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Echinocyte Formation Induced by Potential Changes of Human Red Blood Cells

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Summary. In isotonic 30 mM NaCl-saccharose solution, human red blood cells with intact membrane and normal inside ionic content (C-state) indicate a transmembrane potential between +30 mV (at pH 7.4) and +46 mV (at pH 5.1). After treatment with amphotericin B or nystatin as ionophores, a Donnan equilibrium (D-state) will be reached with the same potential at pH 5.1 but a sharp drop down to −20 mV will occur at pH 7.4. Concerning the erythrocyte shape at these states, a stomatocyte-echinocyte transformation takes place, in correlation with the potential shift. Stomatocytes formed at $\Delta\psi > +25$ mV, echinocytes at $\Delta\psi < +25$ mV. At potentials lower than +5 mV, no further effect can be observed. This process is reversible. Neuraminidase treatment as well as outside EDTA do not influence this process significantly. Human serum albumin in concentrations of 2% stabilizes the stomatocytes.

Key words erythrocyte shape · membrane potential · amphotericin · neuraminidase · ionic states · electric field

Introduction

As we have previously shown (Glaser, 1978, 1979; Glaser, Brumen & Svetina, 1980) the transmembrane potential of human red blood cells strongly influences their stomatocyte-echinocyte transformation. In the case of the well-known pH-dependence of the erythrocyte shape (e.g., Weed & Chailley, 1973), as well as of the stomatocytogenic effect of the outside solution of low ionic strength, the shape transformation correlates with the transmembrane potential. That is positive potentials inside-out produce stomatocytes, and on the other hand negative potentials give rise to echinocytes.

Especially for those experiments we used the method to transfer the cells from their normal state (i.e., with chloride and pH being inside-out in equilibrium = C-state) in a full Donnan equilibrium (D-state) with the aid of ionophores. When this is performed in isotonic 30 mM NaCl-saccharose solution at pH 5.1, despite the shrinkage, no potential change occurs and correspondingly no shape changes are observed. At pH 7.4, under the influence of ionophores, the potential jumps from +30 mV inside-out (C-state) to −20 mV (D-state), accompanied by a stomatocyte-echinocyte transformation.

In the present publication we will demonstrate these circumstances in more quantitative terms and chiefly raise the question, whether the observed phenomena are really induced by the electric field itself or possibly by concentration changes of ions inside the cell. For this reason, mainly the reversibility of the echinocytogenesis has to be checked.

Materials and Method

Preparation of Cells

The method was already described in detail in a previous publication (Glaser, 1979). All experiments were performed with human erythrocytes, obtained from the blood bank and stored no longer than 48 hr in ACD-medium at 283 °K. After centrifugation at 500 × *g* the supernatant plasma and buffy coat were removed by aspiration. Then they were washed twice with isotonic PBS (pH 7.4) and once with isoosmotic-buffered 30 mM NaCl-saccharose solution (centrifugation always at 2000 × *g*).

The transformation of the cells into a full Donnan-equilibrium (D-state) was produced by incubation in solutions containing 100 μg/ml amphotericin B (Fungizone, Squibb) or 50 μg/ml nystatin (Sigma) for 1 hr.

The neuraminidase treatment was performed with α-neuraminidase from Vibrio cholerae (SERVA). Immediately following the second washing process, the cells were incubated for 1 hr at 310 °K in PBS (PCV approx. 0.7) with continuous stirring. The neuraminidase treatment was stopped by immediate cooling in ice water. Subsequently, the cells were washed twice in ice-cooled PBS.

Evaluation of Shapes

In all cases the shapes of echinocytes were investigated by an inverted light microscope after their sedimentation on glass cover slips. To compare the results of different experiments, we needed a method allowing a quantification of observed shapes. In view of the large number of samples to be observed, as well as concerning the limited time available for sample observations (no longer than 10 min), this method has to give a shape index in a sufficiently short time.

