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Electro-Mechanical Properties of Human Erythrocyte Membranes: The Pressure-Dependence of Potassium Permeability

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Summary. Electrical breakdown of cell membranes is interpreted in terms of an electro-mechanical model. It postulates for certain finite membrane areas that the actual membrane thickness depends on the voltage across the membrane and the applied pressure. The magnitude of the membrane compression depends both on the dielectric constant and the compressive, elastic modulus transverse to the membrane plane. The theory predicts the existence of a critical absolute hydrostatic pressure at which the intrinsic membrane potential is sufficiently high to induce "mechanical" breakdown of the membrane. The theoretically expected value for the critical pressure depends on the assumption made both for the pressure-dependence of the elastic modulus of the membrane and of the intrinsic membrane potential. It is shown that the critical pressure is expected at about 65 MPa. The prediction of a critical pressure could be verified by subjecting human erythrocytes to high pressures (up to 100 MPa) in a hyperbaric chamber. The net potassium efflux in dependence on pressure was used as an criterion for breakdown. Whereas the potassium net efflux was linearly dependent on pressure up to 60 MPa, a significant increase in potassium permeability was observed towards higher pressure in agreement with the theory. The increase in the net potassium efflux above 60 MPa was reversible, as indicated by measurements in which the same erythrocyte sample was subjected to several consecutive pressure pulses. Temperature changes in the erythrocyte suspension during compression and decompression were so small (less than 2 °C) that they could not account for the observed effects.

Electrical breakdown of the normally electrically insulating cell membrane is observed when the membrane potential is taken rapidly (say in nsec to μsec) to a critical value of the order of 0.5 to 1 V [32, 34]. Breakdown results in a dramatic, but reversible, decrease in the membrane resistance and is associated with a marked increase in membrane permeability [22, 31]. Due to the temperature-dependent resealing properties of biological membranes, the original membrane resistance and, in turn, the membrane impermeability can be restored by a temperature increase.

A reversible electrical breakdown was demonstrated for membranes of animals [32] and plant cells [3, 4, 26], and for bacteria [34] and artificial bilayers [1] using quite different techniques (i.e., Coulter Counter, discharge chamber, intra- and extracellular electrodes, and charge-pulse techniques.)

The mechanism of electrical breakdown has been the subject of some controversy in the past, since Tsong and Kingsley [24] attempted to explain the observed electrical phenomena in terms of temperature changes in the external medium and within the membrane. Meanwhile, there is ample evidence [12, 31-33] that the electrical field is primarily responsible for the electrical breakdown.

The electrical breakdown of the cell membrane can be interpreted in terms of an electro-mechanical compression of the membrane. The electro-mechanical model introduced by Zimmermann et al. [5, 26, 32] (for the explanation of electrical breakdown) postulates that for certain finite membrane areas the actual membrane thickness depends on the voltage across the membrane and the applied pressure. The magnitude of the membrane compression depends both on the elastic compressive modulus transverse to the membrane plane and the relative dielectric constant of that breakdown area.

Breakdown of the cell membrane occurs on the line of this model once a critical membrane thickness in the breakdown area is reached in response to an external electrical field pulse of short duration.

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The theory predicts that the breakdown voltage of the cell membrane should be pressure-dependent and that a critical absolute hydrostatic pressure should exist at which the intrinsic membrane potential is sufficiently high to induce a reversible "mechanical breakdown" [26, 29].

In walled cells in which the internal pressure, i.e., the turgor pressure, can be manipulated over a large range and measured by an inserted pressure probe, it was recently demonstrated that the breakdown voltage indeed decreases with increasing turgor pressure, and that the decrease was of the expected order [28].

The second crucial prediction arising from the electro-mechanical model is examined in this communication. Human erythrocytes were subjected to increasing absolute pressure for short time intervals in a hyperbaric chamber and the net potassium efflux was used as an indicator for the postulated "mechanical" breakdown. The results have shown that a significant reversible increase in the net potassium efflux is observed beyond about 60 MPa, where the "mechanical" breakdown of the membrane should occur in terms of the electro-mechanical model.

Theoretical Considerations

The electro-mechanical model introduced by Zimmermann and Pilwat [*see* 32] and extended by Coster and Zimmermann [2, 6, 26; *see also* 9, 15] postulates that for certain finite membrane areas the membrane thickness, δ, depends on the voltage across the membrane, V, and the pressure, P. The magnitude of the expected change in membrane thickness depends also on the compressibility and the relative dielectric constant of the membrane, which, in turn, vary according to the heterogeneous membrane topography [23].

The model is based on the assumption that a finite membrane area can be regarded as a capacitor filled with an isotropic elastic dielectric material. At equilibrium the electrical compressive force, P_e, arising either from the intrinsic membrane potential or from an externally applied voltage across the membrane and the mechanical compressive force, P, resulting either from the turgor pressure or from external hydrostatic pressure (hyperbaric chamber), are counterbalanced by the elastic restoring force, P_m, generated by the compression of the membrane:

$$P_e + P + P_m = 0. \tag{1}$$

A quantitative description of the electrical compressive forces and the elastic restoring forces on a molecular scale is difficult. In order to proceed [2], we assume that the macroscopic laws of electrostatics and elasticity (Hooke's Law for the one-dimensional case) are applicable. This means that

$$P_e = \frac{\varepsilon \cdot \varepsilon_0}{2 \cdot \delta^2} \cdot V^2 \tag{2}$$

and

$$P_m = Y_m^o \ln \frac{\delta}{\delta_o} \tag{3}$$

where ε is the relative dielectric constant of the membrane material, ε_o the electric permittivity of the free space ($8.85 \cdot 10^{-12}\,\mathrm{F\,m^{-1}}$), V the membrane voltage, δ the thickness of the stressed membrane and δ_o the thickness of the unstressed membrane ($P=0$, $V=0$). Y_m^o represents the so-called elastic compressive modulus transverse to the membrane plane. For the derivation of Eq. (3) it is assumed that Y_m^o is independent of the membrane thickness, δ, over the whole range of compression.

