J. Membrane Biol. 51, 217–228 (1979)

The Shape of Red Blood Cells as a Function of Membrane Potential and Temperature

Roland Glaser

Bereich Biophysik, Sektion Biologie, Humboldt-Universität zu Berlin, DDR 104 Berlin, Germany

Received 27 February 1979; revised 7 August 1979

Summary. It is well known that a pH shift of the outside medium from 5 to 9 produces a shape transformation of washed human red blood cells from stomatocytes to echinocytes in isotonic salt solutions. In addition, a stomatocytogenic effect is demonstrated here due to solutions of low ionic strength (below 70 mM). An analysis of the true cell state in these situations, proved by measurements of predicted volume changes, indicates a good correlation between transmembrane potential and cell shape. The fact that amphotericin B acts as echinocytogenic agent in low ionic strength medium at pH 7.4 but not at pH 5.1 underlines this explanation. Therefore, a transmembrane potential positive inside produces stomatocytes, slightly negative inside (below −10 mV), normocytes, and strongly negative, echinocytes. The temperature dependence of this process underlines the rigidity-pattern hypothesis of red blood cell shape (Glaser & Leitmannová, 1975, 1977).

Many publications have been concerned with investigations of the isovolumic change of erythrocyte shape induced by various conditions. Some authors used the classification of "echinocytogenic" (i.e., "crenators") and "stomatocytogenic" agents (i.e., "cup formers") (Deuticke, 1968; Brecker & Bessis, 1972; Chailley *et al.*, 1973; Weed & Chailley, 1973; Sheetz & Singer, 1974, 1976). The list of these factors is quite heterogeneous and so apparently are their mechanisms of action. The latter, of course, are closely connected with the fundamental understanding of the mechanism of red blood cell shape transformation in general, and therefore this question is of interest from different points of view.

Obviously some of these agents are directly incorporated into the lipid part of the membrane (e.g., fatty acids) and therefore influence its physical properties directly. Other factors, mainly ions and polyions, interact somehow with the electrostatic structure of the membrane and therefore more or less influence its behavior indirectly.

In the present paper we are interested particularly in the action of pH and ionic strength of the outside solution on erythrocyte shape,

0022-2631/79/0051-0217 $02.40

as pointed out in some papers (Furchgott, 1940; Rand, Burton & Canham, 1965; Brecker & Bessis, 1972; Fujii, Sato & Nakanishi, 1973; Weed & Chailley, 1973).

In contrast to the "bilayer couples" model (Sheetz & Singer, 1974), we have recently proposed a rigidity-pattern hypothesis explaining erythrocyte shape as a combination of two factors: (i) spectrin-contraction state and (ii) membrane fluidity (Glaser & Leitmannová, 1975, 1977). We supposed that, as a result of spectrin contraction, islands of intercalated protein-lipid complexes would be formed. Free lipid regions between the islands would be more flexible than the intercalated regions, giving rise to a heterogeneous response to bending stresses (but not to active bending!). This "island formation" can be kinetically related to a type of planar crystallization. The diameter of the islands formed, as the inverse of its number, therefore depends on their planar mobility. We could show that in case of relatively high fluidity a formation of large rigid areas occurs resulting in stomatocyte formation. If the mobility of the "crystallization points" was low, we obtained a set of numerous but small rigid membrane areas, leading to echinocyte formation. This rigidity-pattern model results from geometrical and physical calculations. It agree well with a body of experimental facts. Marikovsky, Khodad and Weinstein (1978), using various electron-microscopical techniques, indicated differences in the distribution of membrane particles and surface charges by different erythrocyte shapes.

To check this hypothesis, we were interested in some factors directly influencing membrane fluidity. Obviously temperature increase should increase the fluidity of at least the lipid phase of the membrane and therefore lead to an echinocyte-stomatocyte transformation. On the other hand, the mechanical properties of the cell membrane depend on the field strength in it. Träuble (1974, 1977), Forsyth *et al.*, (1977), and Jähnig (1976), e.g., showed that the surface charge, i.e., the surface potential (Ψ_o) of lipid membranes, influences its phase transition region. Anderson *et al.* (1977) indicated similar phenomena for locust muscle membranes, induced by the change of the transmembrane potential difference ($\Delta\Psi$).