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Table 1. Example of an evaluation scheme, indicating the influence of 5 mM EDTA in outside solution on the shape transformation, due to the C→D-state transformation, induced by amphotericin in isotonic 30 mM NaCl-saccharose solution

pH		No.	Control			5 mM EDTA		
			Ech	Norm	Sto	Ech	Norm	Sto
5.1	C-state	1			XXXX			XXXX
		2		XX	XX		XX	XX
		3			XXXX			XXXX
		4		XX	XX		XX	XX
		5		X	XXX		XX	XX
				1.0	3.0		1.2	2.8
	D-state	1			XXXX			XXXX
		2		X	XXX		X	XXX
		3		X	XXX			XXXX
		4		XX	XX			XXXX
		5			XXXX		XX	XX
				0.8	3.2		0.6	3.4
7.4	C-state	1		X	XXX		X	XXX
		2		XXX	X		XXX	X
		3		XX	XX		XXX	X
		4		X	XXX			XXXX
		5		XXX	X		XXX	X
				2.0	2.0		2.0	2.0
	D-state	1	XXXX			XXXX		
		2	XXXX			XXXX		
		3	XXXX			XXX	X	
		4	XXXX			XXXX		
		5	XX	XX		XX	XX	
			3.6	0.4		3.4	0.6	

As we found in previous experiments (Glaser, 1979) for those reasons a count-out of a representative number of different shapes is possible only on the basis of microphotographs produced during the experiment. This, however, provides a pseudoexactness, because in many cases an exact recognition of the shapes, especially of the transition states and their identification on films, is problematic.

Therefore in the following experiments all samples were documented by microphotographs, but nevertheless as a blind test we evaluated them directly by visual observation during the experiment. We differentiated only between the following three shapes: stomatocytes, discocytes and echinocytes and used the predicates: 'exclusively', 'mostly', 'as well as', 'few' and 'no'. This very crude method nevertheless allows some quantification introducing the following system of valuation:

'exclusively':'no' = 4:0

'mostly':'few' = 3:1

'as well as' = 2:2.

Usually only two types of shapes occur in one sample simultaneously. If exceptionally a sample contains all three shapes, then the four dots are distributed over them according to their frequency.

Table 1 demonstrates such an evaluation. Five experiments (on different days with different blood samples) were performed to test the influence of 5 mM EDTA in the outside solution. If we look at the C- and D-states at two different pH values (pH 5.1 and 7.1) for control and EDTA-treated cells, eight different situations result. The crosses in the Table demonstrate the frequency of the shapes. The numbers under them give the average of the five experiments, and therefore a sort of mean frequency factor for the given shape.

Considering the discocyte as an intermediate shape between echinocyte on the one hand and stomatocyte on the other, we defined a further step of abstraction. Subtracting the frequency factor of echinocytes from that of the stomatocytes, we got a shape index, characterizing the situation in a given suspension. In this case +4 means 'exclusively stomatocytes', 0 means 'exclusively discocytes' or a symmetric distribution around them, and −4 means 'exclusively echinocytes'. As will be demonstrated in the following Figures, this index allows a sufficient demonstration of quite different experimental situations.

Calculations

For the interpretation of our experiments, we used the following classification of quasi-equilibrium or equilibrium ionic states of human erythrocytes, already published in detail (Brumen, Glaser & Svetina, 1979*a*, *b*; Glaser 1979; Glaser et al., 1980):

C-state = equilibration of water, chloride and pH,

D-state = Donnan-equilibrium of water and all ions.

Taking into account the conditions of electroneutrality and equilibration of the osmotic pressure as well as the pH dependence of the hemoglobin charge, the osmotic coefficients and the activity coefficients of all constituents, a set of equations indicating the volume, the transmembrane potential and the ionic con-

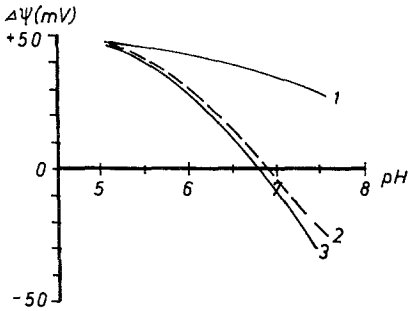

Fig. 1. Transmembrane potential ($\Delta\psi$) of erythrocytes, calculated for the C-state (1), D-state (2), and CD-state (3) as a function of outside pH in isotonic 30 mM NaCl-saccharose solution ($T = 293\,°K$). The CD-state is determined by the D-state at pH 5.1

