > 1$ the system of Eq. (9) is solved numerically. In this work, the tangential method was used to solve Eq. (9).

Fig. 2 shows the dependence of the relative radii r_p and r_d on the relative cell volume v_0 for different numbers of daughter vesicles N . The cell shapes of the maximal relative area difference Δa_ℓ composed of a parent cell and N daughter vesicles exist only for relative volumes v_0 greater than $(N + 1)^{-1/2}$, where at the latter value of v_0 we have $r_p = r_d = (N + 1)^{-1/2}$. It can be seen in Fig. 2 that the relative radius of the parent cell r_p increases while the relative radius of the daughter vesicle r_d decreases with increasing relative cell volume v_0 . It is also shown in Fig. 2 that r_p is an increasing function of N while r_d is a decreasing function of N .

Furthermore, it can be seen in Fig. 3 that the relative area difference Δa_ℓ decreases with increasing relative cell volume v_0 . On the other hand, Δa_ℓ increases with increasing number of daughter vesicles N , which means that the cell shapes corresponding to a larger Δa_ℓ could implicate a larger number of the daughter vesicles.

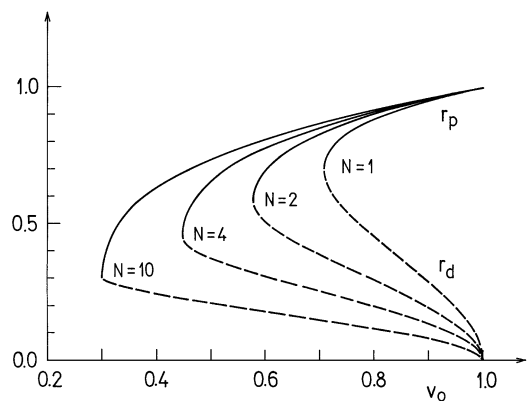


Fig. 2. The calculated relative radius of the parent cell r_p (full line) and the radius of the daughter vesicles r_d (dashed line) as a function of the relative cell volume v_0 for different numbers of daughter vesicles N .

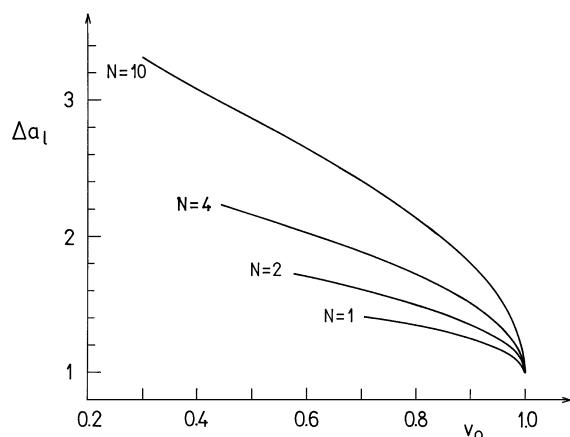


Fig. 3. The extreme relative area difference Δa_l corresponding to the cell shapes composed of a spherical parent cell and N spherical daughter vesicles, as a function of the relative cell volume v_0 .

3. Discussion and conclusions

It has been shown that the bilayer couple model (Sheetz and Singer, 1974) can describe the pH-induced RBC shape changes (Gedde et al., 1997). Lowering of the intracellular pH decreases the difference between the outer and the inner monolayer areas ΔA while elevation of pH increases ΔA (Gedde et al., 1997). However, the molecular mechanism of pH associated change of ΔA remains obscure (Gedde et al., 1997).

The pH mediated change of the state of the RBC skeleton is in direct contrast to the pH dependence of ΔA and the corresponding RBC shape changes (Gedde et al., 1997), since the membrane skeleton contracts at low pH and expands at high pH (Elgsaeter et al., 1976; Svoboda et al., 1992). The membrane skeleton appeared to play a secondary role in ΔA and RBC shape determination since the area expansivity modulus of the skeleton is a few orders of magnitude smaller than the corresponding constant of the bilayer (Mohandas and Evans, 1994). Also, the transmembrane particle aggregation and electrostatic interaction between negatively charged phospholipid headgroups in the inner monolayer do not appear relevant for the pH dependent ΔA changes (Gedde et al., 1997) while the active phospholipid translocation is probably too slow to account for the observed fast pH-induced RBC shape transformations (Gimsa and Ried, 1995). On the other hand, it was proposed recently that conformational changes of membrane protein band 3 induced by the change of pH may rapidly change the ΔA value (Gimsa and Ried, 1995). Since the band 3 occupies about 10% of the total RBC membrane area the latter mechanism seems to be relevant.

