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Electrical Hemolysis of Human and Bovine Red Blood Cells

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Summary. The external electric field strength required for electrical hemolysis of human red blood cells depends sensitively on the composition of the external medium. In isotonic NaCl und KCl solutions the onset of electrical hemolysis is observed at 4 kV per cm and 50% hemolysis at 6 kV per cm, whereas increasing concentrations of phosphate, sulphate, sucrose, inulin and EDTA shift the onset and the 50% hemolysis-value to higher field strengths. The most pronounced effect is observed for inulin and EDTA. In the presence of these substances the threshold value of the electric field strength is shifted to 14 kV per cm. This is in contrast to the dielectric breakdown voltage of human red blood cells which is unaltered by these substances and was measured to be ~1 V corresponding in the electrolytical discharge chamber to an external electric field strength of 2 to 3 kV per cm. On the other hand, dielectric breakdown of bovine red blood cell membranes occurs in NaCl solution at 4 to 5 kV per cm and is coupled directly with hemoglobin release. The electrical hemolysis of cells of this species is unaffected by the above substances with exception of inulin. Inulin suppressed the electrical hemolysis up to 15 kV per cm. The data can be explained by the assumption that the reflection coefficients of the membranes of these two species to bivalent anions and uncharged molecules are field-dependent to a different extent. This explanation implies that electrical hemolysis is a secondary process of osmotic nature induced by the reversible permeability change of the membrane (dielectric breakdown) in response to an electric field. This view is supported by the observation that the mean volumes of ghost cells obtained by electrical hemolysis can be changed by changing the external phosphate concentration during hemolysis and resealing, or by subjecting the cells to a transient osmotic stress immediately after the electrical hemolysis step. An interesting finding is that the breakdown voltage, although constant throughout each normally distributed ghost size distribution, increases with increasing mean volume of the ghost populations.

An external electric field of short duration (μs) superimposed on the intrinsic membrane potential of biological cells induces reversible changes in the membrane permeability [10, 13, 24, 25, 27]. Above a threshold value of the electric field strength, the membrane current increases dramatically as was demonstrated indirectly by measurements with a hydrodynamic focusing Coulter Counter [22, 25, 27] and directly

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by applying a microelectrode technique to giant algal cells [5–7]. The change in membrane permeability is accompanied by the release of soluble materials from the cell interior [10, 12, 24, 25]. This threshold phenomenon was called dielectric or electrical breakdown. However, neither term describes the phenomenon adequately since these terms in solid state physics imply an irreversible change of structure, whereas in the case of a biological membrane the change is reversible; the membrane reseals and the original permeability is restored. This is evident from the experiments where a given cell or cell suspension was subjected several times to an electric field without change in the threshold value of the field strength required for breakdown [6, 7, 25, 26]. Furthermore, release of soluble material from the cell interior can occur gradually and subsequent pulses will release additional amounts of material [10, 26]. Therefore, the term dielectric breakdown as used here implies the reversible restoration of biological membrane properties.

Dielectric breakdown measurements have recently attracted interest as a new tool for studying membrane structure [11]. Furthermore, the transient permeability change of the cell membrane can be utilized to load the cells, after partial depletion from cellular material, with biological active materials (enzymes, drugs, etc.). Such loaded cells may have wide application in clinical therapy [18, 19, 23], e.g., for kidney failure, leukemia, acatalasemia and controlled drug release [26].

A basic requirement for such application is a better understanding of the breakdown mechanism and the secondary processes that are involved in release and uptake of material. It was shown elsewhere [12] that in contrast to bovine red cells, release of material from human red cells occurs in two stages. The dielectric breakdown results in the release of low molecular weight solutes only. Hemoglobin release occurs when the cell suspension is subjected to a pulse of higher electric field strength.

In this communication we investigate the extent to which osmotic processes are involved in electrical hemolysis once dielectric breakdown has occurred. To this end, we studied the influence of anions and uncharged molecules, to which the intact red cell membrane is impermeable, on the dielectric breakdown and on the electrical field strength required for electrical hemolysis of human red cells in isotonic solutions. Furthermore, we investigated the dependence of the mean volume of the resealed ghosts prepared by electrical induced hemolysis and of the corresponding breakdown voltage on transient changes in osmolarity produced immediately after electrical hemolysis has occurred. These experiments were

performed to gain an insight into a possible involvement of osmotic processes during the resealing process of the lysed red blood cells. They were also essential in the light of the clinical application of loaded ghost cells, since ghost cells exhibiting a mean volume which is different from that of intact cells can be used in controlled drug release [26].

Materials and Methods

Human blood samples were taken from healthy donors, preserved with sodium citrate and used on the same day. Bovine blood was collected from the slaughter house.

The red blood cells were gathered in the usual way [3] by centrifuging, removing the supernatant and the white blood cell layer and resuspending the red blood cell pellet in 0.9% NaCl solution. This process was repeated twice.