Y_m^o is a very complex parameter, and the reader is advised to consult the cited literature for a closer definition [2]. Equation (3) describes the direct mechanical compression of the membrane[1] and implies, therefore, that the pressure direction is perpendicular to the membrane surface. This assumption is certainly true for experiments in which the dependence of breakdown voltage on turgor pressure is considered [28], but not for the bulk compression in the hyperbaric chamber. In order to proceed, we assume that pressure effects in parallel to the membrane plane are negligible for a first approximation. Substituting Eqs. (2) and (3) into Eq. (1) yields Eq. (4) for the equilibrium state:

$$\frac{\varepsilon \cdot \varepsilon_o}{2 \cdot \delta^2} \cdot V^2 + Y_m^o \ln \frac{\delta}{\delta_o} + P = 0. \tag{4}$$

Equation (4) relates the membrane potential to the mechanical pressure via the membrane thickness and represents the key equation for the transformation of electrical into mechanical signals and *vice versa* in biological membranes of wall-less cells. At a given pressure, P, Eq. (4) yields the functional relation between the membrane voltage, V, and the membrane

[1] It should be also noted that in walled cells, which regulate the turgor pressure over large pressure ranges, the thickness of the membrane can also be altered indirectly by extension of the cell wall to which the cell membrane is coupled mechanically, electrostatically or chemically. The contribution of both a direct and an indirect decrease in membrane thickness can be taken into account if certain assumptions are made [2, 28].

thickness, δ:

$$V = \left(\frac{2\,Y_m^o}{\varepsilon \cdot \varepsilon_o}\right)^{1/2} \left(\ln\frac{\delta_o}{\delta} - \frac{P}{Y_m^o}\right)^{1/2} \cdot \delta. \tag{5}$$

Upon differentiation of Eq. (5) one obtains the critical thickness, δ_c, at which breakdown occurs (*see also* Eq. (24) in [2]) as a function of pressure:

$$\delta_c = \delta_o \cdot \exp\left(-\frac{P}{Y_m^o}\right) \cdot \exp\left(-\frac{1}{2}\right). \tag{6}$$

The pressure-dependence of the breakdown voltage can be derived from Eqs. (5) and (6):

$$V_c(P) = V_c(P=0) \cdot \exp\left(-\frac{P}{Y_m^o}\right) \tag{7}$$

in which

$$V_c(P=0) = \left(\frac{0.37\,Y_m^o}{\varepsilon_o \cdot \varepsilon}\right)^{1/2} \cdot \delta_o \tag{8}$$

is the breakdown voltage at $P=0$.

Figure 1*a* illustrates the theoretically expected normalized function $V(1-\delta/\delta_o)/V_c(P=0)$ at different pressures, P, for human erythrocytes. Y_m^o was calculated to be 4.5 MPa from the breakdown voltage at $P=0$ (Eq. (8)). For $V_c(P=0)$ a value of 1 V was chosen [31], δ_o was taken to be 4 nm, and ε was assumed to be 3 since there is good evidence that breakdown occurs primarily in the lipid bilayer or, more likely, at the junctions between lipids and proteins [1, 21]. Experiments on the pressure-dependence of the breakdown voltage in *Valonia utricularis* support this conclusion [28]. These experiments have shown that hydrated proteins are not involved in the breakdown event. The water within the membrane and the membrane interfaces therefore does not play a dominant role in the breakdown process. Measurements of the breakdown voltage on solvent-free lipid-bilayer membranes have also demonstrated that the value of the breakdown voltage is not influenced by the solvent included within the membrane (Dr. R. Benz, *personal communication*).

The function $V(1-\delta/\delta_o)/V_c(P=0)$ is only defined for positive changes in thickness (i.e., for compression), but it is valid for both positive and negative potential differences (Eq. (2)). This means that electrical compression can be achieved by electrical field pulses of either polarity. With increasing pressure (*see* arrow in Fig. 1*a*) the membrane becomes mechanically precompressed and thus the breakdown voltage, which corresponds to the maximum in each curve, decreases. The pressure-dependence of the normalized breakdown voltage can be calculated from Eq. (7) or constructed from the family of curves in Fig. 1*a*, as demonstrated in Fig. 1*b*.

In the low-pressure range, a linear approximation of Eq. (7) can be used to describe the pressure-dependence of the breakdown voltage:

$$V_c(P) = V_c(P=0)\left(1 - \frac{P}{Y_m^o}\right). \tag{9}$$

This is indicated by the inset in Fig. 1*b*. For clarity only a pressure interval of 0.5 MPa is considered [28].

For larger pressure ranges, up to 100 MPa, which can be realized in experiments in which cells are exposed to pressure in a hyperbaric chamber (*see* below), the exponential decline of the relative breakdown voltage with increasing pressure (Eq. (7)) must be taken into account. Consequently a critical pressure, P_c, should exist [26, 29] at which the electrical field strenght required for breakdown becomes as low as the electrical field strength arising from the intrinsic membranes potential difference, provided that the intrinsic and externally-applied electric field strengths are linearly superimposed on each other. The critical pressure can be calculated from Eq. (10):

$$P_c = Y_m^o \cdot \ln\frac{V_c(P=0)}{|V_m|}. \tag{10}$$

In Fig. 1*b* the critical pressure P_c is indicated by the arrow at $|V_m| = V_c(P=P_c)$. $|V_m|$, the resting membrane potential difference, was measured in red blood cells directly, using intra- and extracellular electrodes [13, 14]. The most negative value for V_m recorded in these experiments was -13 mV. A similar value was calculated from the passive chloride distribution between the cell interior and the surrounding medium (e.g., in the NaCl buffer used below [7]). Both methods for determining the membrane potential difference in red blood cells were subject to criticism because of intrinsic experimental uncertainties. In order to proceed, we assume that the value of 13 mV reflects the true absolute value of the membrane potential difference. The critical pressure value, P_c, is calculated to be 20 MPa. However, a value of $P_c = 20$ MPa should be too low because one cannot expect that the elastic modulus, Y_m^o, is independent of the membrane thickness over the entire compression range, which should be larger in "mechanical" breakdown experiments compared with "electrical" breakdown experiments (*see also* Eq. (6), and Fig. 1).