Therefore, it would be of interest to examine the influences of ionic strength and composition, as well as pH, on shape transformation. As a well-known effect, pH diminution to below 7 produces stomatocytes, whereas pH increase to above 8 produces echinocytes (Weed & Chailley, 1973). A quick change of these conditions testifies to its reversibility (Rand *et al.*, 1965). The "glass effect" as an echinocytogenic factor seems

to be somewhere connected with such phenomena but has not been explained yet (Brecker & Bessis, 1972).

Materials and Methods

Human erythrocytes stored no longer than two days in ACD medium were used in all experiments. The blood was centrifuged at $500 \times g$ and washed at $2{,}000 \times g$ with isotonic phosphate-buffered Krebs-Ringer solution at pH 7.4. In experiments with low ionic strength solutions, the cells were washed once before in the same solution in which the experiment was carried out to prevent increase in concentration by trapped salt. The NaCl solutions applied were buffered with 5.8 mM phosphate and equilibrated with sucrose at iso-osmotic level (295 mosmol), measured with freezing point osmometer.

The shape of red blood cells was registered with the aid of an inverted microscope after sedimentation on glass. So in all cases the "glass effect" had to be equally taken into consideration.

Amphotericin B (Fungizone, Squibb) was solved in bidistilled water and added to the suspension to obtain a final concentration of 100 μg/ml. Erythrocytes were incubated for 1 hr in this solution (hematocrit = 30...50%) and then transferred to an amphotericin-free solution by pure dilution (hematocrit < 0.1%).

The shrinkage of cells due to amphotericin treatment was measured simply by the hematocrit method ($11{,}000 \times g$). The electrophoretic mobility of cells was measured as usual with a cytopherometer (Zeiss, Oberkochen).

The temperature dependence of red blood cells was investigated in a special transparent chamber which allowed one to observe the cells sedimented on the glass by an inverted microscope. A sufficiently rapid temperature change was attained using a special water-thermostat system and measured continuously with a thermocouple.

Calculation of the Parameters of Erythrocyte States

The normal steady state of erythrocytes ("N-state"), stabilized by ionic pumps, was temporarily lost after a change of the conditions of the outside solution (pH, ionic composition, etc.). In view of the well-known transport parameters of water, chloride, and cations in the membrane of human erythrocytes, which differs drastically, we considered three quasistationary states in which only water ("W-state") or water, chloride and pH ("C-state") or water and all principally permeable ions ("D-state") were equilibrated.

As a result of the very high exchange constant of water, the W-state was reached $t < 1$ sec and was stationary only during a very short period. In the present investigation it may be neglected. The C-state (identical with the state studied by Donlon and Rothstein, 1969) was reached after some minutes and was quasi-stationary for more than 2 hr. If the ionic pumps worked and ionic permeabilities were not changed, a new N-state would be reached later under physiological conditions. With pumps omitted (by inhibitors, temperature decrease, etc.) or cationic permeability highly increased (e.g., by amphotericin channels), the cell will be transferred more or less rapidly to the D-state.

The theoretical basis of these calculations in connection with the process of hemolysis is described in detail in a special publication (Brumen, Glaser & Svetina, 1979). For present considerations, we only need calculations of the C- and D-state.

In all situations, the osmotic pressure difference of inside-outside can be considered as zero. Sucrose is assumed not to penetrate the membrane. The osmotic pressure inside the cell is due to hemoglobin ($c_{Hb} = 7$ mM/liter H_2O) with an osmotic coefficient (g_{Hb}) (Gary-