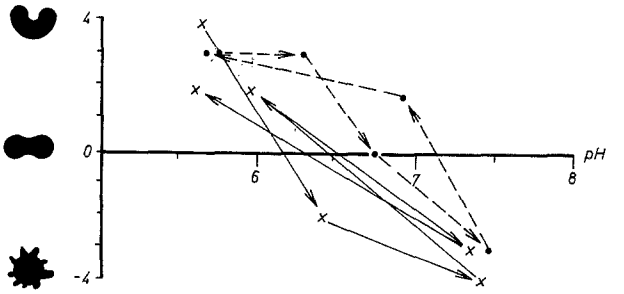

Fig. 2. Shape transformation in two erythocyte suspensions (×⟶×, ●---→●), following repeated pH-changes in the outside solution (isotonic 30 mM NaCl-saccharose solution) after its incubation with amphotericin B at pH 5.1

ditions for a defined state could be written down and solved (*see* Appendix). As a comparison between calculated and measured data indicates, these calculations reflect the real situation sufficiently correctly.

For the following experiments, a special ionic state is to be taken into consideration, which occurred when the membrane was opened for cations only for a time and under definite conditions (CD-state; *see* Glaser et al., 1980). It especially occurred in the following situation: We incubated the cells with 100 μg/ml amphotericin at pH 5.1 in 30 mM NaCl-saccharose solution. Then the amphotericin was washed out and the outside pH of the solution was changed. In this case, we obtained a D-state with a particular inside cation content under incubation conditions. This cation content remains constant after the amphotericin pores have vanished, even if the outside conditions change (the concentration of cations, however, may vary due to volume shift). The CD-state therefore is to be considered as a special type of a C-state (*see* Appendix). Figure 1 indicates the transmembrane potentials as a function of the pH for isoosmotic 30 mM NaCl-saccharose solution for all three situations. This indicates that for this case no great differences between D- and CD-states occur.

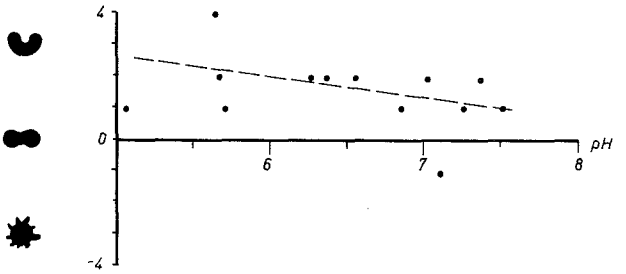

Fig. 3. Erythrocyte shapes in C-state at different pH-values of the outside solution (isotonic 30 mM NaCl-saccharose solution)

Results

Reversibility of Shape Changes

The calculated strong pH-dependence of the transmembrane potential in the D-state as well as in the CD-state enable us to change the potential in both directions, in this way checking the reversibility of the observed shape transformation. For this purpose, we resuspended the cells, which had been treated at pH 5.1 in 30 mM NaCl-saccharose solution with amphotericin in amphotericin-free solution with a low PCV of approximately 0.005 and titrated the suspension to give different pH values. The resulting shapes of two typical experiments are indicated in Fig. 2. In one case, a double time shape change could be induced successively in that way. The time interval from one observation to the other (i.e., between the points), amounts to only a few minutes and is given by the time necessary for the titration process and for shape evaluation. The similarity of the potential function for D- and CD-states (Fig. 1, curves 2 and 3) indicates that it does not seem to be so important whether the amphotericin is totally washed out from the membrane or not.

Shape Transformations as a Function of the Transmembrane Potential

For a better understanding of the potential dependence of shape transformation, we performed numerous experiments with untreated cells in the C-state and those transformed into the D-state by incubation with amphotericin. All experiments were carried out in isotonic 30 mM NaCl-saccharose solution.

