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ELECTROROTATION OF RED-CELLS AFTER ELECTROPORATION

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Electrorotation measurements of human red cells after dielectric breakdown showed a decrease in the first characteristic frequency simultaneously with a decrease in rotation. This behaviour was theoretically analyzed and internal and membrane conductivity were evaluated. It was shown that it is the internal conductivity which even after membrane dielectric breakdown determines mostly the electrorotational behaviour. From the membrane conductivity value followed that the AC-conductance might be realized through band 3 protein. A binding constant at the inner site of 2.1 mM and a membrane ion mobility value of $5*10^{-11}$ m^2/Vs were estimated.

The new method of electrorotation has been successfully applied to a number of systems /1,5,6,8,12/. By means of recording the cell spin the polarizability of a single cell is measured as a function of the frequency of the externally applied rotating electric field. In most cases there are two extrema of the spin of the cell as a function of frequency. The first one occurs in the kHz-range and is characterized by a cell spin against the rotation of the external field. The second falls into the MHz-range. The spin there is with the field direction. The reason of the occurrence of the peaks is that there is a dispersion of the polarizability of the cell. In particular, in the range of the first peak with increasing frequency of the external field the capacitive conductivity of the cell membrane increases. Therefore the membrane will no longer serve as a barrier to current flow, and, consequently, that part of polarization of the particle caused by the existence of the membrane as a nonconductive shell will disappear. Consequently, measurements of rotation in the range of the first peak should be especially sensitive to the impedance properties of the membrane as compared to the internal conductivity.

Now, we tried to measure the electrorotation behaviour of human red cells after dielectric breakdown. The original idea was to investigate whether it would be possible to measure the increase

of membrane conductivity caused by electroporation in order to develop another method /14/ to characterize the breakdown properties of cell membranes. However, a detailed investigation showed that this was not possible, because of the astonishingly high original membrane conductivity. Instead, we were able to measure the decrease of the internal conductivity due to the ion efflux through the breakdown induced pores.

We attributed the large original membrane conductivity to the anion exchange system of the red cell by estimating from the permeability of this system its conductivity. Now, the anion concentration decrease inside the cell after breakdown allowed determining of the kinetic properties of the exchange system, since we could observe for the first time a correlation between membrane and internal conductivity. This should have conceptional significance, while now a reduction of the number of independent parameters incorporated in the theory of electrorotation becomes possible.

Materials and methods

Erythrocytes. Human citrate blood from healthy donors was supplied by the blood bank. Plasma and buffy coat were removed after centrifugation at 2,000 g for 5 min, followed by washing in 300 mOsm sucrose, 30 mM NaCl, 5.8 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 6.8 for 10 min at 2,000 g. The cells were resuspended in the same solution at a hematocrit of 10% and stored at 4°C.

Electroporation. The erythrocyte suspension was subjected to a single exponential field pulse by discharging a capacitor between a pair of steel electrodes. The discharge chamber had two compartments, one containing the cell suspension and the other containing an electrolyte solution to adjust the resistance, and therefore, the pulse time constant desired. Pulses of $3*10^5$ V/m and a duration of 7 μs were applied. Immediately after pulsation the cells were diluted in 4° C 300 mOsm sucrose solution containing various concentrations of Na_2HPO_4/NaH_2PO_4 buffer (0, 0.5, 1.0 mM) at a hematocrit of 0.05 %.

Electrorotation. Measurements of electrorotation were performed by means of a four-electrode chamber applying a rotating field of approx. 6,000 V/m, which was generated by square-topped pulses of a key ratio of 1:1. The chamber containing 0.1 ml of the

suspension was placed under an inverted microscope. Measurements were carried out before the cells sedimented onto the bottom of the chamber. By means of the compensation method /2,3,9/ for each cell the first characteristic frequency f_{c1} and the spin at this frequency were measured. The temperature was controlled by cooling the microscope stage to 4°C.