For the electrical hemolysis, the red blood cells were suspended in isotonic solutions of different composition as described in each experiment (*see* below). The suspension density was about 10^8 cells per ml. The electrical hemolysis was carried out in an electrical discharge chamber constructed out of plexiglass with two flat uncoated platinum electrodes (3×3 cm^2) placed 1 cm apart [12]. This chamber filled with the red blood cell suspension was part of a high voltage circuit (Universal Voltronics Corp., Mount Kisco, New York). A capacitor was charged up to a specified voltage and then discharged through the suspension by means of a spark gap. The voltage pulse applied to the suspension decayed exponentially with a time (decay) constant $\tau = RC$ (where R equals the resistance of the suspension and C the capacitance of the discharge circuit). The length of the voltage pulse was defined to be equal to the decay constant of the system. For electrical hemolysis of human red blood cells τ was equal to 40 μs and for that of bovine red blood cells equal to 30 μs. It should be noted that the external field strengths presented in this paper are the peak values E_0. As outlined in detail elsewhere [20] the actual field strength in the membrane leading to breakdown and hemolysis depends on the various time effects (such as the time constant of the membrane, the time constant of the breakdown process, and the decay time constant of the voltage pulse) and on the shape factors of the cell. The shape factor depends on the orientation of the cell to the external electric field.

Since these parameters are unknown it seems plausible at this experimental stage to use only the peak values.

10 min after application of the electrical field at 0 °C the suspension was centrifuged ($4000 \times$ g, 5 min) and the concentration of hemoglobin in the supernatant was determined photometrically at 415 nm. The percentage hemoglobin release was referred to the amount of hemoglobin released by complete hemolysis of the same number of red blood cells in distilled water. The preparation of ghosts by electrical hemolysis is described elesewhere [25, 26]. Some detailed information which is required for an understanding of the experiments reported here are given below in Results.

The breakdown voltage of the cell membranes of intact red blood cells and ghost cells was determined using a hydrodynamic focusing Coulter Counter (AEG-Telefunken, Ulm, W. Germany). As described in detail in several previous publications [21, 22, 25] the dielectric breakdown voltage V can be calculated from the shift of the size distributions of a cell population induced by increasing field strengths in the orifice of the Coulter Counter system. For a critical reappraisal of the connection between the breakdown and the membrane potential measured in experiments using a Coulter Counter the reader is referred to [20]. It should be noted that the Laplace equation used in this calculation

is solved for elliptical cells with one of the three semiprincipal axes parallel to the external field. The potential for the endpoints of the elliptical cells (in respect to the field direction) is $V=f_i a_i E$, where E is the electrical field strength, a_i the semi-axis parallel to the electric field, and f_i the shape factor [1].

The size distributions of red blood cells and ghost cells were determined by suspending the cells in an isotonic buffered solution (IPB solution[1] in mM): NaCl, 138.6; Na_2HPO_4, 12.3; NaH_2PO_4, 2.7; pH 7.4.

Results

Electrical Hemolysis

Using an electrolytical discharge chamber, as previously described [11, 21], dielectric breakdown of the cell membrane of human red blood cells occurs at external electric field strengths E_0 of 2 to 3 kV per cm depending on the size of the red blood cells. The electrical breakdown of the cell membrane is coupled with a redistribution of potassium and sodium ions between the external medium and the cell interior. At these field strengths hemoglobin release does not occur. Much higher electric field strengths are required to cause hemolysis. The dependence of the hemoglobin release on the external electrical field strength in isotonic solution containing only sodium chloride is presented in Fig. 1 (*crosses*). The dependence shows a typical sigmoid behavior with a threshold value of about 4 kV per cm. At 10 kV per cm hemolysis is complete. In the following the field strength at 50% hemolysis will be used for characterizing the effects of various substances on the electrically induced hemolysis of the red blood cells. For hemolysis in NaCl solution this value is 6 kV per cm. As indicated by the filled triangles, the curve for electric hemolysis in isotonic KCl solution coincides with that for the NaCl solution. If the chloride ions are partly replaced by fluoride ions (30 mM) the points fall also on the same curve (*open squares*).

In IPB solution the hemoglobin release curve is shifted to higher field strengths (*asterisks*, 50% hemolysis at 8 kV per cm) and also in this case partial replacement of 30 mM chloride by fluoride (*triangles*) or addition of 30 mM sodium fluoride to the IPB solution has no effect (*circles*).

The effect of phosphate on electrical hemolysis was investigated in more detail and the results are shown in Fig. 2. When phosphate (*open*

1 This isotonic phosphate buffer was used in previous work concerning electrical hemolysis [11, 26] and it is also used in this communication as the reference solution for electrical hemolysis experiments and for dielectric breakdown measurements unless stated otherwise. For clarity and shortness, therefore, the abbrevation IPB is used for the reference solution.