As Fig. 3 demonstrates, the control cells in the C-state are chiefly stomatocytes and discocytes. The regression line of the points merely has a low slope. In contrast to this, the amphotericin-treated cells, which are in the CD- or the D-state, indicate a clear shape transformation from stomatocytes to echinocytes due to an increasing outside pH (Fig. 4). The regression line in this case crosses the abscissa at about pH 6.3.

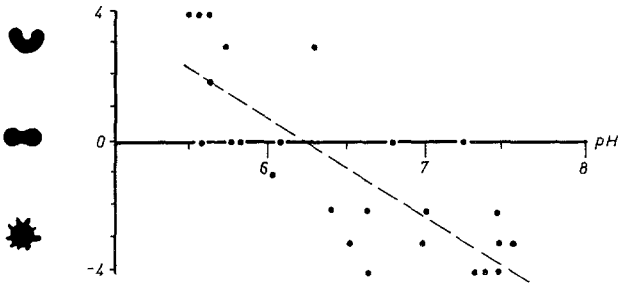

Fig. 4. Erythrocyte shapes in D-state (i.e. amphotericin treated) at different pH-values of the outside solution (isotonic 30 mM NaCl-saccharose solution)

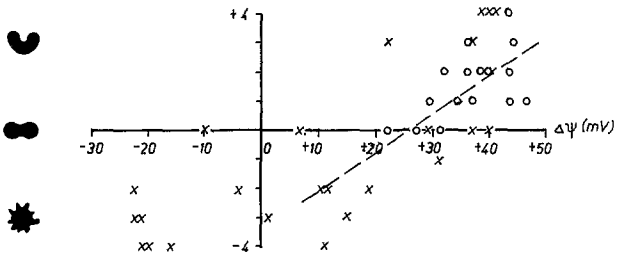

Fig. 5. Erythocyte shapes as a function of transmembrane potential: ●-untreated cells in C-state (values from Fig. 3). ×-amphotericin-treated cells in D-state (values from Fig. 4)

With the aid of our model mentioned above all shape indices of these two types of experiments can be plotted versus the transmembrane potential. The potentials for the C- and D-states as functions of the pH for these outside conditions are represented in Fig. 1. As Fig. 5 indicates, there is no difference between the controls (C-state) and the amphotericin-treated cells using this plot. Supposing that at potentials smaller than +5 mV the effect reaches a saturation; we calculated the following regression lines for the points with a transmembrane potential $\Delta\psi > +5$ mV:

for untreated cells:

$N = 0.102\,\Delta\psi - 2.12, \quad r = 0.61$

for amphotericin-treated cells:

$N = 0.145\,\Delta\psi - 3.63, \quad r = 0.72$

for all points together:

$N = 0.136\,\Delta\psi - 3.39, \quad r = 0.73$

(N-shape index, $\Delta\psi$-potential in mV, r-correlation coefficient).

Obviously, no differences occur between shapes of amphotericin-treated cells and controls as a function of the transmembrane potential. The correlation line crosses the abscissa at +25 mV.

Influence of Various Factors on the Process of Shape Transformation

To indicate the influence of various additional factors on the shape transformation caused by potential