The relative dielectric constant, ε, is generally, among other parameters, also a function of material density and, in turn, of the compression state of the membrane. However, it can be readily shown that the influence of variations in ε with changes in membrane thickness on the function $V_c = f(P)$ is negligible, since the decrease of the breakdown voltage with increas-

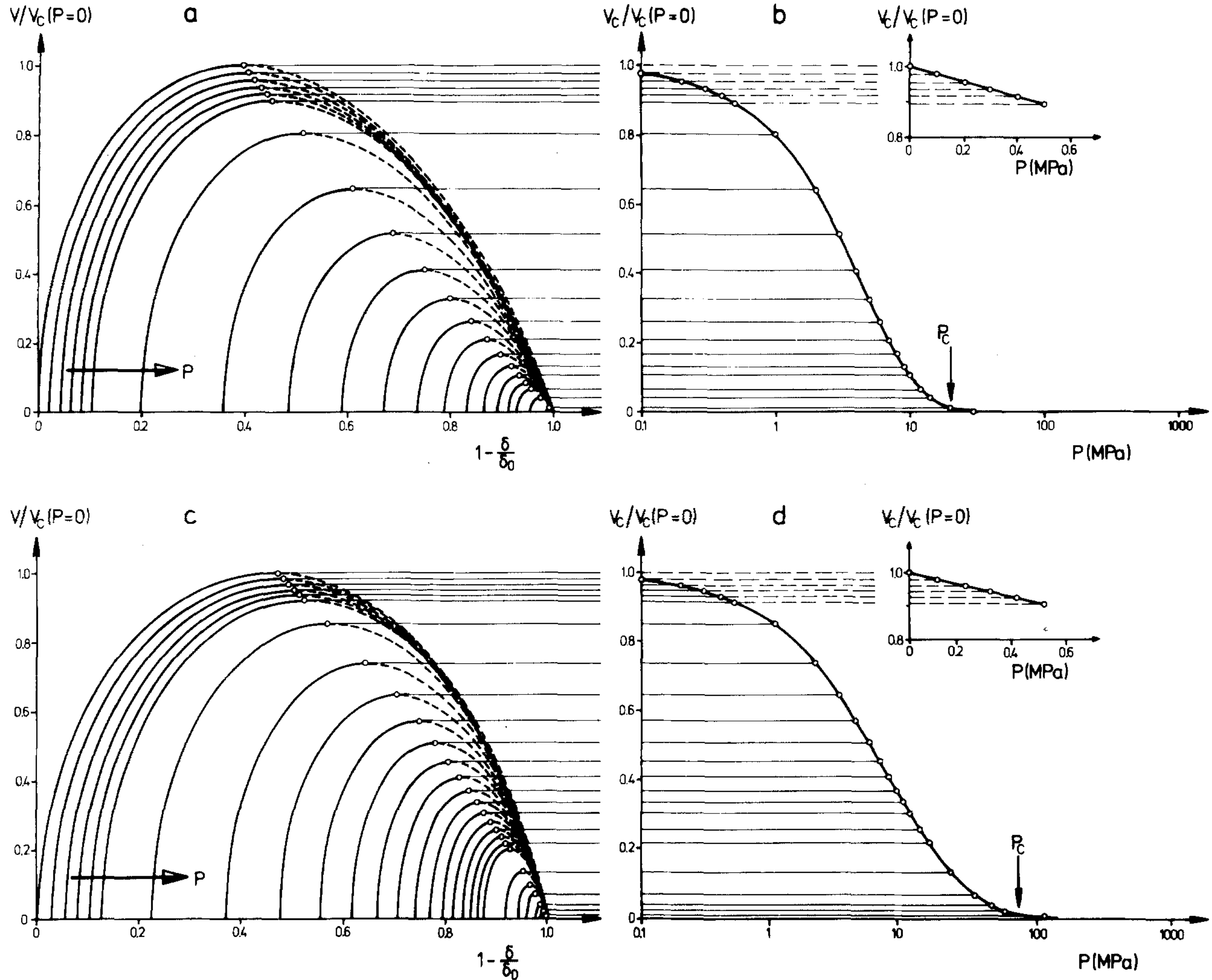

Fig. 1. (*a*): Normalized membrane potential difference, $V/V_c(P=0)$ as a function of the normalized change in membrane thickness $\Delta\delta/\delta_o = 1 - \delta/\delta_o$ at different values of the external pressure (*see* Eqs. (5) and (8)). Y_m and ε are assumed to be independent of the actual membrane thickness. The curves are calculated for $V_c(P=0)=1$ V and $Y_m^o=4.5$ M Pa (for the calculation of Y_m^o *see* Eq. (8)). The precompression of the membrane, increasing from 0 to 30 MPa, is indicated by the arrow. The maximum corresponds to breakdown by definition [5]. (*b*): The maxima of the equilibrium curves in *a*, corresponding to the normalized breakdown voltages $V_c/V_c(P=0)$, are plotted against the external pressure, P (semi-logarithmic plot). The value of the critical pressure, P_c, at which the intrinsic membrane potential is sufficiently high to induce a mechanical breakdown, can be taken from this curve. With $V_c/V_c(P=0)=0.013$, i.e., for the membrane potential $|V_m|=13$ mV and the dielectric breakdown voltage $V_c(P=0)=1$ V (*see* text), a critical pressure of $P_c=20$ MPa is obtained, indicated by the arrow. *Inset:* The normalized breakdown voltage $V_c/V_c(P=0)$ is plotted against pressure in the low pressure range (0–0.5 MPa), which can be realized experimentally in breakdown measurements with giant algal cells in which the cell turgor can be manipulated [28]. (*c*): Normalized membrane potential difference $V/V_c(P=0)$ as a function of the normalized change in membrane thickness $\Delta\delta/\delta_o$ as described in *a* but for $Y_m = Y_m^o\left(1+\ln\frac{\delta_o}{\delta}\right)$ with $Y_m^o=3.5$ M Pa (*see* text and Fig. 2). Precompression up to 100 M Pa. (*d*): The maxima of the equilibrium curves in *c* are plotted against pressure as described in *b*, yielding a critical pressure of $P_c=65$ MPa, when using the data for V_m *and* V_c as in *b*. *Inset:* Normalized breakdown voltage $V_c/V_c(P=0)$ as a function of pressure in the low-pressure range as described for the inset in *b*