Bobo & Solomon, 1968):

$$g_{Hb}=1.048+0.115\ c_{Hb}+0.0119\ c_{Hb}^2.$$

The osmotic coefficients of cations in physiological concentrations (g_K) are taken as 0.92. The buffer capacity of hemoglobin is 10.5 and the iso-electric point (pH_{isoel}) is a function of temperature (T in K) (Dalmark, 1975):

$$pH_{isoel}=\frac{1327}{T}+2.339.$$

In the C-state the inside-outside Cl^- distribution as well as the pH gradient are determined by the Nernst equation and additionally by the claim of electroneutrality. The content of actions and hemoglobin in the C-state, however, remains constant, but its concentration depends on actual cell volume. As normal means for human red blood cells, we found a hemoglobin concentration of $c_{Hb}=7$ mM/liter H_2O, a summed Na^+ and K^+ concentration of $c_{Ko}=146.2$ mM/liter H_2O and a relative content of free water of $q=0.72$ (Gary-Bobo & Solomon, 1968). For such cells we postulated a standard volume V_o. Changing the volume (V) by swelling or shrinking, the concentrations of nonexchangeable components have to be corrected by a dilution factor α.

$$\alpha=\frac{q\cdot V_o}{V-(1-q)\,V_o}.$$

For the C-state, therefore, the iso-osmolarity holds:

$$\pi_a-\alpha(g_K\ c_{Ko}+g_{Hb}\ c_{Hb})-g_C\ c_{Ci}=0$$

(π_a–osmolarity outside; g–osmotic coefficients; c_{Ci}–inside Cl^- concentration).

In the D-state, as a true Donnan-equilibrium state, all ions are equilibrated and their concentrations are therefore determined by the fixed charges of hemoglobin. In this case the dilution factor (α) can be applied only to hemoglobin.

Therefore we obtained a set of equations determining inner pH, volume, transmembrane potential ($\Delta\Psi=\Psi_{in}-\Psi_{out}$), and ionic distribution. These could be solved iteratively for any given condition. The actual volume changes are controlled by experiment. The values of $\Delta\Psi$ agree with those calculated and measured by Donlon and Rothstein (1969).

Results

Temperature Dependence of Red Cell Shape

Washed erythrocytes in isotonic NaCl solution at pH 7.4 and 37 °C were highly unstable with respect to shape. Sedimented on glass surfaces, they mainly became echinocytes. Lowering the temperature, we obtained only echinocytes even in free suspension. In isotonic NaCl sucrose solutions with diminishing ionic strength, the number of stomatocytes increased. As shown in Fig. 4, in isotonic 30 mM NaCl solution, at pH 5.1 (Fig. 4*A*) as well as at pH 7.4 (Fig. 4*B*), only stomatocytes occurred