Eruthrocyte shape. 100-120 μM chlorpromazine (Sigma) was added to the washed red cells kept in 300 mOsm sucrose, 1 mM phosphate buffer. This guaranteed the appearance of all shapes ranging from the advanced stages of stomatocytes to advanced echinocytes within the same suspension after a couple of minutes. Rotation measurements of stomatocytes and echinocytes followed. At fixed frequencies (50, 100, 150, 200, 300 kHz) the spin of both types was compared in order to elucidate the possible influence of cell shape on rotation.

Results

Rotation after electroporation. At three different external conductivities (0.96, 4, 7, mS/m) the first characteristic frequency of control cells was measured. Open and closed circles in Fig. 1 represent the mean values of the rotation and of the characteristic frequencies of controls at 7 and 0.96 mS/m, respectively. Decreasing the external conductivity shifted the first peak towards lower frequencies and decreased the rotation.

Immediately after pulsation the cells were transferred to the chamber. As long as possible rotation and the first characteristic frequency were followed. It was necessary carefully to select duration and strength of the pulse. At too large pulses the cells immediately stopped to rotate. At too small pulses no effect could be observed. The finally selected pulse conditions compare to relatively weak pulses in other studies /13,14/. By no means the rotational behaviour of cells was homogeneous. There was always a certain percentage of cells without rotation, which can be easily explained by different sensitivities of the cells to the pulse caused by different original orientation. Cells which did not rotate were not included in the data in Fig. 1.

The crosses in Fig.1 represent the typical behaviour of a single cell in 0.96 mS/m , while the squares represent mean values in 7 mS/m external solution conductivity. From these data it is

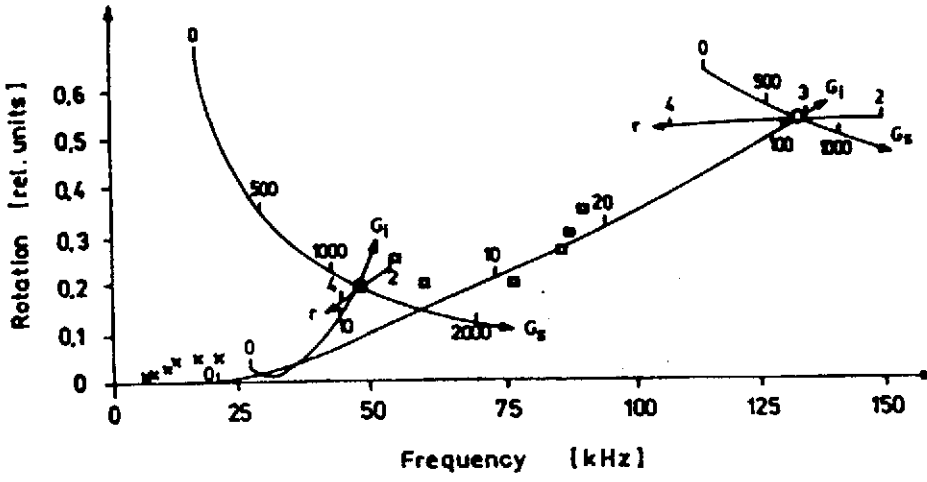

Fig. 1. Mean value of rotation at the first characteristic frequency of control cells (,) and behaviour as a function of time of representative pulsed cells (,) at 7 and 0.96 mS/m, respectively. Curves show variations in Gi = internal conductivity (mS/m), Gs = membrane conductivity (S/m²), r = radius (μm).

obvious that breakdown leads to a decrease in both, first characteristic frequency and rotation. The experiments in 4 mS/m external conductivity yielded the same results (not shown in Fig. 1).

Shape influence. Microscopic observation revealed that in any case pulsation was accompanied by shape transformation to echinocytes. To investigate a possible influence of shape on the electrorotational behaviour we studied separately the shape effect with control cells. In Fig. 2 the results of these experiments are summarized. At all frequencies measured the rotation of echinocytes was higher as compared with stomatocytes by approx. 20%. This difference was significant (α=5%). As Fig. 2 also demonstrates, a shift in the characteristic frequency could not be observed.

