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THE EFFECT OF PRESSURE ON THE ELECTRICAL BREAKDOWN IN THE MEMBRANES OF *VALONIA UTRICULARIS*

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Summary

The interpretation of electrical breakdown in terms of electro-mechanical instabilities, predicts that the breakdown potential should decrease with increasing cell turgor pressure.

Experiments were conducted to test this hypothesis on cells of *Valonia utricularis* over a turgor pressure range of $0.5 \cdot 10^5$—$5.0 \cdot 10^5$ N/m^2. Electrical break down was measured using intracellular electrodes and 500 μs current pulses. The pressure was monitored by an intracellular micropipette pressure transducer. The results obtained show a linear decrease in the critical breakdown potential with pressure. The effective compressive modulus of the cell membrane, γ, is calculated from the slope of this line to $69 \pm 10 \cdot 10^5$ N/m^2 (average value of seven measurements). This is consistent with the theoretical prediction of the electromechanical model using our previously determined values of the elastic modulus of the membrane.

A theoretical analysis is given of the effects of pressure on the breakdown. This includes also considerations of the indirect effect of pressure on the membrane via stretching of the cell wall with a possible coupling of such strains to the cell membrane. The results and analysis presented allow us to conclude on the basis of the experimentally determined breakdown P.D. of 959 mV that the region of membrane where electrical breakdown occurs is a dielectric with one of the following combinations of parameters: (A) a thickness $\delta = 7$—9 nm with a dielectric constant $\epsilon = >10$, e.g. a hydrated protein spanning the whole membrane. (B) $\delta = 4$—5 nm with $\epsilon = 3$—8, e.g. a lipoprotein of lipid bilayer dimensions. (C) $\delta \approx 2$ nm with $\epsilon = 2$—3, e.g. a half lipid bilayer.

If we assume that the breakdown P.D. of the tonoplast and plasmalemma are identical, that is 480 mV, then there is only one reasonable choice for the mem-

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brane thickness and the dielectric constant: δ = 2 nm, ϵ = 3—8, e.g. a (lipo-) proteinaceous module facing a half lipid bilayer.

Introduction

In living cells electrical breakdown occurs when the cell membrane is taken rapidly, say in microseconds, to a critical voltage. The latter is of the order of 1 V for most cells examined so far [1—3]. A voltage of this magnitude can be built up across the cell membrane either directly with the giant cells of algae like those of *Valonia utricularis* using intra- and extracellular electrodes [3—5] or indirectly with microscopic cells by applying sufficiently large external fields to a suspension of such cells [1,2,6,7]. It is possible to interpret the electrical breakdown in terms of an electro-mechanical collapse of the cell membrane which can occur at sufficiently large membrane potentials [1—3]. In this model the membrane is considered to be a compressible structure in which an equilibrium exists between the electrical compressive forces due to the electric field present in the membrane and the mechanical forces (elastic restoring forces and external pressure). It can be shown that for sufficiently large compressions (decrease in membrane thickness) the compressive force due to the electric field in the membrane can increase more rapidly with decreasing membrane thickness than the elastic restoring forces. This leads to a catastrophic collapse (perhaps only locally) of the membrane, which then also results in the electrical breakdown phenomenon. Previously we have shown [3] that the electrical characteristics (for very short current pulses) leading up to breakdown can be accurately accounted for by electro-mechanical compression of the membrane. In this communication we extend the theoretical analysis and present experimental results obtained with cells of *V. ultricularis* of the effect of external turgor pressure * on the critical potential required for breakdown. The dependence of the breakdown potential on the turgor pressure expected from the analysis of electro-mechanical forces is also a crucial factor in any consideration of other possible mechanisms for the breakdown.

Materials and Methods

The cells used in the present experiments were the giant cells of the marine algae *V. ultricularis* which were originally collected from the Mediterranean near Naples and were cultivated in the laboratory in sea water.

The electro-physiological methods and apparatus used in the experiments were the same as those described previously by us [3,4] for the studies on electrical breakdown in these cells, with the exception of the intracellular pressure probe (see below).

Current pulses of 500 μs duration were injected into the cell via a platinum/iridium microwire introduced into the cell through a micropipette with a tip diameter of $\approx$5 μm which was inserted longitudinally into the cell (see Fig. 1).

* In this communication we shall express the pressure in units of 10^5 N/m^2. Note that 10^5 N/m^2 = 1 bar.