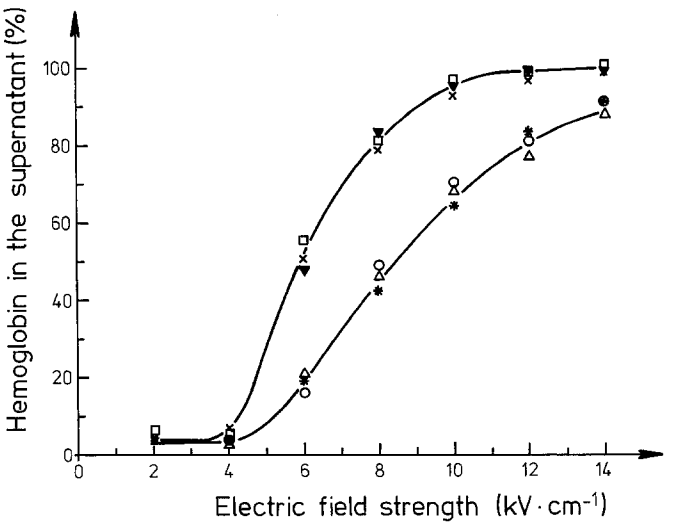

Fig. 1. The effect of increasing external electric field strengths on the hemoglobin release from human red blood cells suspended in isotonic solutions of different composition (in mM): ×–×, 154 NaCl; ▼–▼, 154 KCl; □–□, 124 NaCl+30 NaF (replacement of NaCl by NaF); *–*, 138 NaCl+12.3 Na_2HPO_4+2.7 NaH_2PO_4 (IPB solution); △–△, 108 NaCl+30 NaF+12.3 Na_2HOP_4+2.7 NaH_2PO_4 (replacement of NaCl by NaF); ○–○, 138 NaCl+12.3 Na_2HPO_4+2.7 NaH_2PO_4 +30 NaF (addition of NaF to the IPB solution). The suspension density was 10^8 cells per ml suspension, the pH of the solution was 7.4. The values for the electrically induced hemoglobin release are referred to the amount of hemoglobin released by complete osmotic lysis in distilled water. The data points are average values from two different sets of measurements

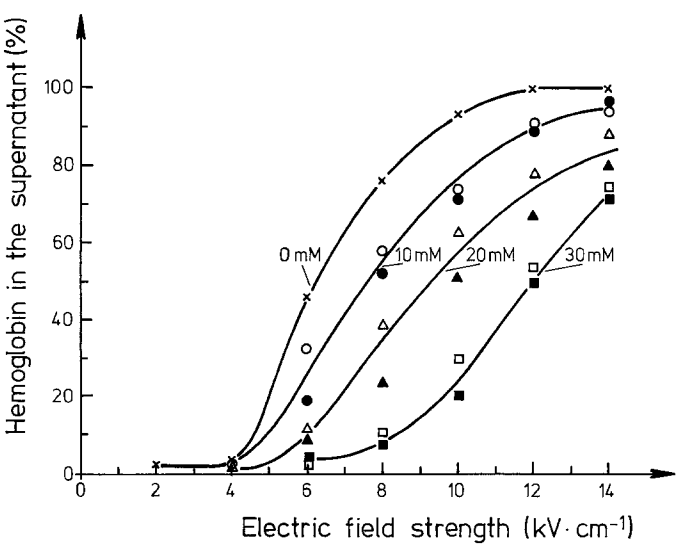

Fig. 2. The effects of increasing concentrations of phosphate and sulphate on the electrical field strength required for electrical hemolysis of human red blood cells. The intact cells were suspended under the experimental conditions given in Fig. 1 in an isotonic NaCl solution in which NaCl was subsequently replaced by increasing amounts of phosphate and sulphate. The open symbols refer to phosphate, the filled symbols to sulphate; the crosses refer to the control experiment in isotonic NaCl solution. The data points are average values from two different sets of measurements

symbols in Fig. 2) replaces chloride in increasing amounts to keep the tonicity roughly constant, the 50% value is shifted successively to higher electric field strengths (no phosphate: 6 kV per cm; 10 mM phosphate: 7.5 kV per cm; 20 mM phosphate: 9 kV per cm; 30 mM phosphate: 12 kV per cm).

Identical shifts are observed if chloride is replaced by increasing concentrations of sulphate (*filled symbols*). It should be noted that in both sets of experiments the dielectric breakdown voltage is nearly unaltered as indicated by measurements with the hydrodynamic focusing Coulter Counter (~ 0.95 V). One possible explanation for the observed effects may be that the membrane to which a field strength of about 8 kV per cm was applied is permeable to univalent ions as mentioned above, but less so to bivalent anions. If this is true then the osmotic pressure of the hemoglobin inside the cell would be partly counterbalanced by the osmotic pressure of phosphate and sulphate and thus hemolysis would be prevented at electric field strengths at which hemolysis occurs in the presence of the permeable chloride ions alone. This interpretation implies that hemolysis occurs osmotically once the membrane permeability to the solutes has gradually increased by increasing electric field strength.

This can be tested by partly replacing the sodium chloride of the IPB-solution by increasing concentrations of sucrose. As shown in Fig. 3 (*open symbols*) sucrose indeed shifts the 50% value to higher field strengths (no sucrose: 9 kV per cm; 10 mM sucrose, 12 kV per cm; 30 mM sucrose, 13.5 kV per cm) to an extent comparable to that found with the bivalent anions. If instead of replacing part of the sodium chloride by sucrose one adds sucrose directly to the IPB solution the same results are obtained (*filled symbols*). It should be pointed out also that under these experimental conditions the dielectric breakdown voltage is unaltered. The interpretation of these observations in terms of osmotic effects was further tested by studying the effect of the large uncharged molecule inulin on the electrical hemolysis. The data are plotted in Fig. 4. In these experiments inulin was added to the IPB solution. As indicated in Fig. 4 a very pronounced effect is observed. At 14 kV per cm only 20% hemolysis occurs.