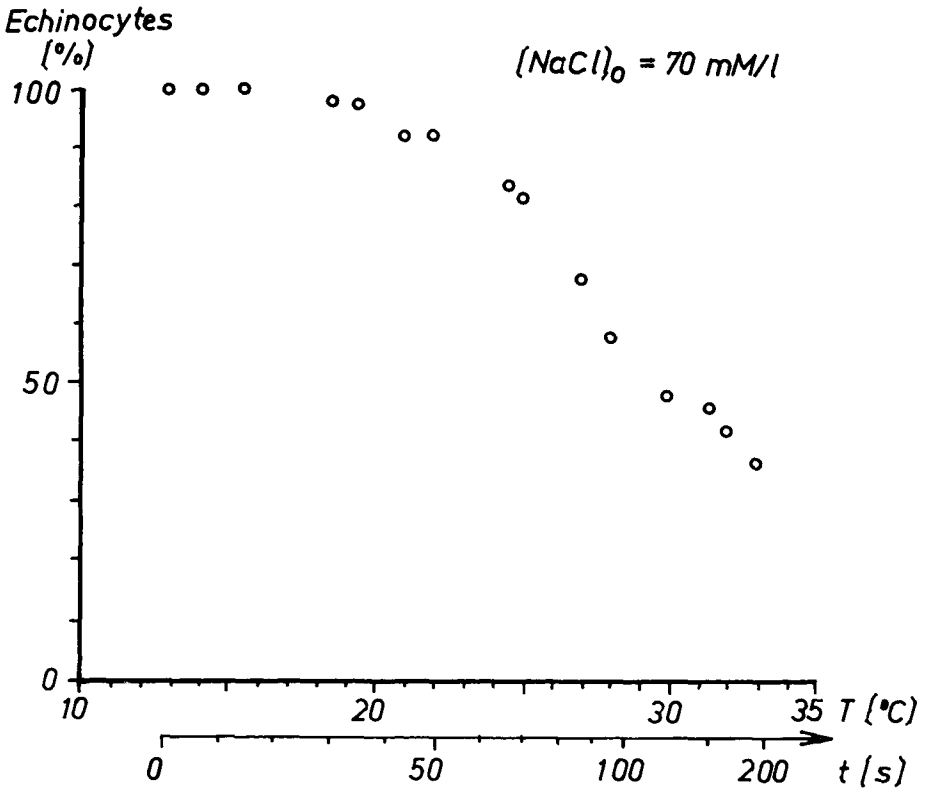


Fig. 1. Temperature dependence of relative amount of echinocytes in isotonic 70 mM NaCl-sucrose solutions (pH 7.4). The lower abscissa shows the time course of the experiment. The points result from a number of 570 registered cells of one representative experiment

(T=20 °C). At an intermediate concentration of 70 mM, a temperature-dependent shape formation developed (Fig. 1). Raising the temperature produced a transformation of echinocytes to normocytes and stomatocytes (a differentiation between the two types could not be made exactly).

In this case the temperature rise was quick enough to avoid irreversible ionic changes in the cell. Otherwise, as indicated by experiments with stopped temperature increase, the transformation time of cells was shorter than the time course of temperature change.

Influence of Electric Potential Difference ($\Delta\Psi$)

The observations mentioned above show that, in isotonic solutions near a NaCl concentration of 70 mM, a temperature-dependent shape

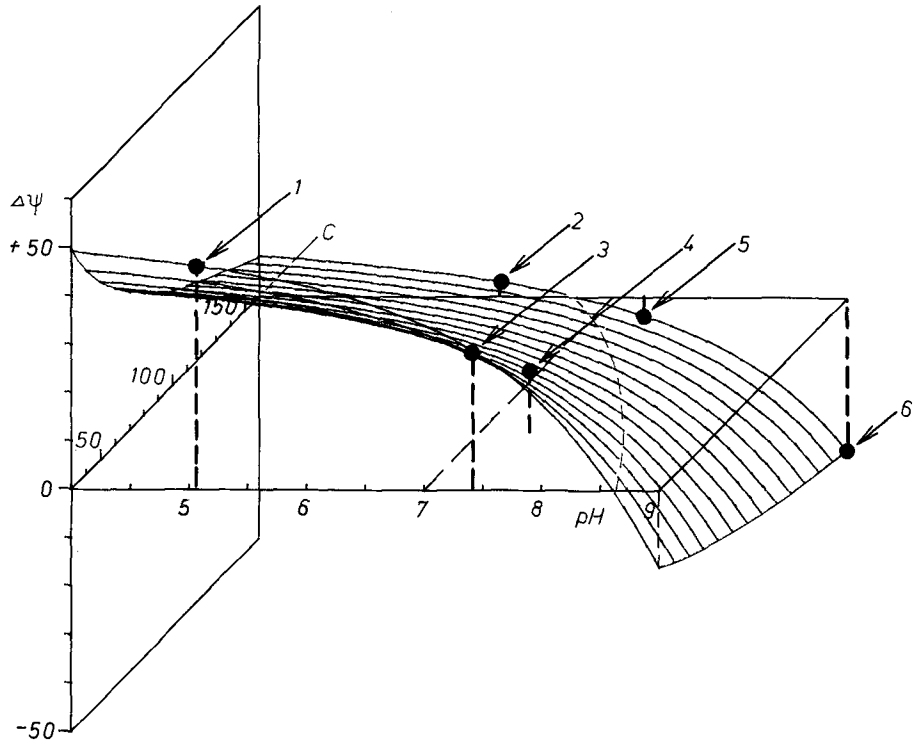


Fig. 2. Calculated potential difference ($\Delta\Psi$ in mV) of human red blood cells in C-state as a function of NaCl concentration (c in mM) and pH in isotonic NaCl-sucrose solution ($\pi = 300$ mosmol, $T = 293$ K). The points at different pH and c indicate the following erythrocyte shapes: *1*—stomatocytes (Fig. 4*A*); *2*—stomatocytes (Weed & Chailley, 1973); *3*—stomatocytes (Fig. 4*B*); *4*—temperature-dependent transition of echinocytes to stomatocytes and normocytes (Fig. 1); *5*—normocytes (Weed & Chailley, 1973); *6*—echinocytes (Weed & Chailley, 1973)