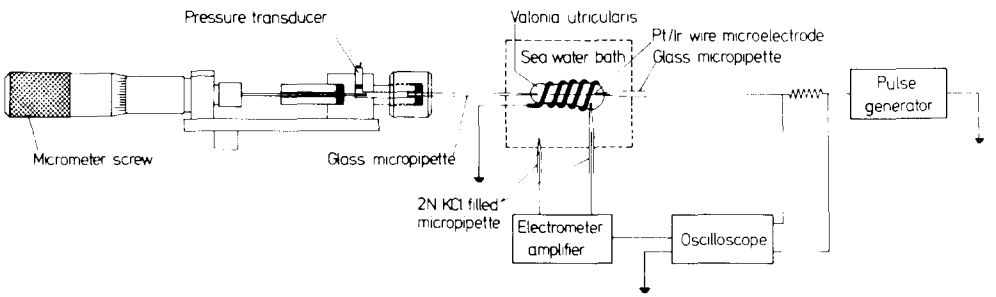

Fig. 1. The experimental set-up for measurements of the pressure dependence of electrical breakdown in cells of *V. utricularis*. The set-up is identical to that described previously [3] except for the addition of the micropipette probe. For details of the measurements see the text.

The membrane potential difference (P.D.) was monitored with the aid of intra- and extracellular glass micropipettes filled with 2 M KCl. The plexiglass cell holder and the flowing sea water bathing the cell were temperature controlled. All experiments were done at 16 ± 0.2° C. The turgor pressure in the cell was monitored using the intracellular pressure probe described previously by Zimmermann and Steudle [8,9]; (see also ref. 10). This probe was inserted longitudinally into the cell from the end opposite to where the current injecting electrode was inserted (see also Fig. 1). The turgor pressure of the cell was changed by adding either distilled water or a concentrated NaCl solution to the external sea water flowing over the cell; the osmolarity being adjusted gradually with the aid of a gradient mixer (LKB Instrument Co.). The pressure inside the cell was so adjusted in the range of $0.5 \cdot 10^5$—$5.0 \cdot 10^5$ N/m^2. At each pressure the breakdown P.D. was determined by injecting a series of current pulses of increasing magnitude into the cell; the current pulses and the voltage responses were displayed on a storage oscilloscope. Breakdown can be clearly discerned by the discontinuous increase in the pulsed current when the critical breakdown P.D. is reached. Further, the membrane P.D. also does not generally increase beyond this critical value upon injection of current pulses of greater magnitude (see also Figs. 2 and 3 of Coster and Zimmerman [3]). There are intrinsic difficulties in calculating the correct value of the breakdown P.D. from the oscilloscope voltage tracings. The shape of the voltage pulses is not rectangular, particularly in the supercritical range (beyond the critical breakdown P.D.). In the experiments reported here, the maximum value of each voltage pulse was taken for evaluating graphically the critical breakdown P.D. as described by Coster and Zimmermann [3]. It should be further noted that the breakdown event is related to two membranes, that is the tonoplast and the plasmalemma. Thus the breakdown P.D. comprises both the breakdown P.D. of the tonoplast and of the plasmalemma. The temporal built up of the potential across both membranes depends in a complex manner on the resistance, capacitance and resting membrane P.D. of each membrane [11]. In addition the recorded membrane P.D. is delayed by the rise time of the potential measuring system resulting in an underestimation of the breakdown P.D. At the temperature of 16° C no splitting of the membrane P.D. signal into two peaks was observed over the whole pressure range. This finding leads us to conclude that the breakdown P.D. of both mem-

branes is reached simultaneously for all pressures used.

Note that this conclusion is derived from the evaluation of the data at hand and that future studies, which are able to increase the limits of resolution, may find a difference in the breakdown P.D. times at this temperature (see also ref. 11). On the basis of simultaneous breakdown, one may assume that the breakdown P.D. of at least one membrane is half that determined experimentally by vacuolar electrode. The electrical breakdown does not lead to global damage of the cell or of the membrane (the membrane reseals rapidly and the process can be repeated many times). A slow deterioration of the cell, however, does occur over a period of, sometimes, 10 h when insufficient time (<20 min) was allowed between measuring sequences. The time between current pulses in each such sequence was about 10 s. This type of deterioration may be due to the formation of toxic electrolysis products. For instance some simulation experiments we have conducted have revealed that significant amounts of hypochlorite are formed when the pulse frequency and amplitude reach sufficiently high values.