Increasing concentrations of the chelating agent EDTA (up to 10 mM) have a dramatic effect on electric hemolysis similar to that of inulin (Fig. 5). As can also be seen from Fig. 5 the effect of EDTA is nearly independent of the pH in the range between 5.6 and 7.4. Experiments in which the effect of the above substances on the electric lysis of bovine

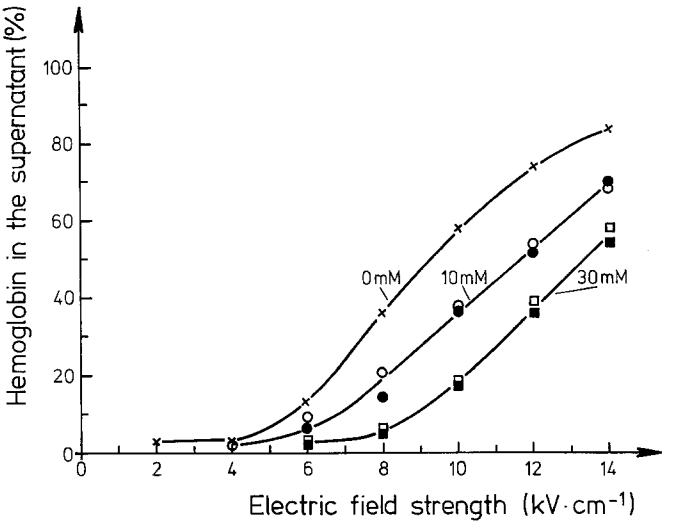

Fig. 3. The effect of increasing concentrations of sucrose on the electric field strength required for electrical hemolysis of human red blood cells. The intact cells were suspended under the experimental conditions of Fig. 1 in IPB solution to which either sucrose of increasing concentrations was added (filled symbols) or in which NaCl was replaced by the osmotic equivalent amount of sucrose (open symbols). The crosses refer to electrical hemolysis of the control experiments in which the cells were suspended in IPB solution. The data points are average values from two different sets of measurements

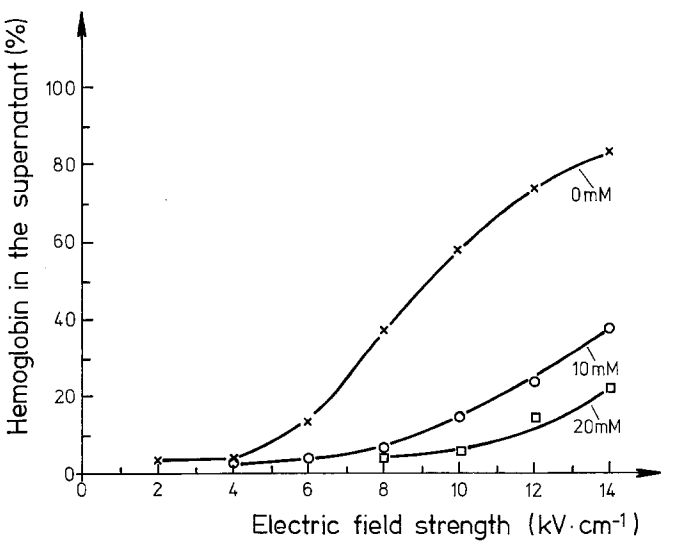

Fig. 4. The effect of inulin on the electric field strength required for electrical hemolysis of human red blood cells. Experimental conditions as described in Fig. 1. The increasing concentrations of inulin were added to IPB solution. The crosses refer to electrical hemolysis of cells suspended in IPB solution (control experiment). The data points are average values from three different sets of measurements

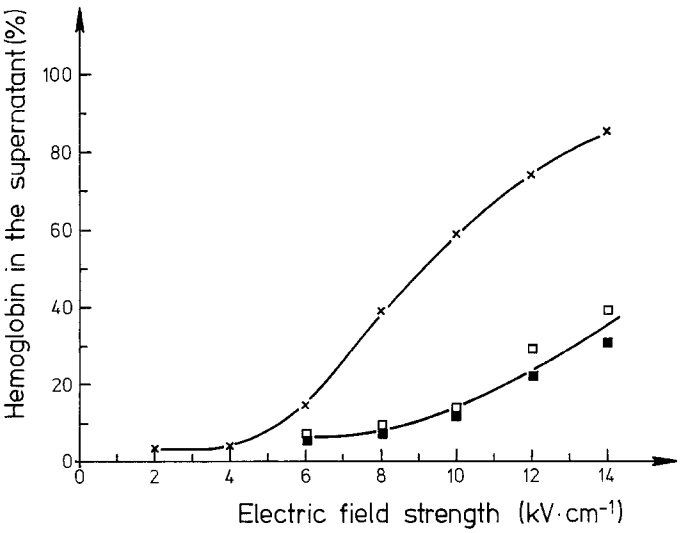

Fig. 5. The effect of increasing concentrations of EDTA on the electric field strength required for electrical hemolysis of human red blood cells. The control experiment was performed in IPB solution (crosses); experimental conditions as described in Fig. 1. EDTA was added to IPB solution. In one set of experiments the pH of this solution was 5.6 (open squares), in the other one 7.4 (filled squares). The data points are average values from three different sets of measurements