ing pressure is always predominantly determined by Y_m^o (*see also* Eq. (7)). Thus, it is justified to assume that ε is constant. The assumption of Y_m^o being pressure-dependent means that the membrane material is not linearly elastic but shows nonlinear elastic behavior. It may also be viscoelastic. The viscous part of the behavior may be due to the viscous behavior of the interior lipid molecule tails. If this conclusion is true, a dependence of the electrical breakdown voltage on the duration of the applied external field should be expected if the mechanism of breakdown is interpreted in terms of the electromechanical model.

A dependence of breakdown voltage, V_c, and, in

turn, of the compressive eleastic modulus, Y_m^o, on the duration of the applied electric field pulse could indeed be demonstrated recently with cells of *Valonia utricularis* and artificial bilayers made up of oxidized cholesterol (U. Zimmermann & R. Benz, R. Benz & U. Zimmermann, *in press*). The breakdown voltage increases by a factor of 2 and more below a pulse length of about 10 µsec. Above a pulse length of 10 to 100 µsec a constant value of the breakdown voltage seems to be reached asymptotically. It is notable that towards very short pulse lengths (below about 0.8 µsec) the breakdown voltage, at least, of bilayers did not increase further but reached a constant value.

These findings, together with results concerning the pressure dependence of the elastic moduli of cell walls [26, 30] support the assumption that Y_m^o is not constant over the whole compression range. The exact relationship between Y_m and the membrane thickness, δ, is not known. However, it seems reasonable to assume a logarithmic relationship as

$$Y_m = Y_m^o \left(1 + \ln \frac{\delta_o}{\delta}\right) \tag{11}$$

where Y_m is the elastic modulus at a given membrane thickness, δ. Equation (11) represents the simplest reasonable function between Y_m and δ yielding an analytical solution.

With Eq. (11) we obtain the modified Eqs. (12), (13), and (14), in direct analogy to the derivation of Eqs. (4), (7), and (8), respectively, for Y_m being pressure independent.

$$\frac{\varepsilon \cdot \varepsilon_o \cdot V^2}{2\delta^2} + Y_m^o [(1 + \ln \delta_o) \ln \frac{\delta}{\delta_o} - \frac{1}{2}(\ln \delta)^2 + \frac{1}{2}(\ln \delta_o)^2] + P = 0 \tag{12}$$

and

$$V_c(P) = V_c(P=0) \cdot 1.03 [(1+P') e^{(1-P')}]^{1/2} \tag{13}$$

whereby

$$P' = \left(5 + \frac{8P}{Y_m^o}\right)^{1/2}$$

and

$$V_c(P=0) = \left(\frac{0.47 \cdot Y_m^o}{\varepsilon \cdot \varepsilon_o}\right)^{1/2} \cdot \delta_o. \tag{14}$$

The modified function $V(1-\delta/\delta_o)/V_c(P=0)$ is plotted in Fig. 1*c* for increasing pressure (indicated by the arrow). ε^o and δ_o were chosen to be 3 and 4 nm, respectively. Y_m^o was calculated from Eq. (14) to be 3.5 MPa using $V_c(P=0) = 1$ V.

It is evident from a comparison of the insets in Fig. 1*b* and *d* that in the low pressure range (up to 0.5 M Pa) the breakdown voltage should depend linearly on the pressure, irrespective of whether Y_m is assumed to be independent or dependent on δ. The assumption that Y_m depends on δ is therefore not in contradiction to the experimental finding in cells of *Valonia utricularis* [28]. The expected linear relationship between breakdown voltage and pressure at low pressures is also immediately obvious from Eq. (13) if the following approximations are made:

With

$$\left(5 + \frac{8P}{Y_m^o}\right)^{1/2} \sim 5^{1/2} + \frac{4}{5^{1/2}} \cdot \frac{P}{Y_m^o} \quad \text{for } P/Y_m^o \ll 1 \tag{15}$$

we obtain

$$V_c(P) = V_c(P=0) \cdot 0.556 \left(\frac{1.81 + P/Y_m^o}{0.56 + P/Y_m^o}\right)^{1/2}. \tag{16}$$

If we further assume that P/Y_m^o can be neglected in the nominator in the root, it follows that