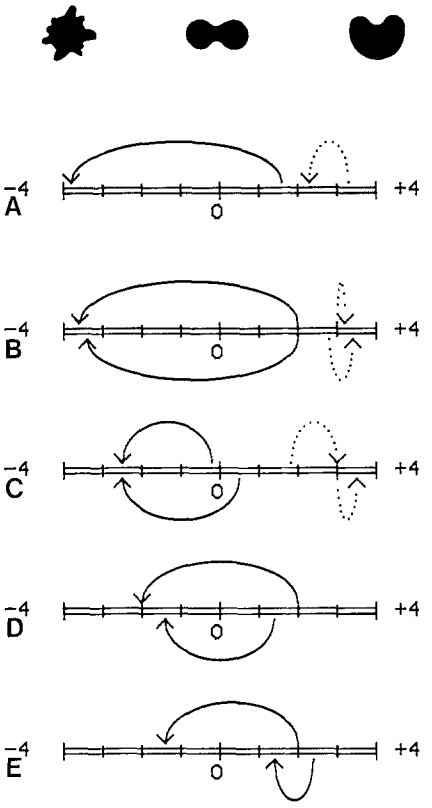

Fig. 6. Schematic representation of the change of the shape index by the transformation of erythrocytes from C-, into D-state at pH 7.4 (——›) and pH 5.1 (·····›). Apart from case *A*, all the transformations were performed by amphotericin B. The control experiments (i.e. without the agents, indicated) are expressed above the axis; the results of the experiments with special treatment are indicated below it. *A* - nystatin treatment (5); *B* - 5 mM EDTA (6); *C* - neuraminidase treatment (5); *D* - 0.2% HSA (5); *E* - 2% HSA (5). (In brackets the number of experiments, performed with different blood samples at different days.)

changes, we performed several series of experiments, using the C→D-state transformation of the cells by ionophores at outside pH-values of 5.1 and 7.4. The results of these experiments are summarized in Fig. 6. For every situation a control test was carried out. In the graphs in Fig. 6, the shift of the shape index at pH 7.4 (full lines) and 5.1 (dotted lines) during the C→D-state transformation caused by the influence of ionophores, is demonstrated. The arrows above the axes always refer to the controls; those below, the experiments with the special conditions.

The results of Fig. 5 always indicate that shape transformations are not due specifically to amphotericin. This fact is underlined additionally by experiments performed with nystatin instead of amphotericin. As the corresponding graph (Fig. 6*A*) indicates, the results are generally the same as given by amphotericin.

To test the role of surface charges for the observed process, the shape change during the C→D-state transformation is performed with neuramini-

dase-treated cells. There is no reason to believe that the transmembrane potential is changed by neuraminidase treatment, but following electrophoretic measurements, the surface charge in our preparation was reduced down to 20–30%. However, as demonstrated in Fig. 6*C*, this treatment has no significant effect on the process of potential-induced shape transformation.

The reversibility of the stomatocyte-echinocyte transformation (Fig. 2) already demonstrates that obviously the effect could not be induced by a possible calcium leak in the membrane, produced by ionophores. Nevertheless, we tested the role of calcium in this process, performing experiments with 5 mM EDTA as chelating agent in the outside solution. Figure 6*B* indicates that no difference could be observed between the control solution and those containing EDTA.

A complete stabilization of the stomatocytes occurs in solutions containing 2% human serum albumin (HSA) (Fig. 6*E*). A concentration of 0.2% HSA, however, is not enough to avoid the stomatocyte-echinocyte transformation (Fig. 6*D*).

Apart from special influences of different factors listed in the graphs of Fig. 6, it is striking that the controls of these experiments (arrows above the axis) differ among themselves. When one looks at the Table where the data for all five EDTA-experiments are listed in detail, which are summarized in Fig. 6*B*, it seems unlikely to believe that the differences between the average values for the different series are produced only by the variations of the individual experiments. It has to be pointed out that all these series of experiments are performed one after the other, and therefore in different seasons of the year. It seems that the differences are given by a systematic error. It looks like a seasonal variation, but another systematic methodical error cannot be excluded.

Discussion

In our first publication (Glaser, 1978) we indicated a correlation between the transmembrane potential of human erythrocytes and its shape transformation, analyzing the dependence of the shape from the pH and the ionic strength of the outside solution. To check this idea, we changed the transmembrane potential of the cells experimentally, producing a full Donnan equilibrium (D-state) with the aim of amphotericin as a relatively unspecific cationic ionophore (Glaser, 1979). The experiments presented here confirm this correlation, as demonstrated clearly in Fig. 5. In this case control cells with a physiological inside cation content are compared with cells in the D- or the CD-state, which contain an inside cation concentration equilibrated to 30 mM NaCl outside in isotonic NaCl-saccharose solution. These results suggest the following conclusions:

1. It cannot be excluded that the amphotericin itself, building up special membrane domains (Meyer, 1979) somehow influences this process but this influence does not seem to be responsible for the observed shape transformation.