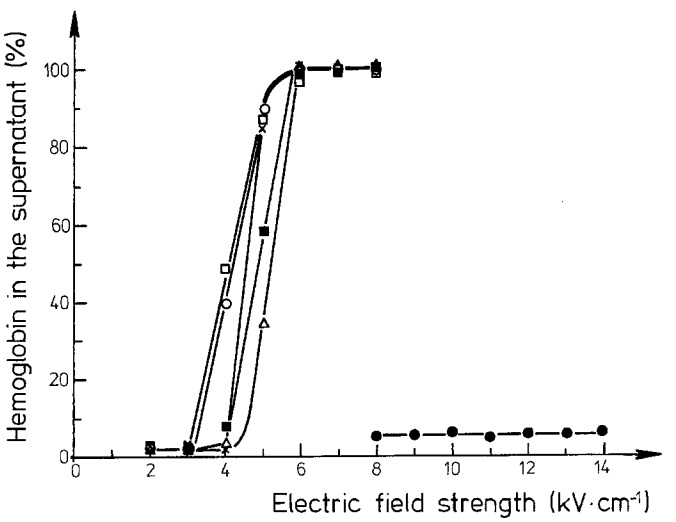

Fig. 6. The effect of phosphate, sulphate, sucrose, inulin and EDTA on the electric field strength required for electrical hemolysis of bovine red blood cells. Inulin, sucrose and EDTA were added to IPB solution, whereas the phosphate and sulphate solutions were prepared by replacing part of NaCl by osmotic equivalent amounts of phosphate and sulphate in an isotonic NaCl solution. In mM: ○–○, 30 phosphate; □–□, 30 sulphate; △–△, 30 sucrose; ●–●, 20 inulin; ■–■, 10 EDTA. Control experiments performed in IPB solution are indicated by crosses. The data points are average values from two different sets of measurements

red blood cells was tested are shown in Fig. 6. In contrast to the findings for human red blood cells, sucrose and the bivalent anions do not shift (or only slightly shift) the hemoglobin release to higher electric field strengths. The same negative result is found with EDTA, whereas with inulin the hemoglobin release is suppressed up to electric field strengths of about 15 kV per cm.

Ghost Preparation by Electrical Hemolysis

In the following set of experiments we investigated the properties of the ghost cells obtained by electrical hemolysis from human red blood cells in solutions containing different concentrations of phosphate and resealed by a procedure described previously [25, 26]. According to this procedure the hemolysis was performed in an isotonic solution containing 105 mM KCl, 35 mM NaCl and 4 mM $MgCl_2$ ("KCl solution"). Different phosphate concentrations were achieved by replacing part of NaCl in the "KCl solution" by sodium phosphate at constant tonicity. Phosphate concentrations higher than 15 mM were not investigated because of the hemoglobin retention in the red blood cells at high phosphate concentrations (*see* Fig. 2 for the phosphate effect and also Fig. 1 which shows that no difference in the onset of electrical hemolysis was observed when NaCl was replaced by KCl). The experiments were performed at an external electrical field strength of 12 kV per cm. At this field strength retention of hemoglobin is only about 5 to 10% up to concentrations of 15 mM phosphate.

Resealing of the lysed red blood cells was performed for 10 min at 0 °C and then, after raising the temperature to 37 °C, for a further 20 min. The size distributions were measured with the ghost cells suspended in IPB solution.

In Fig. 7 the mean volumes of the ghost preparations are plotted as a function of increasing phosphate concentrations. The figure also represents the breakdown voltage of the ghost populations obtained under these conditions. It should be pointed out that only the size distributions of ghost cells which were prepared in "KCl solutions" containing phosphate concentrations above 5 mM are normally distributed. These size distributions were also electrically homogeneous [26] since the breakdown voltage was constant throughout each single size distribution.

On the other hand, the size distributions of ghost cells prepared in "KCl solutions" containing phosphate concentrations below 5 mM

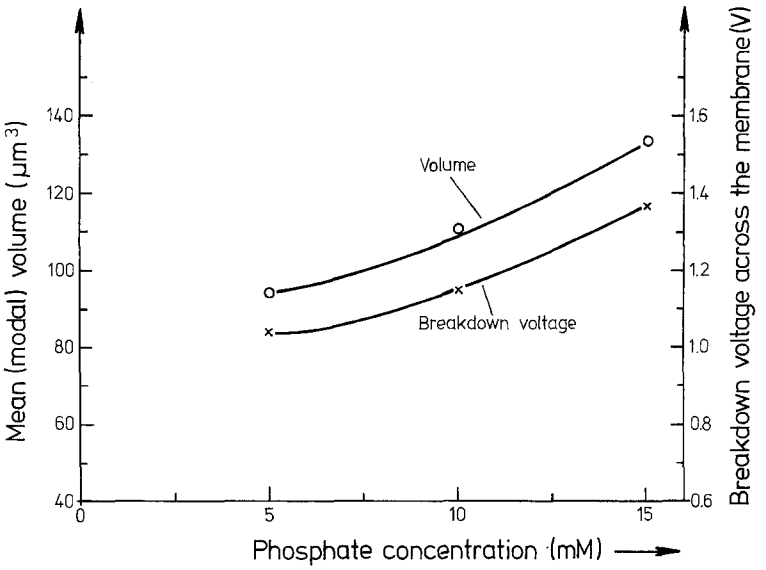

Fig. 7. The effect of increasing phosphate concentrations on the mean volume (circles) and the breakdown voltage (crosses) of ghost cells prepared from human red blood cells by electrical hemolysis. Electrical hemolysis was performed in "KCl solution" containing (in mM) 105 KCl, 35 NaCl and 4 $MgCl_2$. Different phosphate concentrations were achieved by replacing part of the NaCl of this basic medium at constant tonicity. The electrical field strength was 12 kV per cm. Resealing of the electrically lysed cells was performed for 10 min at 0 °C and for 20 min at 37 °C. The data points are average values from four different sets of measurements

were skewed indicating two different ghost populations with two different breakdown voltages. The ratio between these two ghost populations varied from experiment to experiment and it was not possible to obtain reproducible data. Changes in pH of these "low"-buffered solutions could be definitely excluded as the possible effect for the skewness. Therefore, for the graph in Fig. 7, data are presented only for experiments performed in solutions containing phosphate concentrations above 5 mM. As indicated by Fig. 7 the mean volume increases with increasing phosphate concentrations above 5 mM. An interesting finding is that a parallel trend is observed for the critical breakdown voltage, suggesting that the breakdown voltage is directly related to the mean volume of the ghosts.