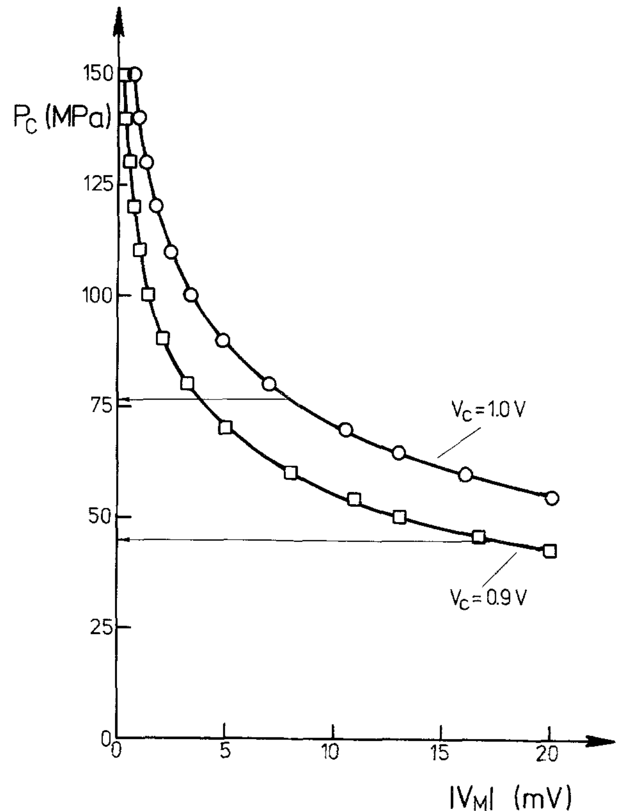

Fig. 2. The absolute value of the critical pressure, P_c, in dependence on the assumed membrane potential, $|V_m|$. The curves were calculated according to Eq. (16) for two different values of the breakdown voltage, $V_c(P=0)$, 1 and 0.9 V. The range of the P_c-value, indicated by the two horizontal lines, reflects the most likely values for P_c (*see* text)

$$V_c(P) \sim V_c(P=0)\left(1-0.895\frac{P}{Y_m^o}\right). \tag{17}$$

Towards higher pressures the breakdown voltage decreases at a much lower rate, if viscoelastic behavior is assumed. The critical pressure that is indicated by the arrow at $|V_m|/V_c$ $(P=0)$ in Fig. 1*d* can be numerically calculated from Eq. (13) to be $P_c=65$ MPa with $|V_m|=13$ mV, $V_c(P=0)=1$ V, and $Y_m^o=3.5$ MPa.

The calculation of the absolute value of the critical pressure depends on the values taken for the absolute value of the intrinsic membrane potential difference, $|V_m|$. There is evidence from other biological objects that in both the low and high pressure range the membrane potential is pressure-dependent [17, 27, 35, 36]. Since breakdown is a very rapid event [1, 4], we can expect that small changes in the membrane potential with pressure will not occur. In addition, large changes in the membrane potential of erythrocytes are not very likely, since the potential is controlled by the passive chloride distribution which, in turn, is unaltered during pressure application below the critical pressure.

Nevertheless, in order to get insight into the expected range of the critical pressure value, according to Eq. (13) we calculated P_c as a function of the membrane potential (Fig. 2). The calculations were made both for a critical breakdown voltage of 1 and of 0.9 V because very recent experiments (*unpublished data*) using long orifices in the hydrodynamically focusing Coulter Counter showed that the breakdown voltage of erythrocytes seems to be slightly smaller than 1 V due to a pulse-length dependence. The two horizontal lines in Fig. 2 indicate the expected range for the values of the critical pressure if we allow changes in the membrane potential of ± 5 mV.

Materials and Methods

Human blood was obtained from apparently healthy donors and diluted with ACD buffer (1:1). The blood was stored at 4°C for not more than 24 hr. Every 4 hr an aliquot was taken, centrifuged, and the buffy leucocyte layer carefully removed. For pressure application the cells were washed twice with a solution containing (in mmol/liter): 145 NaCl, 5 $MgCl_2$, 5 Tris HCl, 10 glucose. The pH was adjusted to 7.4. The cells were resuspended in the same solution at a suspension density of 1:14 (packed cells to solution). The cells were allowed to equilibrate for 10 min at 22 °C. For each experimental run three aliquots of 4 ml were taken from the suspension and subjected to pressure at 22 °C, whereas for control two aliquots were exposed to atmospheric pressure in a water bath at 22 °C. Pressures between 20 and 100 MPa were applied for 10, 20 or 40 min. The time to reach a given pressure varied between 10 to 30 sec, depending on the magnitude of the applied pressure. Five min after decompression the samples were centrifuged at 10,000 × g for 20 min at 4 °C. The supernatant was carefully collected, the sediment was lysed with distilled water and diluted to a volume of 200 ml. The concentrations of potassium both in the supernatant and the sediment were determined by flame photometry. The values for the pressure-induced potassium release were corrected for the potassium loss observed in the control experiments under identical conditions except for the pressure application. The efflux values per unit time are referred to the amount of potassium released by complete osmotic lysis of the same number of cells in distilled water.

The pressure device used in these experiments was developed by Péqueux and the technical details are described elsewhere [18, 19]. Briefly, the hyperbaric chamber (Fig. 3) consists of a steel cylinder with an inner plexiglass vessel, filled with the same solution as used for the cell suspension. The cylinder is open at the top to insert 4 tubes containing the cell suspensions. Silicone oil is layered on top of the surface of the suspensions in the tubes and on the surrounding solution in the plexiglass vessel. The chamber can be closed pressure tight by a steel screw cap and the sealing is achieved by means of a rubber o-ring to which a steel disc is pressed. Silicone oil (Dow Corning Silicone 200 Fluid 1 cs.) is used to transmit the hydrostatic pressure to the sample in the centrifuge tube placed within the chamber. The value of the hydrostatic pressure is determined by means of an oil-filled high-pressure manometer with a steel pipe spring (not shown). The