Control experiments were also performed to determine any possible effects of turgor pressure on the tip potentials of the intracellular potential-measuring electrode. This was done in separate experiments in which one electrode with a large tip diameter (≈ 20 μm) (which was also sealed at the other end) and one electrode with a small tip diameter (≈ 5 μm) as used in the breakdown experiments were inserted into cells together with the pressure probe. The effect of pressure on the tip potential of the fine electrode was found to be <2 mV over the investigated pressure range.

The pressure probe inserted into the cell is fitted with a micrometer-driven plunger by which the volume and hence the pressure inside the cell may be varied. In this way it is also possible to determine, directly, the volumetric elastic modulus of the cell wall [9,10,12,13] which is defined by the following equation [14]:

$$Y_w = \frac{\Delta P}{\Delta v} \cdot v, \tag{1}$$

where Y_w is the cell volumetric elastic modulus of the cell wall. The subscript "w" here refers to the cell wall. (We shall use a subscript "m" to denote the cell membrane). Usually the symbol "ϵ" has been used for the volumetric elastic modulus but in this paper we reserve the latter symbol for the dielectric constant. ΔP is the change in cell (turgor) pressure. Δv is the corresponding change in cell volume v. Usually Y_w is a function of pressure and cell volume [8,9,12,13,15].

Electro-mechanical stresses

(a) Transverse stresses. The presence of an electric field in a membrane creates a compressive stress, P_e. This stress together with the compressive stress due to the external pressure, P, (e.g. the turgor pressure) is counterbalanced by the elastic restoring force, P_m, generated by strains induced in the membrane. Thus at equilibrium

$$P + P_e + P_m = 0 \tag{2}$$

We will consider the effect of each of these forces on the thickness of the membrane. Further, we will below also consider the effects of longitudinal strains. Therefore, we shall denote the mechanical transverse stress by the subscript "t", the electric stress by the subscript "e". When no strains are present at all, i.e. also no longitudinal strains, the thickness is denoted by δ_o (see also Glossary of symbols). The stress due to the electric field in a homogeneous membrane is given by (assuming a constant field)

$$P_e = \frac{\epsilon\epsilon_0 V^2}{2\delta^2} \quad , \tag{3}$$

where ϵ is the dielectric constant of the membrane material, ϵ_o, the electric permittivity of free space, V, the membrane potential difference and δ, the stressed membrane thickness. The elastic restoring force P_m for an ideal elastic membrane is given by:

$$P_m = Y_m \ln \frac{\delta}{\delta_{t=0,\, e=0}} \tag{4}$$

where Y_m is the elastic modulus * for compression of the membrane in a transverse direction, $\delta_{t=0,\, e=0}$ is the membrane thickness in the absence of both mechanical and electrical transverse stress. When $V = 0$ and hence the electric stress $P_e = 0$ the turgor pressure is (from Eqns. 2 and 4) given by

$$P = -P_m(V = 0) = -Y_m \ln \frac{\delta_{e=0}}{\delta_{e=0,\, t=0}} \tag{5}$$

where $\delta_{e=0}$ is the thickness of the membrane when $V = 0$ (and $P_e = 0$) but we consider here the case when other strains may be present. When a P.D. is present substitution of Eqns. 3, 4 and 5 in the equilibrium condition Eqn. 2 then yields

$$\frac{\epsilon\epsilon_0 V^2}{2\delta^2} = Y_m \ln \frac{\delta_{e=0}}{\delta} \tag{6}$$

When the compression of the membrane is sufficiently large, the rate at which the electric stress (Eqn. 3) increases with the decreasing membrane thickness exceeds the rate at which the elastic restoring force (Eqn. 4) increases. At this point the membrane becomes electro-mechanically unstable and electrical breakdown will result.

The breakdown P.D. can be obtained from the instability condition

$$\frac{\delta P_e}{\partial\delta} = -\frac{\partial P_m}{\partial\delta} \tag{7}$$

At a given, constant, pressure this occurs when $V = V_c$, where from Eqn. 6, the critical breakdown P.D., is given by

$$\frac{\epsilon\epsilon_0 V_c^2}{\delta^3} = \frac{Y_m}{\delta} \tag{8}$$

* The elastic modulus is here operationally defined as the rate at which the internal restoring forces increase with a change in thickness, δ, expressed per unit thickness of the membrane. Y_m in general will be a complex quantity [16].