instability of washed erythrocytes occurred. In solutions with lower NaCl concentration, we obtained stomatocytes, in those of higher concentrations, echinocytes. Temperature stability increased in parallel with the increasing distance from this point. In iso-osmotic salt solutions, a pH dependence of red cell shapes developed as described by Weed and Chailley (1973). It seems reasonable to assume that in all of these experiments the cells were in the C-state according to the above nomenclature. Fig. 2 shows the calculated electric potential difference ($\Delta\Psi$) of human erythrocytes as a function of pH and NaCl concentration in isotonic solutions at $T = 293$ K. The localization of the situations in which this shape behavior was observed indicates a correlation between shape and potential difference for pH experiments as well as for experiments with different ionic strength.

Table 1. Electrophoretic mobility (EPM), transmembrane potential ($\Delta\Psi$) and volume changes of cells in C-state (control) and D-state (after amphotericin treatment) at different pH in the medium [a]

	pH = 5.1		pH = 7.4	
	1 C-state	2 D-state	3 C-state	4 D-state
EPM (meas.) ($m^2 V^{-1} sec^{-1} \times 10^{-8}$)	1.092 ± 0.058	1.060 ± 0.044	1.204 ± 0.011	1.181 ± 0.027
$\Delta\Psi$ (calc.) (mV)	+46.1	+46.3	+29.1	−19.5
Shrinkage by C-D-transition:				
(calc.)	59.4%		68.8%	
(meas.)	$61.8 \pm 1.3\%$		$67.2 \pm 3.1\%$	

[a] The values measured are averages of 6 independent experiments.

It is well known that not only transmembrane potential difference but additionally the surface potential is a function of ionic strength and pH. We therefore need experiments which allow us to differentiate between these two possibilities. For this purpose we transformed the cells, Na-K permeability by amphotericin B from the C- to D-state. The cells were incubated for 1 hr at $T = 293$ K in the solution containing amphotericin B and shape changes were observed (*see* Fig. 4). Both electrophoretic mobility and volume change were measured (*see* Table 1).