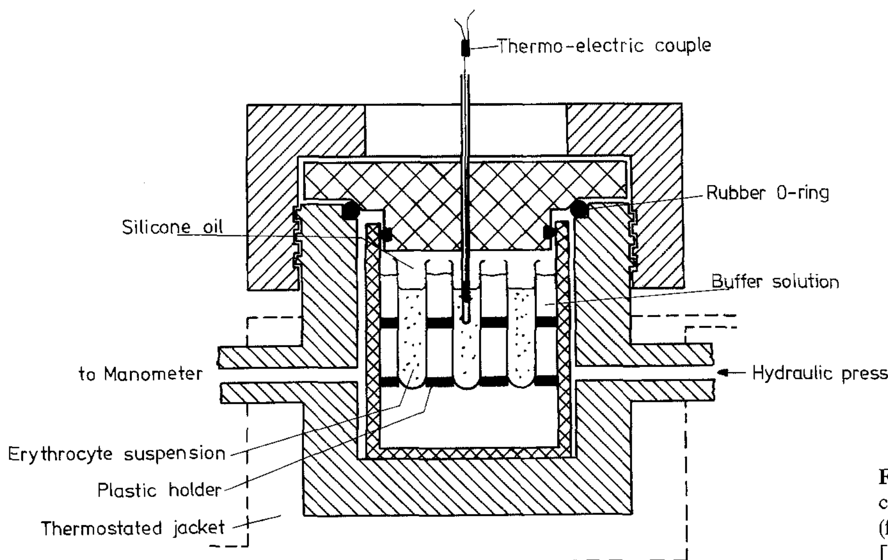

Fig. 3. Schematic diagram of the hyperbaric chamber showing the principle procedure (for explanation and for technical details, [18, 19]; *see also* text)

temperature of the chamber is controlled by a thermostated jacket and measured by means of a thermo-couple introduced into the tubes (*see* below).

Results

A typical experimental run is shown in Fig. 4. As indicated, the relative amount of potassium released from the cell during the application of pressure is almost linearly dependent on time over the whole pressure range (up to 100 MPa). The values are corrected for the potassium release observed in control experiments in which the cell suspension was kept at 22 °C without pressure application. The measurements were performed on erythrocytes obtained from the same blood. Experiments on blood samples taken from different donors, or on different days from the same donor, showed that the potassium loss from the cells in response to pressure may vary too much in order to evaluate accurately that pressure range at which the potassium release is increased significantly. Thus, it was attempted to measure the kinetics of the net potassium efflux at different pressures on the same blood sample although this experimental procedure requires more than 12 hr due to the limited capacity of the hyperbaric chamber. In order to exclude changes in cellular parameters during the storage of the blood and pressure treatment, the mean volume of the erythrocyte population in each sample was measured before and after pressure application using a hydrodynamically focusing Coulter Counter [34]. Independent of the magnitude of pressure and of the storage time of the erythrocytes suspended in ACD buffer, no change in cell volume and in the shape of the normally distributed erythrocyte size distribution could be detected, despite the high resolution of the hydrodynamically focusing Coulter Counter. Measurements of the size distribution at different electric field strengths in the orifice of the Coulter Counter were used to determine the electrical

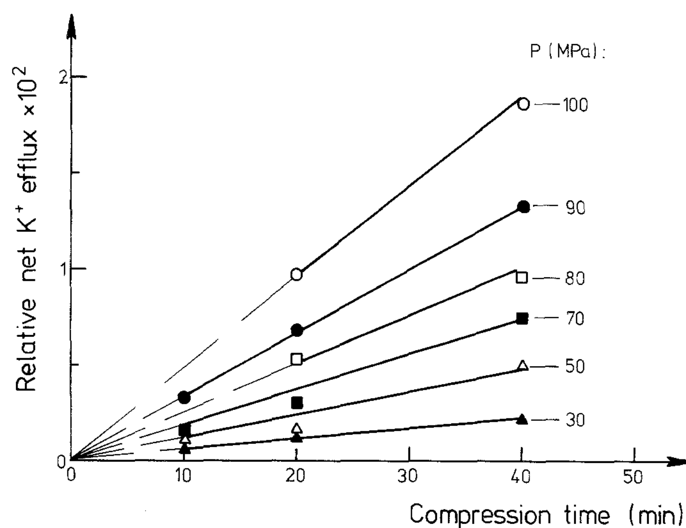

Fig. 4. The pressure-induced net potassium efflux from human erythrocytes as a function of time at a given hydrostatic pressure. The experiments were performed on the same blood sample. The values for the pressure-induced potassium release are corrected for the potassium loss observed in control experiments under identical conditions but with no pressure application. The efflux values are referred to the amount of potassium released by complete osmotic lysis of the same number of cells in distilled water. The time required to establish a certain pressure varies between 10 to 30 sec, depending on the magnitude of the applied pressures. The final pressure values are maintained for 10, 20 or 40 min. Five min after decompression the samples are centrifuged at 10,000 × *g* and the amount of potassium in the supernatant and in the sediment is determined by flame photometry. The experiments were carried out at 22 °C

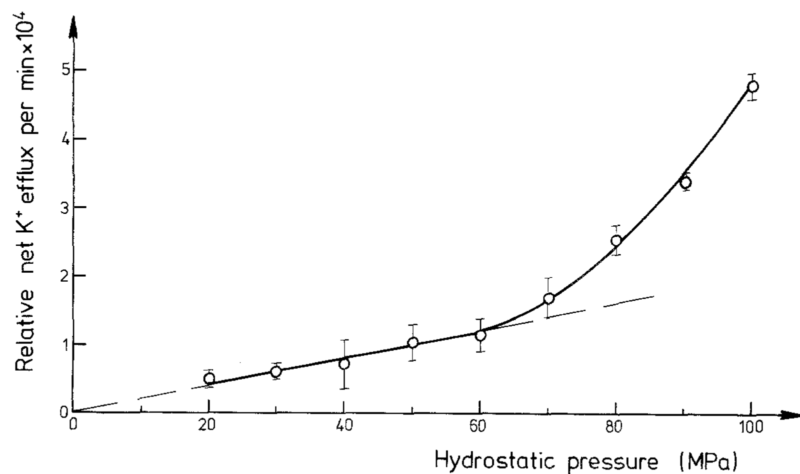

Fig. 5. The pressure-induced net potassium efflux per min from human erythrocytes is plotted as a function of hydrostatic pressure. Data were taken from Fig. 4 using the values for 20 and 40 min pressure application. The bars indicate the error in the determination of the potassium release, that has been calculated by the method of error propagation