2. The shape transformation does not depend upon the inside cation concentration.

The latter point is underlined by the reversibility of the effect illustrated in Fig. 2. There are no changes in the cell during the experiments, which would need an ionic pump for their reversal. A concentration change of Na, K, or even Ca cannot be repaired by pumps in a few minutes, particularly at room temperature and in low ionic strength solutions with glucose omitted. The influence of Ca ions on membrane viscosity and erythrocyte shape, obviously coupled by the ATPase system (Quist & Roufogalis, 1976; Quist, 1980) therefore seems to be insignificant in our experiments.

The question of whether the observed effects are only correlations between the electrical transmembrane potential and the erythrocyte shape or actually based on a true causal relation, cannot yet be answered. Until now, we have neither had an approved theory about the mechanism of the erythrocyte shape, nor a clear idea of the electric field in the membrane and its influence on the membrane behavior.

Evidently, the transmembrane potential is only one of many other factors influencing erythrocyte shape. A large number of drugs are known as stomatocytogenic or echinocytogenic agents (Deuticke, 1968; Weed & Chailley, 1973; Sheetz & Singer, 1974, 1976; Fujii, Sato, Tamura, Wakatsukir Kanaho, 1979; Fujii & Tamura, 1979). These drugs are quite different in their chemical structure and biochemical activity. Even for these interactions the actual molecular mechanisms are still unknown.

All discussions about possible molecular mechanisms underlying the process of erythrocyte shape transformations must be based on the following biophysical alternatives, explaining them as a result of:

positive or negative intrinsic membrane curvature, produced by bending active molecules or by the bilayer couples effect (="bilayer couples"-model) (Sheetz & Singer, 1976; Brailsford, Korpman & Bull, 1980*a*, *b*; Svetina, Ottova-Leitmannová & Glaser, 1981).

A dynamic pattern of membrane areas with different bending resistances influenced by membrane-spectrin interactions (="rigidity pattern" model) (Glaser & Leitmannová, 1975, 1977; Markin & Glaser, 1980).

In any way, looking for possible mechanisms of shape transformations, obviously, one has to take into consideration the following molecular properties of the red cell membrane:

The mean area of the individual phospholipid molecules in the inner or outer membrane leaflet, or the intercalation of additional molecules in the membrane.

The interaction of membrane components with the spectrin network.

Now the question arises: How may the transmembrane electric field influence these properties? First of all the actual field profile in the membrane and near it should be analyzed. For this purpose, the surface potentials at both surfaces have to be taken into consideration. We calculated the potential profile on both sides of the membrane, assuming the following charge distribution (for detail *see* Heinrich, Gaestel & Glaser, 1981*a*, *b*):

Glycoproteins, forming a 5.5 nm outside surface coat with negatively charged sialic acid residues, homogeneously distributed (charge density: -3.5×10^6 As/m^3).

Phosphatidylserine, producing negatively charged groups in the inner leaflet of the membrane (surface charge density: -0.09 As/m^2).

Spectrin, forming a 3.5 nm inside layer with negative charges (charge density: -1.23×10^7 As/m^3).

This simplified model indicates a strong inside surface potential, produced by charged phospholipid head groups, located in normal human erythrocytes nearly exclusively in the inner membrane leaflet. In all cases, it is greater than the outer surface potential as well as the transmembrane potential calculated for all situations described here. On the one hand, this explains why the neuraminidase treatment did not influence shape transformations (Fig. 6*C*); on the other, it leads to the conclusion that the calculated transmembrane potential is not identical with the actual electric field in the membrane.

Considering the recent knowledge of the molecular basis of shape transformations as well as the structure of the electric membrane field, we come to the conclusion that obviously the following effects could occur:

1. The electric field generates local ionic conditions, influencing the behavior of membrane molecules (lipids, spectrin, e.g.).

2. The electric field directly influences the membrane behavior, interacting with dipoles of its molecular constituents.

On the basis of the present experiments it seems hard to explain the results in the sense of the first possibility. In this case it is difficult to understand why the results obtained by quite different inner cation concentrations look so similar. The direct field effect on membrane components, on the other hand, will have to be investigated further with specific methods.