To test this hypothesis ghosts were subjected to a transient osmotic stress immediately after the electrical hemolysis step, i.e., in the very early stage of the resealing at 0 °C. This was done by lowering the tonicity of the "KCl solution" containing 10 mM phosphate by addition of distilled water immediately after electrical hemolysis and by restoring

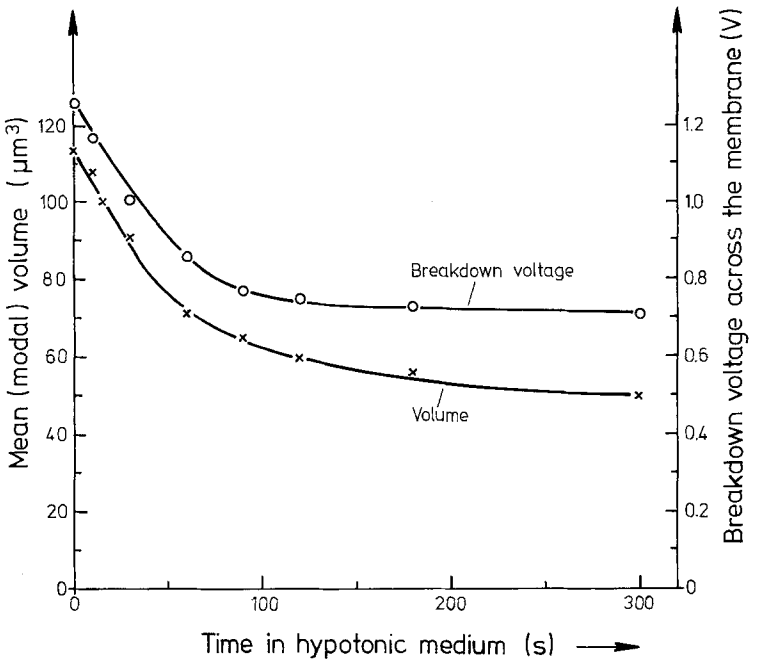

Fig. 8. The mean volume (crosses) and the breakdown voltage (circles) of ghost cells prepared electrically from human red blood cells after a transient osmotic stress in dependence on the time interval between lowering and restoring isotonicity. Cells which were lysed electrically under the conditions given in Fig. 7 were subjected immediately after the electrical hemolysis step to osmotic stress by addition of distilled water to the "KCl solution" (final osmolarity: 30 mosM). After a certain time had elapsed, isotonicity was restored by addition of a hypertonic phosphate-buffered "KCl solution". Then the temperature was increased from 0 °C to 37 °C for 20 min to complete the resealing process. The data points are average values from two different sets of measurements

the isotonicity after a certain time had elapsed. Isotonicity was restored by addition of a hypertonic phosphate-buffered "KCl solution" to give a final isotonic solution of the original composition. Then the temperature was raised to 37 °C for 20 min to complete the resealing process. After centrifugation the cells were suspended in IPB solution and the size distributions were determined.

In Fig. 8 the time interval between the lowering and restoring of isotonicity was varied for a given value of osmolarity (30 mosM). As indicated in the figure, the mean volume of the normally distributed ghost populations depends on the time interval during which the suspensions were kept at low tonicity. It is also evident that the volume dependence of the breakdown voltage is the same as that found for the ghost populations prepared in "KCl solutions" containing phosphate concentrations between 5 and 15 mM. The volume and the breakdown voltage

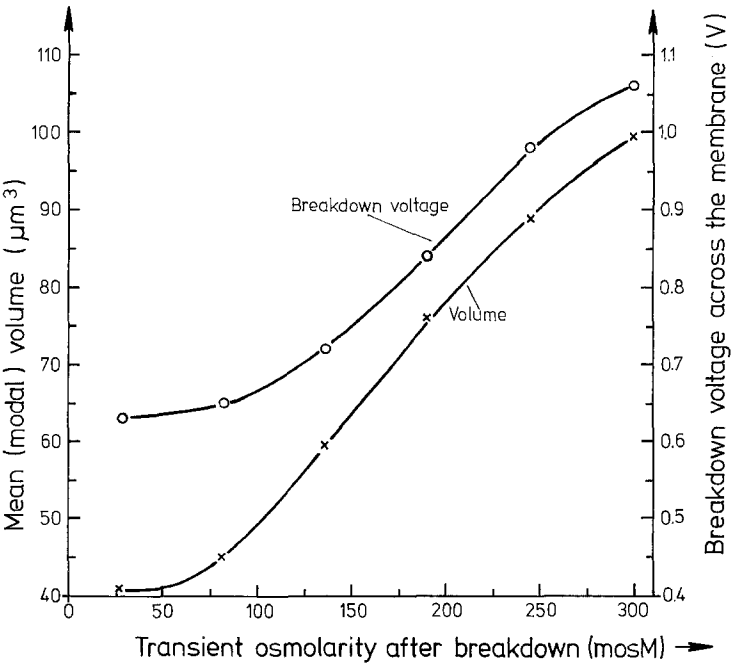

Fig. 9. The effect of different transient osmotic changes on the mean volume (crosses) and the breakdown voltage (circles) of ghost cells prepared from human red blood cells suspended in "KCl solution" by electrical hemolysis. Experimental conditions as described in Fig. 8. The time interval between lowering and restoring the isotonicity after electrical hemolysis had occurred was 5 min. The osmolarity of the solutions to which the lysed cells were subjected immediately after electrically induced hemolysis was varied between 28 and 300 mosM. The data points are average values from five different sets of measurements