breakdown voltage of the cell membranes [32]. The breakdown voltage was used as a second criterion for the membrane integrity after pressure application. As shown previously [21, 33], the breakdown voltage should change if changes in the membrane structure and composition occur. The electrical breakdown voltage was calculated from the underestimation of the size distribution beyond a certain critical external field strength; from this underestimation in size, conclusions concerning the internal conductivity can also be drawn [31]. These measurements performed on each erythrocyte sample subjected to pressure did not show any significant change in breakdown voltage and internal conductivity. Thus, we can conclude that the storage of the blood for several hours has no effect on the cellular parameters and that changes in volume which may occur during the pressure application are completely reversible.

In another set of experiments, we examined whether the potassium release at a given pressure and at a given time interval is also reversible. To this end, human erythrocytes were exposed to several consecutive pressure applications (100 MPa for 20 min at 22 °C). Between each pressure application the cells were kept at atmospheric pressure for 20 min. The potassium release per unit time for each subsequent pressure application was indeed the same.

It should also be noted that in the presence of ouabain (0.1 mmol/liter) no significant change in the potassium release from the cells could be detected, thus excluding the possibility that the active potassium-sodium transport system known to be pressure-dependent [18, 20] is involved in the observed potassium net efflux. A serious problem when studying membrane transport under high pressure is the possibility of temperature changes during compression and decompression, particularly in the light of the very small amounts of potassium released from the cells. Therefore, the temperature changes in the tubes containing the suspension and in the silicone oil layer on the top of the suspension were measured in control experiments very carefully by means of thermo-electric couples introduced both into the suspension and the silicone oil. In order to avoid a misrecording of the thermo-electric couple under pressure, the couple was inserted into steel tubes of 1.5 mm diameter filled with mercury to improve the heating transition between steel wall and thermo-electric couple, and the steel tubes were connected with the atmospheric pressure. Whereas a temperature increase in the oil layer of about 15 °C at maximum pressure (100 MPa) and maximum duration (40 min) was measured, only an increase of 2 °C could be recorded in the solution phase. This finding is in agreement with results reported by Distèche [8]. Thus, temperature changes cannot account for the observed effects.

In Fig. 5 the relative potassium release from the erythrocytes per unit time is plotted against pressure. Data were taken from Fig. 4. In Fig. 6 the same plot is given presenting the average values from 5 independent experiments. Both figures demonstrate that the net potassium efflux increases almost linearly with the pressure up to about 60 to 70 MPa. Beyond this pressure range a significant rise in the potassium efflux occurs. This increase in the net potassium efflux is considerably higher than the experimental error indicated by the vertical bars.

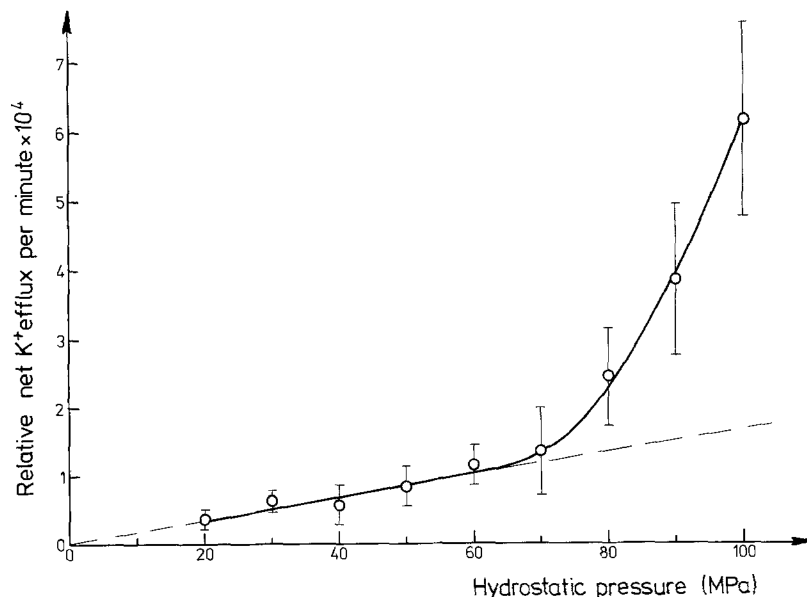

Fig. 6. Same plot as in Fig. 5. Data points are the average of five independent measurements performed on erythrocyte samples taken from different donors. The experimental conditions are the same as described in Fig. 4. The bars indicate the standard deviation from the mean. Despite the larger fluctuations in the potassium release at a given pressure of about 60 to 70 MPa, a pronounced increase in potassium release from the erythrocytes is observed

Discussion

The existence of a critical pressure, at which the membrane becomes compressed to the extent that the intrinsic electrical field is sufficiently high to induce mechanical breakdown of the cell membrane, is a second criterion for the validity of the basic assumptions of the electro-mechanical model, although it has to be pointed out that the model was primarily conceptualized for low pressure ranges [26]. The observation of a distinct permeability increase for potassium at pressures higher than 60 to 70 MPa for human erythrocytes is in the predicted range and, therefore, a good argument in favor for the model. The pressure-induced potassium release from the erythrocytes is reversible, i.e., the original membrane impermeability is restored after pressure release. This conclusion is also supported by Coulter Counter measurements, by which no changes in cell volume, internal conductivity, or electrical breakdown voltage could be detected. Repeated pressure application to the same cell suspension induces a reproducible permeability increase. These reversibility criteria, which have also been proved in electrical breakdown experiments with a variety of living cells using quite different techniques, also favor an electro-mechanical interpretation of the experimental results. Reversible changes in shape of the erythrocytes under pressure cannot be excluded with certainty (*see also* [10]). On the other hand, a pressure-induced transition from the biconcave shape to a more spherical one during pressure application cannot be responsible for the increase in the net potassium efflux, because in this case a decrease in the potassium efflux is expected due to the decrease in the membrane area. Comparing the sharp transition of the membrane from the low conductance state into the high one observed at a critical field strength in the orifice of a hydrodynamically focusing Coulter Counter [32], the more exponential increase in the potassium efflux in a pressure interval of about 10 MPa seems to be contradictory to the interpretation of this effect in terms of a reversible mechanical breakdown of the membrane at a definite critical pressure.