The regulative role of the membrane potential for various cellular functions is now being discussed in many papers. Quite different functions are correlated with transmembrane and surface potential. The interpretation of the underlying mechanisms will in most cases be much more complicated compared to the erythrocyte as a simplest "model cell".

The competent and responsible assistance by Mrs. Jutta Donath is gratefully acknowledged.

Appendix

Basic Conditions and Equations for the Calculation of Parameters of the Ionic States[1]

We defined the "normal" conditions of human erythrocytes as *in vivo* conditions. For this situation, a cell volume of 100% was defined, the relative water content was assumed to be 71%, the sodium-potassium concentration, $c_{Ko}=146$ mmol per liter cell water, and the hemoglobin concentration: $c_{Hbo}=7$ mmol per liter cell water.

C-State. In the C-state (as a quasiequilibrium state) the distribution of water, chloride and pH inside-out is given by the thermodynamic equilibrium. The content of the sodium + potassium ions per cell corresponds to the "normal" conditions (their concentration, of course, changed with the corresponding cell volume).

Basic Equations:

$$\alpha\left[c_{Kio}-c_{Hbo}\beta\left(\mathrm{pH}_a+\frac{\Delta\psi F}{RT\ln 10}-\mathrm{pH}_{isoel}\right)\right]-c_{Ci}=0 \tag{CI}$$

$$\Delta\psi=\frac{RT}{F}\ln\frac{a_{Ci}}{a_{Ca}} \tag{CII}$$

$$\pi_a-\left(g_K\,\alpha c_{Kio}+g_C f^{-1} a_{Ca}\exp\frac{\Delta\psi F}{RT}+g_{Hb}\,\alpha c_{Hbo}\right)=0. \tag{CIII}$$

(CI = electroneutrality conditions; CII = equilibrium for chloride; CIII = osmotic equilibrium.)

D-State. The D-state is a full Donnan equilibrium for all ions.

Basic Equations:

$$\exp\left(\frac{2\Delta\psi F}{RT}\right)-\frac{\rho_{Hb}}{a_{Ca}}\exp\left(\frac{\Delta\psi F}{RT}\right)-1=0 \tag{DI}$$

$$\rho_{Hb}=-\alpha c_{Hbo}\beta\left(\mathrm{pH}_a+\frac{\Delta\psi F}{RT\ln 10}-\mathrm{pH}_{isoel}\right) \tag{DII}$$

$$\pi_a-\left[g_K f^{-1} a_{Ka}\exp\left(-\frac{\Delta\psi F}{RT}\right)+g_C f^{-1} a_{Ca}\exp\left(\frac{\Delta\psi F}{RT}\right)+g_{Hb}\,\alpha c_{Hbo}\right]=0. \tag{DIII}$$

[1] For detailed information *see* Glaser et al., 1980.

(DI = electroneutrality conditions; DII = hemoglobin charge density (ρ_{Hb}) as a function of pH; DIII = osmotic equilibrium.)

CD-State. The CD-state is a C-state with a definite change of the inner cation content. Therefore the equation set CI...CIII has to be solved inserting a definite "non-normal" value of c_{Ko}. This value can be calculated by Eqs. (DI)-(DIII) for the conditions where the cells were equilibrated to reach the D-state:

$$c_{Ko} = \frac{a_{Ki}}{\alpha f} = \frac{a_{Ka}}{\alpha f} \exp\left(-\frac{\Delta\psi F}{RT}\right). \qquad \text{(CD I)}$$

Additional Equations:

$\alpha = 71/(V - 29)$
$g_{Hb} = 1.048 + 0.115\, c_{Hb} + 0.0119\, c_{Hb}^2$
$pH_{isoel} = 2.339 + 1327/T.$

Symbols:

a	activity
c	molar concentration
f	activity coefficient
F	Faraday
g	osmotic coefficient
R	gas constant
T	Kelvin temperature
V	relative volume (in %)
β	buffer capacity of hemoglobin (10.5 eq/mol)
$\Delta\psi$	transmembrane potential
ρ	charge density (eq/liter)
π	osmotic pressure

Indices:

i = inside, a = outside, o = corresponding to the "normal" conditions, K = cations (Na + K), C = chloride, Hb = hemoglobin.

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