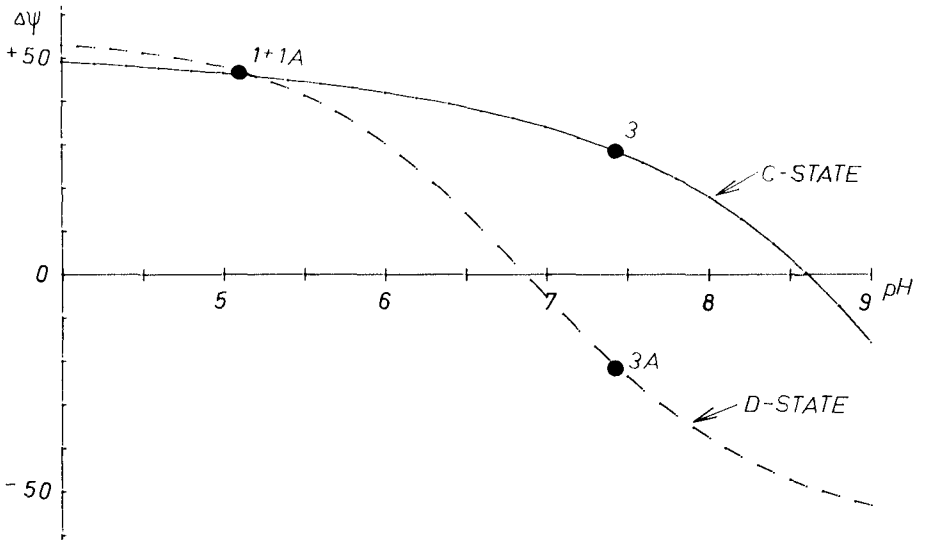
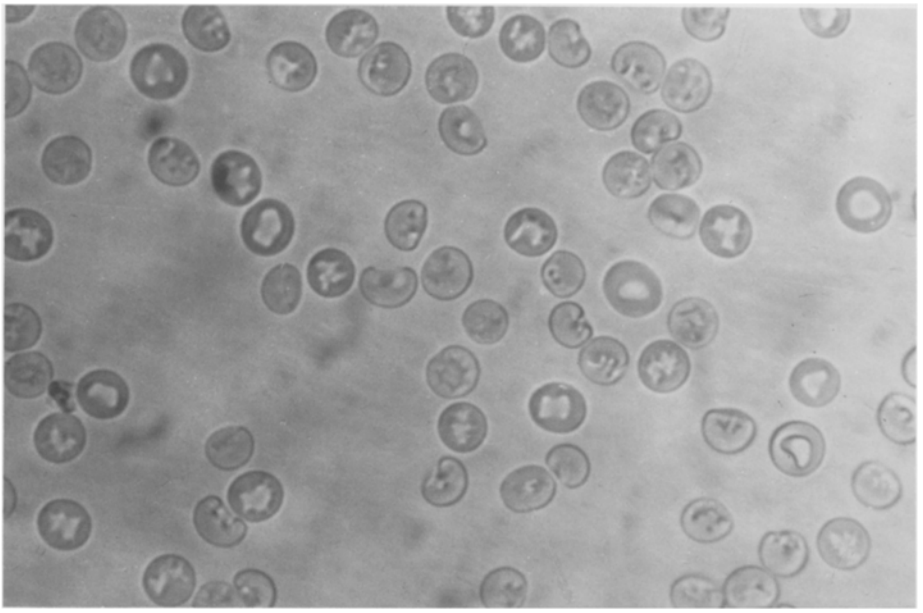
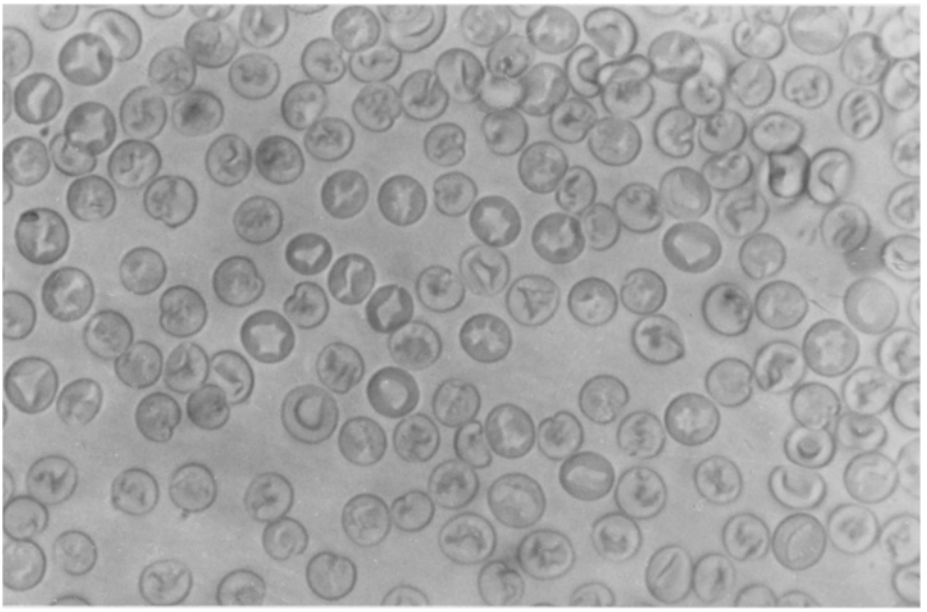


Fig. 3. Calculated potential difference ($\Delta\Psi$ in mV) of human red blood cells in C-state (— · —) and D-state (– – – –) as a function of pH. Points *1* and *3* correlate with those of Fig. 2. *A:* position after treatment with amphotericin