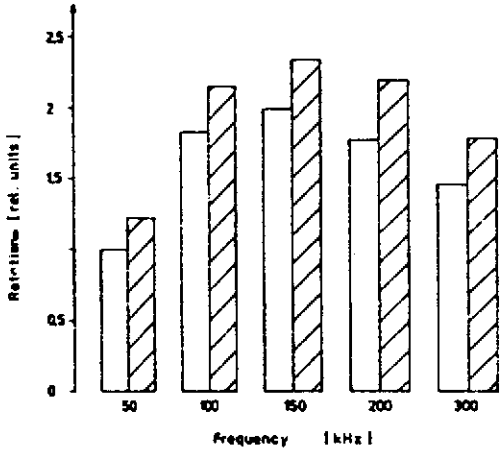

Fig. 2. Influence of cell shape on rotation as a function of frequency of erythrocytes treated with 120 μM chlorpromazine. Hatched columns - echinocytes, blank columns - stomatocytes.

Evaluation of dielectric parameters. For data evaluation it was necessary to use the complete theory of single shell spheres,

given by Fuhr & Kuzmin /7/. We estimated the influence of surface conductivity. At 0.96 S/m there was almost a 20% influence of surface conductivity on the calculated membrane conductivity. At higher external conductivities the surface conductivity could be safely neglected.

First we estimated the membrane conductivities of control cells. In contrast to earlier studies /11/ our measurements at low external conductivities provided us with the possibility to do this. The idea was that the lower the external conductivity the higher the influence of a given membrane conductivity on the position of the peak. At higher external conductivities this position as a function of frequency can be used to determine internal conductivity and membrane capacity. Assuming that these values will not significantly change when decreasing the conductivity outside we were then able to calculate the membrane conductivity of control cells. These data are represented in the second column of Tab 1.

	control		pulsed	cells
Ge	Gs	Gi	Gs	Gi
[mS/m]	[S/m²]	[mS/m]	[S/m²]	[mS/m]
0.96	1200	150	373 ± 127	1.1 ± 0.63
4	625	150	700 ± 232	3.5 ± 1.1
7	750	150	686 ± 344	17.3 ± 12.2

Tab. 1. Changes of specific membrane conductivity (Gs) and internal conductivity (Gi) after dielectric breakdown as a function of external conductivity (Ge). The G_i-values of control cells are averages from several fits.

We further assumed that breakdown will not change the membrane capacity. It is reasonable, however, to assume that membrane conductivity and internal conductivity will change after the electric pulse has been applied. Since there are two unknowns for the pulsed cells membrane conductivity and internal conductivity two equations to determine these parameters were needed. We selected the dependence of the first characteristic frequency on both conductivities as well as the dependence of the rotation at the first peak. Solving this system of two algebraic equations numerically we were able to obtain membrane and internal

conductivity for a number of cells after pulsation. Mean values togather with the standard deviations characterizing these parameters are also given in Tab. 1.
The most surprising result of Tab. 1 was that the membrane conductivity after pulsation did not increase. There is even a significant decrease of membrane conductivity at the lowest external conductivity studied. The internal conductivity decreased to values slightly above the external conductivity, which was a quite expected result.

Discussion

Before explaining the results with pulsed cells it is useful to compare the internal conductivity value of control cells with literature data obtained by means of other methods. PAULY and SCHWAN /17/ found from impedance measurements a value of 0.518 S/m. PILWAT and ZIMMERMANN /18/ obtained by breakdown measurements a value in the order of 0.526 S/m. Our value was three times less than these data. However, the rotational behaviour strongly predicted this value, since inserting higher internal conductivities yielded bad fits.
A possible explanation for this discrepancy might be that the cells in our experiments are suspended in very low ionic strength solution. This changes the internal electrochemical state of hemoglobin and the small ions as well as pH /10/. It is well known that the ion-protein interaction is critical for the conductivity /19/. Another reason was that the cells in low ionic strength solution show a loss of small ions as a function of time. This phenomenon, at present, is under more detailed investigation.
Now, in order to understand the breakdown behaviour of red cells it is quite useful to interpret the membrane conductivity in terms of membrane permeability. Although it might be very problematic there is a straightforward way to perform this task. Standard electrochemical considerations will yield the following relation between membrane conductivity and permeability.