reach a constant value after a time interval of about 5 min. In Fig. 9 the time interval between lowering osmolarity and restoring the isotonicity was kept constant at 5 min and the osmolarity was changed by adding different amounts of distilled water to final osmolarities between 28 and 300 mosM. It is obvious that the mean volume of the resealed ghosts depends on the osmolarity to which the cells were brought after electrical hemolysis at 0 °C. Furthermore, the same relationship between breakdown voltage and mean volume is observed as in Fig. 8 and in Fig. 7 for concentrations of phosphate higher than 5 mM. It should be noted that the ghost populations obtained by transient osmotic changes after electrical hemolysis are normally distributed and electrically homogeneous over the whole range of osmolarity.

A possible specific effect of phosphate which may be suggested by the experiments in Fig. 7 can be ruled out since the same results as in Fig. 8 are obtained if the transient osmotic changes are induced at

constant phosphate concentrations, i.e., by addition of a solution containing 10 mM phosphate instead of distilled water.

Discussion

The data described here reveal a well-defined critical strength of electrical field required for hemolysis of human red blood cells to low molecular weight solutes present in the isotonic solution. This is remarkable in view of the finding that bovine red blood cells lack this sensitivity. Electrical hemolysis of cells of this species can be suppressed (up to high electric field strengths of the order of 14 kV per cm) only by larger molecules like inulin. These differences may be linked with the different response of the red blood cell membrane of these two species to the electric field [12]. This conclusion is strengthened by the fact that EDTA suppresses the electrical hemolysis of human red blood cells to an extent comparable to inulin, whereas the electrical hemolysis of bovine red blood cells is unaffected.

On the other hand, the secondary processes which are induced by the change in membrane structure in response to the electric field, and which lead to hemolysis, seem to be of an osmotic nature with both species. The effects of the charged and uncharged molecules on electrical hemolysis as well as the change in the mean ghost cell volume with variation of the experimental conditions can be satisfactorily explained by the assumption that the reflection coefficients of the membranes to various substances are field-dependent.

The reflection coefficient (σ), introduced by Staverman (*cf.* [8]), defines the degree of semipermeability of membranes. The reflection coefficient is 1 if the membrane is impermeable to a given solute and zero if the membrane cannot distinguish between water and solute flow. Therefore, the osmotic pressure exerted by a given solute depends both on the concentration and on the reflection coefficient. It is well known that the reflection coefficients of the substances tested here are 1 for the intact red blood cell membrane. On increasing the electric field strenght σ of the various substances decreases. Considering human red blood cells, at the electrical breakdown voltage (corresponding to an external electric field strength of about 2 to 3 kV per cm) the redistribution of sodium and potassium observed experimentally [12] indicates that σ_{NaCl} and σ_{KCl} have decreased significantly. Since there is no hemolysis at the breakdown potential σ_{NaCl} must at least still be different from

zero, since for $\sigma_{NaCl}=0$ the osmotic pressure gradient exerted by the hemoglobin would not be counterbalanced and hemolysis would occur. For bovine red blood cells at the critical breakdown voltage of the membrane (corresponding to an electric field strength of 4 to 5 kV per cm) the redistribution of the alkali ions is linked with hemoglobin release. Therefore, we can conclude that the permeability change in bovine red blood cell membranes induced by this electric field strength is so dramatic that the membrane cannot distinguish between these ions and water (σ_{NaCl} and σ_{KCl} are equal to zero). For human red blood cells, the reflection coefficients of both salts must vanish at 8 kV per cm where in isotonic NaCl and KCl solution electrical hemolysis occurs. Furthermore, it seems reasonable to assume that at this electric field strength the reflection coefficient for larger bivalent anions like phosphate, sulphate, and uncharged molecules like sucrose are still not equal to zero so that these substances will exert an osmotic pressure gradient counterbalancing that of hemoglobin. With further increasing field strength the reflection coefficients of the various substances will also tend toward zero. Therefore, it is not unexpected that inulin, which has the largest molecular radius (1.2 nm [15]) shows the largest shift of the electrical hemolysis towards higher electrical field strengths. The hypothesis presented also provides a straightforward explanation for the observations that a change of the osmolarity of the isotonic solution (by addition or replacement, *see* Figs. 1 and 3) results in the same shift of the onset of electrical hemolysis. The hypothesis is also consistent with the finding that sucrose and the bivalent anions have only a slight effect on electrical hemolysis of bovine red blood cells. The reflection coefficients of the bovine red blood cell membrane to these substances seem to decrease very rapidly with increasing electric field strength; an assumption which is supported by the experimental observation that dielectric breakdown in NaCl solution immediately leads to hemolysis. It is surprising that inulin suppressed electrical hemolysis of bovine red blood cells more effectively than that of human red blood cells, whereas EDTA has no effect.

These differences of σ in the dependence of electric field strength observed for both species may be related to differences in the membrane structure and may also point to different properties of the local areas in the membrane where dielectric breakdown occurs.