Eqn. 8 with the equilibrium condition Eqn. 6 then yields the following expression for the critical breakdown P.D.

$$\frac{\epsilon\epsilon_0 V_c^2}{2\delta^2}\left(1 - 2\ln\frac{\delta_{e=0}}{\delta}\right) = 0. \tag{9}$$

Substitution of Eqn. 8 back into Eqn. 9 yields

$$V_c^2 = 0.3679\,\frac{\delta_{e=0}^2 Y_m}{\epsilon\epsilon_0}\,. \tag{10}$$

In this equation $\delta_{e=0}$ is a function of pressure (Eqn. 5) and hence V_c in Eqn. 10 is also a function of P. Thus substituting for $\delta_{e=0}$ from Eqn. 5, Eqn. 10 yields the following expression for the breakdown P.D. as a function of pressure:

$$V_c = V_c(t = 0)\exp(-P/Y_m) \tag{11},$$

where

$$V_c(t = 0) = \left[\frac{0.3679\ Y_m}{\epsilon\epsilon_0}\right]^{1/2} \cdot \delta_{e=0,\ t=0}$$

(b) Longitudinal stresses. In our analysis so far we have not included any possible effects of longitudinal and concomitant transverse strains induced in the membrane due to stresses in the cell wall. The cell membrane could be coupled closely to the cell wall (mechanically, chemically or electrostatically). In this case strains induced in the cell wall due to turgor pressure will induce longitudinal strains in the cell membrane. The relative change in the area of the cell wall and the cell membrane will depend on the degree of mechanical coupling between these structures. The exact degree of this coupling is unknown. To proceed we will make the simplifying assumption here that the ratio of the strains in area of the cell membrane and cell wall will remain constant with changes in pressure. This ratio we will refer to as the degree of coupling α (the values of α will therefore be in the range of 0—1). To see what possible effect this might have on the breakdown P.D. we can make the crude approximation that for such longitudinal strains the density of material in the membrane remains constant. This would mean that the thickness of the membrane would decrease as the turgor pressure increases. There is some evidence [17] that longitudinal strains in cell membranes might indeed account for the decreased thickness of the supposed hydrophobic layer in cell membranes relative to that of an artificial bilayer. The changes in cell volume are related to changes in turgor pressure and the elastic modulus of the cell wall by the Philip equation (Eqn. 1).

To persue this argument consider a spherical cell of radius r_0 when the turgor pressure is zero and a radius r at any other pressure. The change in the thickness of the membrane, under our assumption of constant density of the membrane material, can then be obtained with the aid of the integrated Eqn. 1. Details of this are given in the Appendix A. It is shown there that

$$\delta_{e=0,\ t=0} = \delta_0 \exp\left(-\frac{2\alpha P}{3Y_w}\right) \tag{12}$$

δ_0 is the thickness when no strains at all are present. The stressed thickness in

Eqn. 10 should therefore now include the effect of the strain due to the longitudinal stress. When this is included the critical breakdown P.D. is given by

$$V_c = V_c(P = 0) \exp(-P/\gamma) \tag{13}$$

where

$$V_c(P = 0) = \left[\frac{0.3679\ Y_m}{\epsilon\epsilon_0}\right]^{1/2} \delta_0$$

and

$$\frac{1}{\gamma} = \frac{2\alpha}{3Y_w} + \frac{1}{Y_m} \tag{14}$$

Here V_c $(P = 0)$ is the breakdown P.D. when the pressure $P = 0$, at which point there is no compressive mechanical stress in the membrane and there are no longitudinal strains induced due to the stretching of the cell wall. In the investigated pressure range Y_m is of the order of $50 \cdot 10^5$ N/m^2 [3] and $Y_w \simeq 300 \cdot 10^5$ N/m^2 [15]. Even with the two extreme cases of $\alpha = 0$ and $\alpha = 1$ the expression 14 predicts a nearly linear decrease in V_c with P. The slope of the curve of V_c as a function of P yields then the complex quantity $V_c\ (P = 0)/\gamma$.

If due to some peculiarities of the cell membrane direct compression is not possible, compression by the electric field (which terminates on charges in the membrane matrix) is still possible. In this case the effect of the cell wall remains and Eqn. 13 is still applicable but with the term in Y_w predominating.

Using the intracellular pressure probe we can determine directly, for a given cell, the elastic modulus of the cell wall with the aid of Eqn. 1. In this way we could, in principle, separate out the effects of direct compression of the membrane due to the turgor and an indirect thinning due to longitudinal strains induced through the coupling to the cell wall for the case $\alpha = 1$.

Results

At each pressure the critical P.D. for electrical breakdown was determined by injecting a sequence of current pulses of increasing magnitude into the cells as described elsewhere (see also Materials and Methods). While at breakdown the electrical conductance increases sharply, the breakdown did not lead to a decrease in the turgor pressure except after prolonged experimentation on a cell.