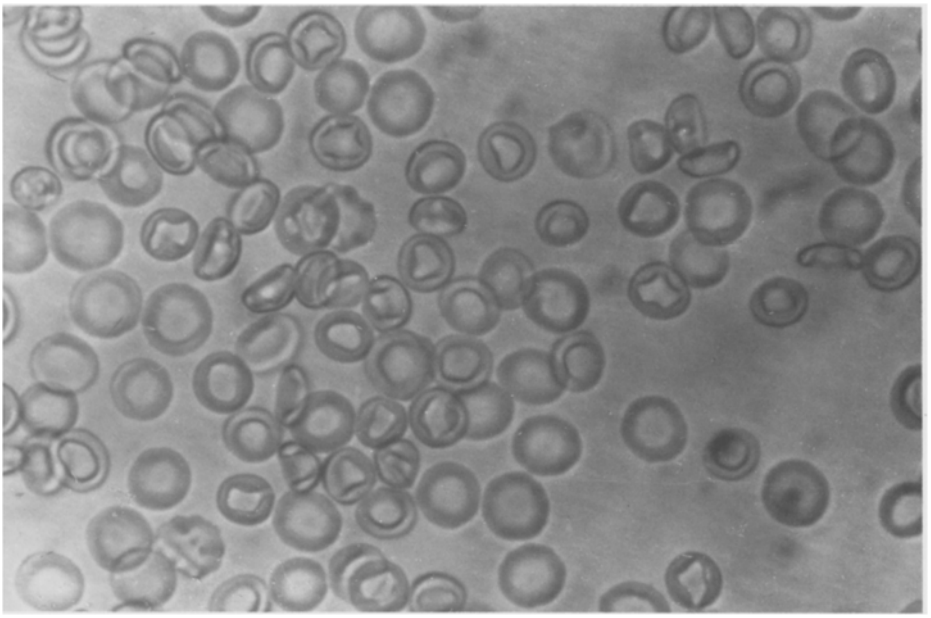


A

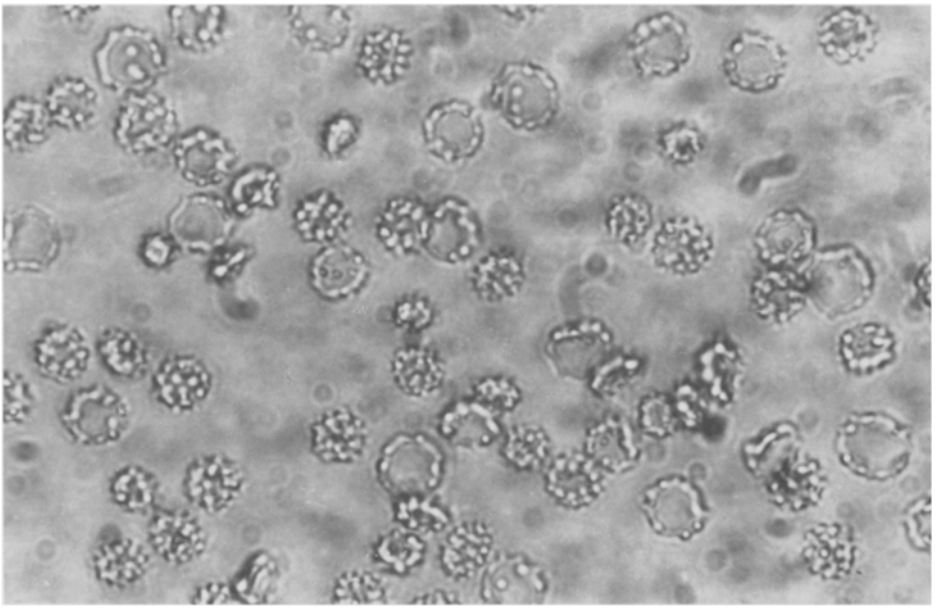


B

Fig. 4. Shapes of human red blood cells in isotonic 30 mM NaCl-sucrose solution sedimented on glass surface. (*A*): pH 5.1, control; (*B*): pH 7.4, control; (*C*): pH 5.1, with amphotericin; (*D*): pH 7.4, with amphotericin



C



D

Six independent experiments with different blood samples were made, showing identical behavior in both qualitative and quantitative terms.