It should be noted that the effect observed with EDTA argues against a role of chelation or sequestration of Ca^{++} ion as a primary cause for the shift to high values of the hemolytic field strength. This is so

because Ca^{++} ion chelation should destabilize the red cell, as is well known for divalent cations (*cf.* [4]). Furthermore, this view does not account for the observation that fluoride ions are ineffective in shifting the electrical hemolysis to higher field strengths. If Ca binding is the primary process then this result is unexpected considering the high binding constant of fluoride and EDTA to Ca^{++} ($K_{Ca\text{-}EDTA} = 10^{11}$, $K_{CaF_2} = 10^{11}$) [2, 14]. Also, as shown by Bodèmann and Passow [3], when EDTA permeates the membrane during a hemolytic event, divalent ions are removed only at elevated temperatures, but not at 0 °C.

Thus far we have assumed a field dependence of the reflection coefficient in order to explain our results concerning the shift in electrical hemolysis. Furthermore, if we make the reasonable assumption that the reflection coefficient for the various solutes, also, at 0 °C, increases with time from zero to higher values once the critical field pulse is over then we can also explain the time dependence of the mean ghost volume on osmolarity after electrical hemolysis (Fig. 8).

After lowering the osmolarity of the external isotonic "KCl solution" in which electrical hemolysis was performed, the lysed cells swell. The degree of swelling depends on the effective osmotic pressure of the dilute solution, i.e., both on the concentration and the reflection coefficient. At this time σ will be very low but different from zero. According to the assumption made above, when isotonicity is restored the reflection coefficient will be the higher and consequently cell shrinkage larger, the longer the time interval that has elapsed. Therefore, it is expected that the mean cell volume of the ghosts decreases with increasing time and also with decreasing osmolarity of the solution in which the ghost suspension was kept (Fig. 9).

The dependence of the mean ghost volume on phosphate concentration in isotonic solution (Fig. 7) can be explained along the same lines if we assume that the reflection coefficient of the resealed ghosts to phosphate salt is slightly larger than that of chloride salt. After breakdown the amount of phosphate trapped inside the ghosts decreases with decreasing phosphate concentration in the external solution. When transferred to isotonic NaCl solution containing 15 mM phosphate the cells will shrink until the phosphate concentration inside the cell matches the concentration outside the cell.

The phenomenological considerations presented here imply that electrical hemolysis is a secondary process of osmotic nature induced by the transient permeability change of the membrane in response to the applied external electric field. This conclusion would trace back the pheno-

menon of electrical hemolysis to the so-called colloid osmotic pressure theory introduced by Wilbrandt [17] and extended by several authors [9, *cf.* 16] for explanation of hemolysis induced by hypotonic osmosis and UV radiation.

Of prime importance is the question concerning the molecular processes leading to the field dependence of the reflection coefficients. The data obtained in previous work [5, 6] using intra and extracellular electrodes indicate that dielectric breakdown leads to a localized electromechanical collapse of the membrane. In the area concerned the electrical resistance decreases drastically. The values of the breakdown voltages quoted so far [11, 21, 22, 25, 26] from Coulter Counter measurements were calculated for the two endpoints of the cell (spherical or elliptical) with respect to the field direction. These are the points of the maximal membrane potential difference in the external field. Once the critical potential difference is reached, breakdown will occur at these points leading to a reduction of the potential difference in their vicinity (due to the decreased membrane resistance). Increasing the electric field strength leads to an increase in the current density. Possibly the attending local heating effects may enlarge the breakdown area. Alternatively it is also possible that increasing external field strength above the critical level leads to creation of several locally separated breakdown centers. The number of the breakdown centers may be increased either simultaneously or consecutively, depending on several time effects involved in the breakdown process.

First, the time of charging the membrane depends on the rise time of the external field and on the time constant of the membrane. Secondly, the membrane is discharged due to breakdown when the critical membrane potential difference is reached with a time constant which may be different from that of charging the membrane. The dynamics of the breakdown process itself may also influence the discharging process.

If the rise-time of the external field and the time constant of charging the membrane is smaller than the time constant of discharging the membrane following local breakdown, then the critical membrane potential difference is reached simultaneously over larger membrane regions. This follows simply from the angular dependence of the voltage distribution determined by the solution of the Laplace equation as mentioned above, and previously published [21, 22].

Although this is very plausible it is also not unreasonable that the rise time of the membrane potential difference can be larger than the time of the breakdown itself [12] and the time constant of discharging

the membrane [6]. In this case the voltage distribution over the membrane becomes very complex when the external field is increased above the critical field strength.

Qualitatively this leads to several locally separated breakdown centers occuring consecutively with increasing external field.

An increase of the number of the breakdown centers or in the area of breakdown centers with increasing electric field strength, in the presence of additives (like inulin and EDTA) protecting the cell against hemolysis, would have the important consequence that the detection of the breakdown centers by electronmicroscopic techniques may be feasible.

It should be pointed out that a suitable interpretation of the dependence of the breakdown voltage on the mean volume of the ghost populations is still lacking. Since preliminary experiments with intact red blood cells subjected to hypotonic stress here revealed the same relationship of the breakdown voltage on mean volume, we can assume that this relationship is either of general nature or reflects a gradual change in the membrane permeability and/or in the shape factor of the cell with cell volume. Notwithstanding the above, it is important to consider the volume-dependence of the critical breakdown voltage in experiments concerning possible effects of membrane structure on the breakdown potential.

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