The cell shapes of an extreme ΔA composed of two differently sized spheres, exist only for the relative cell volumes v_0 larger than $2^{-1/2} \approx 0.71$, where at this value

of v_0 we have $r_p = r_d = 2^{-1/2}$ (Fig. 2). It can therefore be proposed that in the case of the RBC shape presented in Fig. 1b (RBCs at pH ≈ 11) the relative cell volume $v_0 > 0.71$. Since the normal value of the relative cell volume v_0 would be around 0.6 (Beck, 1978) it follows that at pH ≈ 11 the cell volume would be increased or/and the cell area is decreased relative to the normal values, respectively. However, the experimental results show that the RBC volume monotonously decreases with increasing pH (Nakao, 1990; Weed and Chailley, 1973). The relative cell volume around 0.5 has been detected at pH 10 (Weed and Chailley, 1973). Therefore, the second possibility, i.e. the decrease of the membrane area with increasing pH, seems to be more probable. A possible decrease of the membrane area with increasing pH could be the consequence of the release of microvesicles (diameter about 0.15 μm) during stages of incubation where the skeleton-bilayer interactions are only locally disturbed.

Microvesiculation has been detected under many different experimental conditions (see, for example, Hägerstrand and Isomaa, 1989) and is thought to be induced by the elevation of the area difference ΔA . Microvesicles shed from the RBCs were normally depleted in major membrane skeletal components spectrin and actin (Hägerstrand and Isomaa, 1994). Recently, a possible physical mechanism was proposed to explain the membrane skeleton depletion of small RBC daughter vesicles (Igljč et al., 1995; Igljč and Hägerstrand, 1996). A disturbance in the interactions between the skeleton and the lipid bilayer may be general characteristics of the RBC vesiculation. The present work supports this supposition. It is shown that RBC shapes at pH ≈ 11 correspond to the shapes of a closed lipid bilayer having the maximal difference between the outer and the inner monolayer areas. To explain these RBC shapes it might be supposed that the skeleton-bilayer interactions are heavily disturbed or completely abolished at this pH. This agrees well with experimental observations (Low et al., 1991).

It should be stressed at this point, that only if the skeleton-bilayer interactions are not seriously disturbed, a transformation of the discocytic shape to the echinocytic one can be induced by increasing the area difference ΔA (Igljč, 1997). As it has been observed, under such circumstances a discocyte can be, (Bessis, 1973; Bretcher and Bessis, 1972; Isomaa et al., 1987; Sheetz and Singer, 1974), continuously transformed by continuously increasing ΔA , first into an echinocyte, then into a spherocytic echinocyte and finally into a spherocyte. The final spherical shape arises because of the irreversible loss of membrane in the microvesiculation process (Liu et al., 1989), presumably due to the local disturbances in the skeleton-bilayer interactions. Accordingly, we detected a shift of the RBC shape towards the echinocytic shapes in samples equilibrated to pH ≈ 8 . In samples equilibrated to

pH 9.2, where the value of ΔA is expected to be even higher, sphero-echinocytes and spherocytes were the predominant RBC shapes. This indicates that the microvesiculation may have occurred.

Unlike RBCs, artificial giant lipid vesicles have never been reported to attain true echinocytic shapes, i.e. shapes with many spicula (Berndl et al., 1990; Svetina and Žekš, 1996). This indicates that the skeleton of the RBC membrane is responsible for the formation and stability of the echinocytic shape characterized by many spicula (Igljč, 1997). This theoretical prediction is also supported by the recent preliminary observations from our laboratory showing that the lamprey RBCs, which are deficient in the membrane skeleton (Ohnishi and Asai, 1985), do not form true spicula upon treatment with echinocytogenic amphiphiles.

Therefore, as expected, the RBC shapes at pH ≈ 11 are not sphero-echinocytic or spherocytic since at this high pH the skeleton-bilayer associations are abolished and the skeleton is probably at least partly disintegrated (Low et al., 1991). Instead, the RBC shapes at pH ≈ 11 are predominantly composed of a spherical parent cell and one or several large spherical daughter vesicles.

It is shown that the ansatz $y = [R^2 - (x - x_0)^2]^{1/2}$ representing a system of spheres of two different radii given by Eq. (8) can solve the Poisson–Euler Eq. (7). However, this is not the only solution of the equation (7). The constant $y = \lambda_A/\lambda_B$ representing the cylinder and the circle $y = y_0 + (R^2 - x^2)^{1/2}$ representing the plate are also analytical solutions of Eq. (7) and are therefore also the shapes of the extreme area difference. In determining which of these solutions would be relevant in a particular case, the process leading to the shape of the extreme area difference is of importance. In this work we consider the solution representing a system of the spheres, as they adequately explain the observed phenomenon. The tubular vesicles released by the RBC membrane after incubation with exogeneously added amphiphiles (Hägerstrand and Isomaa, 1992) were also observed indicating that the principle of the extreme area difference may be in some cases a general principle in explaining the shapes of bilayer structures.

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