$$G_s = c_i z^2 F^2 P/RT \qquad (1)$$

where G_s is the specific membrane conductivity measured in S/m², P is the permeability, c_i is the internal concentration of the

transported ion, that z, F, R, and T stand for charge number, Faraday's constant, gas constant and absolute temperature, respectively. In deriving equation (1) we made use of the assumptions that there is only one species of ion responsible for conductivity and, second, that outside concentration of this ion is very small. With the help of equation (1) the membrane conductivity of the control cells compares to permeability values of $1.5\text{-}3*10^{-4}$ m/s. This value almost exactly corresponds to the chloride exchange permeability of red cells /16/, and is orders of magnitude larger than any other ion permeability of the red membrane except proton and hydroxyl ion permeability. Since the latter two ions have very small concentrations we concluded that it is the chloride ion flow through the membrane which determines the membrane conductivity. Although we are aware of the fact that chloride ion exchange does not contribute to DC-membrane conductivity we were compelled to take this view by the above consideration. The solution of this discrepancy might be that, in contrast to other studies, we are dealing with alternate current. If the chloride exchange is sequential /16/ alternate current flow via the anion exchange system might be possible at large enough frequencies. A lower limit of the frequency might be obtained if we consider the transport rates through the band 3 protein. As a function of temperature it was found that 230 - 50000 ions per site may pass the membrane per second /4,15/. Consequently, frequencies in the kHz-range are above a possible membrane conductivity dispersion range.

From this viewpoint it becomes also quite clear that the pores induced by dielectric breakdown should cause only a negligible increase of membrane conductivity, since their permeability under the pulse conditions applied in this study is at least two to three orders of magnitude less than the anion exchange permeability /13/. Now it is also obvious that the first characteristic frequency was shifted towards lower values because it is solely the internal conductivity decrease after breakdown which influences the change of the cell rotational behaviour. This is demonstrated by the theoretical curves shown in Fig. 1. Neither a radius decrease due to osmotic shrinkage nor a membrane conductivity increase could shift the first peak towards lower frequencies. From the shape experiments it follows that the shape change is not responsible either for the frequency shift, nor will

it change the rotation very much.

In summary, there is the following curious situation: The electric pulses induce a membrane permeability increase sufficiently large for the efflux of the internal electrolyte within minutes but too small to increase the membrane conductivity significantly. On the other hand, the large enough salt permeability will change the electrorotation behaviour because it changes the internal conductivity.

Now let us look in closer detail at the membrane conductivity of the cells after breakdown shown in Tab. 1. How could the decrease of the membrane conductivity in 0.96 mS/m external conductivity solution be explained? To interpret these data we tried to simulate the electrorotation behaviour as a function of internal conductivity (Fig. 3). A first approximation would be a decrease of the internal conductivity together with a constant membrane conductivity. Such an assumption is in contradiction to the experimental results as shown in Fig. 4, curve 1. Therefore we concluded that there has to be a decrease of the membrane conductivity together with the internal conductivity after dielectric breakdown. This assumption is supported by the fact that the concentration of ions in the membrane has to be correlated to the concentration of both adjacent phases. We tried also a linear relationship of membrane conductivity and internal conductivity which compares to a simple ion two-phase distribution law. The result was that the membrane conductivity decrease was too fast, and, consequently, this would lead to an increase of rotation instead of the observed decrease, while the peak frequency shift was described correctly.

Finally, we tried Michaelis-Menten-like connection between membrane conductivity and internal conductivity which yielded rather good fits of the rotational behaviour (curve 2 in Fig. 4). The behaviour of the membrane conductivity was the following: It remained fairly constant over a large range of internal conductivity. Only if the internal conductivity reached small enough values the membrane conductivity began to drop quickly. In this context it is necessary to make two remarks. First, the membrane conductivity, although it drops, is still large as compared with the pore-induced conductivity. Second, this is also the reason why a resealing of the pores could not explain the observed behaviour.