However, experimental differences in principle exist between "electrical" and "mechanical" breakdown experiments. Consecutively to an "electrical" breakdown event, high electric current densities occur in the local breakdown areas, which may cause local heating effects and thereby phase transitions or melting of the surrounding lipid phase [1, 31]. Such secondary processes lead to an increase in membrane permeability. Thus, the electrical field strength required for dielectric breakdown of microscopic cells in the Coulter Counter is similar to the critical field strength required in the discharge chamber [31] to induce a measurable ion exchange between the cell interior and the external medium (provided the differences in length and shape of the applied field pulse are taken into account). The ion equilibration should not be disturbed by the onset of resealing after field application, because even at higher temperatures the restoration of the original ion permeability is still a relatively slow process for erythrocytes, lasting for several minutes.

In the "mechanical" breakdown experiments, however, the primary permeability change is not amplified by effects of high current densities. Therefore, the change in membrane permeability due to a mechanical breakdown is rather low, and higher pressure values have to be applied in order to enlarge the breakdown areas within the membrane and, in turn, induce a measurable ion exchange. In addition, the resealing time, being a function of the size of the breakdown areas in the membrane, should be much shorter in the pressure-induced breakdown experiment than that after the application of an electric field pulse.

As outlined above, it is somewhat difficult to calculate theoretically the precise value of the critical pressure due to the scarcity of data for the electrical and mechanical membrane parameters. In general, it has to be questioned how far a macroscopic model, in which the membrane is considered as a solid body, can explain biological processes at a molecular level. The intrinsic problems involved in such considerations are quite obvious if the critical thickness to which the membrane has to be compressed is calculated for both electrical and mechanical breakdown. In the electrical breakdown the membrane thickness has to be changed by about 40% [5], in the mechanical one by more than 90% (*see also* Fig. 1*a* and *c*), which seems to be unrealistic if they represent volumetric compressions. However, if the membrane area simultaneously extends so the volume does not have to change as much as the thickness, then these large thickness compressions could be realistic.

Furthermore, changes in membrane thickness can also be brought about by injection of membrane molecules into the adjacent bulk phases in response to pressure, resulting in the formation of conducting channels. Such processes are equivalent to compression of membrane parts from a phenomenological standpoint and could formally be described by the same equations. Furthermore, in the derivation of the critical pressure it was assumed that the membrane potential linearly drops across the membrane. This is certainly not true, particularly if a membrane bearing fixed charges is considered in which high electric field strength can locally exist within the membrane. It is

therefore quite conceivable that mechanical breakdown occurs at a much smaller degree of compression, as also discussed for the electrical breakdown in lipid bilayers [1]. These considerations show that a macroscopic model should not be overinterpreted. A macroscopic model is a description and explanation of some aspects of biological processes, which is rather useful if new correlations between parameters of a complex cellular system are predicted. Despite these objections to the theory delineated above, it has to be noted that a very good agreement exists between the experimentally determined value for the critical pressure range and the theoretically predicted value, particularly if it is assumed that Y_m is dependent on the actual membrane thickness, δ, and if the values for the intrinsic membrane potential, V_m, and for the breakdown voltage, V_c, reported in the literature are used.

On the other hand, we cannot completely exclude alternative mechanisms and explanations for the observed high-pressure effect. For example, a pressure-induced phase transition in the lipid bilayer of the membrane could be responsible for the permeability change [11]. Recently, experiments by Wattiaux-de Coninck et al. [25] have shown that high hydrostatic pressure (above 75 MPa) can cause reversible changes in the membrane structure of rat-liver mitochondria, triggering a release of malate dehydrogenase, an enzyme located within the matrix of the organelle, into the external medium. The authors interpret their results in terms of a pressure-induced liquid-crystal gel phase transition, which is accompanied by a decrease in volume.

Murphy and Libby [16] studied the incorporation of radioactively labeled phosphate into the rabbit erythrocyte under high hydrostatic pressure. They report a very sharp change in incorporation at a pressure of about 60 MPa. On the one hand, this finding would be consistent with the predictions of the electro-mechanical model; on the other hand, it cannot be excluded that a phase transition in the lipids is responsible for this observation, as discussed by the authors. It is notable, that the decrease in uptake of phosphate at 60 MPa was reversible. It is also conceivable that some of the protein units normally do not reach completely through the cell membrane but might do so if the membrane thickness is reduced as a consequence of an increase in either the electric field or the pressure. Thus, at certain critical values for the applied membrane potential or pressure these units would then form a conducting channel through the membrane [5].

Furthermore, the different compressibilities of water, lipids, and proteins (e.g., the volume of water decreases by about 3% between 0 and 70 MPa) may cause tensions along the membrane surface, which could then result in the observed permeability increase. For further possible mechanisms for electric field and pressure effects involved within membranes, the interested reader is referred to the recent review by Zimmermann [26].

Although we have not yet completely disproved every possible explanation alternative to the "reversible mechanical" breakdown, we can say at the present that the results so-far obtained from electrical-breakdown experiments and from pressure effects on membrane transport [26] are consistent with the electro-mechanical model.

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