As indicated by the values of the table, the measured shrinkage of cells after amphotericin treatment (D-state) is in good agreement with calculated values. This shows that the set of equations (Brumen *et al.*, 1979) satisfactorily reflects reality and the calculated $\Delta\Psi$ values are therefore at least near the true values, too (volume and electric potential result from the same set of equations). Besides, the shrinkage values testify to the action of amphotericin at pH 5.1 as well as at pH 7.4. The EPM values show differences between pH 5.1 and 7.4 in the well-known manner. No difference, however, can be observed which is induced by amphotericin action, i.e., during C-D-transformation of cells.

As shown in Fig. 3, the pH values have been chosen especially to obtain one C-D-transformation without $\Delta\Psi$ change (pH = 5.1) and another with a drastic difference of $\Delta\Psi$ (pH = 7.4). As shown in Fig. 4, only at pH 7.4 does a shape change occur. This underlines the hypothesis mentioned above, namely, the assumption that transmembrane potential but (under the conditions applied here) not surface potential is responsible for the shape transition of cells.

Discussion

In the introduction we assumed that, according to our explanation of the mechanism of shape transformation of human red blood cells (Glaser & Leitmannová, 1975, 1977), an increase of membrane fluidity should promote the echinocyte-stomatocyte transformation. The temperature dependence of cell shape in iso-osmotic NaCl sucrose solutions with 70 mM NaCl as indicated in Fig. 1 clearly underlined this hypothesis. In this case the increase of temperature obviously enhances the fluidity of some membrane compounds (possibly lipids) up to a critical point, which leads to the "melting" of echinocytes. The absence of such a temperature dependence in solutions of other ionic strength may be interpreted as a shift of this critical point out of the temperature interval applied here. Träuble & Eibl (1974), Jähnig (1976), Forsyth *et al.* (1977) found that the phase-transition temperature of lipid membranes depended on ionic conditions in the solution. It should be noted, however, that they discussed only the case of membranes without electrochemical gradients and therefore stressed only the influence via surface potential but not transmembrane potential. The effects demonstrated here, particu-

larly under the influence of amphotericin, include very strong changes of transmembrane potential including depolarization. It may be assumed that at least the electric field strength inside the membrane is responsible for the shape changes observed. In general, this field is built up by both transmembrane potential and surface potentials inside and outside the cell. In the present situation the contribution of transmembrane potential change to field strength change, however, seems to be more important than that of surface potential.

Based on these results we cannot yet follow up the causal chain of shape transformation for all situations. To this end, we need further direct measurements of the mechanical properties of the erythrocyte membrane under these conditions. Without any speculations, at present we have only to accept the fact that transmembrane potential, as one of certainly many other factors, influences the shape transformation of human red blood cells. This includes, however, the connection of the influence of ionic strength of the medium as well as its pH. The well-known fact that erythrocytes in hypertonic salt solutions shrink to form crenated echinocytes and flattened normocytes can, in view of the extrapolation of the curves of Fig. 2 to $c > 160$ mM, also be interpreted as an effect of lowering transmembrane potential. As shown in Fig. 4*C*, along with the corresponding values of Table 1 in the case of high transmembrane potential ($\Delta\Psi > 0$), it is quite possible to produced shrunk stomatocytes.

If we have a look at a list of "echinocytogenic" and "stomatocytogenic" agents (e.g., Weed & Chailley, 1973), we may find drugs affecting even ionic permeability or the activity of ionic pumps. Their action possible also functions via the change of transmembrane potential.

I thank Mrs. Jutta Donath for her most competent and conscientious technical assistance and Mrs. Margit Wünsche for electrophoretic measurements.

This paper is dedicated to my revered teacher in cell biology, Prof. Manfred Gersch (Friedrich-Schiller-Universität Jena), on the occasion of his seventieth birthday.

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