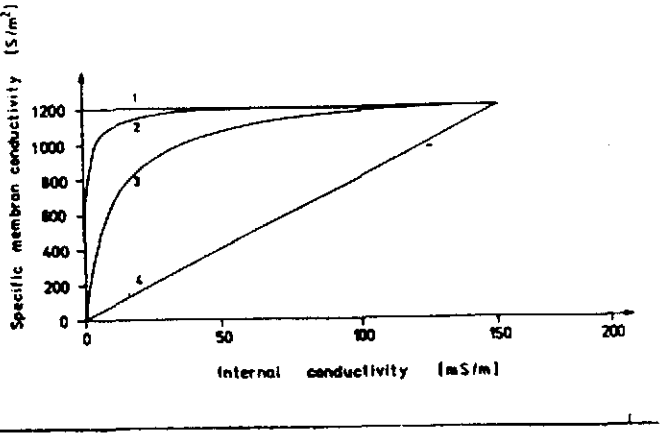

Fig. 3. Membrane conductivity as a function of internal conductivity. A theoretical model (Eq. 2) similar to Michaelis-Menten kinetics was used. Curves 1-4 refer to $k_m Fu_i$ values of 0, 1, 10, 1000 mS/m.

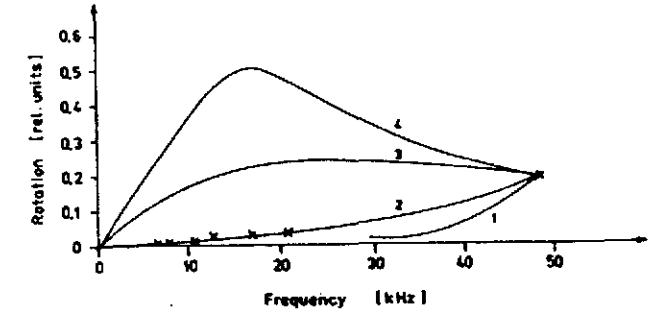

Fig. 4. Fit of rotational behaviour at 0.96 mS/m external conductivity using the dependencies on internal conductivity given in Fig. 3. x - refers to a representative single cell before (rightmost value) and after breakdown.

Now, let us investigate the relationship between membrane and internal conductivity from a theoretical point of view. Assuming Michaelis-Menten kinetics of the anion binding to the transport protein at the inner surface of the membrane the relation between membrane ion concentration (bound to enzyme) c_m and the inside concentration c_i is as follows:

$$c_m = E_t c_i / (k_m + c_i) \qquad (2)$$

where k_m is the Michaelis-Menten constant, and E_t is the total enzyme concentration. This relation can be transformed to an expression containing conductivities.

$$G_s = E_t u_m F G_i / d / (k_m F u_i + G_i) \qquad (3)$$

where u_m and u_i are the ionic mobilities in the membrane and in the cell interior, respectively. d is membrane thickness.
Taking the membrane enzyme concentration to be $1.2*10^4$ copies per cell /16/, d taking 8 nm and the red cell surface $1.4*10^{-10}$ m² we obtained from the best fit in Fig. 3 an ion binding constant of 2.1 mM and a membrane ionic mobility of $5*10^{-11}$ m²/Vs. This ionic membrane mobility is 100 times less than the ionic mobility of the interior which was determined to be $5*10^{-9}$ m²/Vs. The latter value is 16 times smaller than the ionic mobility of chloride in water.

This sequence of mobility values seems to be quite reasonable because the resistance to ion movement in the red cell should be significant due to the presence of hemoglobin (PAULY and SCHWAN /20/ assumed half of the ions to be completely immobile), and the membrane mobility appeared to be even lower which should be attributed to the mechanisms of the chloride ion transport by the protein. Both this membrane ion mobility value and also the binding constant could not be compared to data in the literature, since we were not able to find these particular constants. The only available data were exchange transport equilibrium constants obtained under different conditions ranging from 3 to 60 mM /16/. Since ion binding is possibly not the rate limiting step /16/ our value being less than the equilibrium constant seems to be also quite reasonable. We would like to note also that different cells yielded slightly different kinetic constants.

In summary we would like to emphasize, if the above concept proves to be applicable in future, too, that electrorotation offers a new way to study this and similar fast transport systems.

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