The breakdown P.D. for various values of the turgor pressure in one cell is shown in Fig. 2. For these results the turgor pressure was adjusted cyclically to increasing and decreasing values. This particular cell originally had a pressure of $1.4 \cdot 10^5$ N/m^2 and a volume of 45 mm^3 (45 μl).

The line shown in Fig. 2 is an exponential curve fitted to the data by the method of least squares in accordance with the theoretically expected variation (Eqn. 13) from which γ_m is calculated to be $71 \cdot 10^5$ N/m^2. The correlation coefficient for the fit is 0.9. Considering the experimental difficulties involved in the simultaneous measurement of turgor pressure and the critical breakdown P.D., this result seems good evidence that the breakdown P.D. does indeed decrease with turgor pressure, as predicted by Eqn. 13.

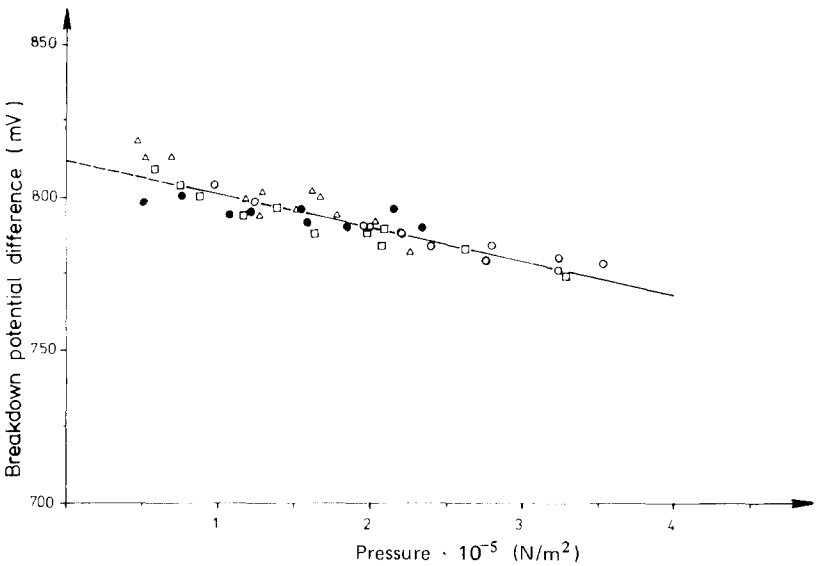

Fig. 2. A plot of the critical potential for electrical breakdown as a function of pressure in a single cell of *V. utricularis*. The pressure was varied in steps by dilution of the sea water with distilled water or by the addition of NaCl. The different symbols indicate separate experimental runs. △, □, decreasing pressure: ○, ●, increasing pressure. The line drawn is a plot of Eqn. 13 with $\gamma = 71 \cdot 10^5$ N/m^2.

Similar variations of the breakdown P.D. with turgor were obtained for all cells examined (13 cells). The experiments could not always be conducted over the whole pressure range; in many cases, for instance, the cells ruptured when the pressure was increased [8]. While all cells displayed a similar dependence of V_c on P the breakdown P.D. at $P = 0$ for different cells were different, sometimes by more than 200 mV. The average value of the breakdown P.D. V_c ($P = 0$) of seven cells (see also footnote) was 959 ± 141 mV.

In order therefore to present the cumulative data on the changes in V_c with turgor pressure obtained with different cells we had to normalize our data. For this purpose we extrapolated the breakdown P.D. data for each cell to zero pressure (V_c ($P = 0$)) and determined the ratio of $V_c(P)/V_c(P = 0)$. The cumulative data for the relative decrease in this breakdown P.D., $V_c(P)/V_c(P = 0)$ as a function of turgor pressure for seven cells is shown in Fig. 3 *. For such a normalized plot the slope of the line gives the value of $1/\overline{\gamma}$ (as can be easily shown from Eqn. 13 by expanding the exponential function). The slope of the line in Fig. 3 has a value of $1.45 \cdot 10^{-7}$ N$^{-1} \cdot$ m^2 (correlation coefficient 0.8). Hence the average value of $\overline{\gamma}$ so calculated is $69 \pm 10 \cdot 10^5$ N/m^2. It is obvious, also considering the relative decrease of the single cells in Fig. 3, that the breakdown P.D. decreases with pressure in agreement with the electro-mechanical model.

* The seven experiments were selected from the 13 measurements in the following way: The data of the relative decrease in the breakdown P.D. of each cell was fitted to a straight line by the method of least squares, the slope of each line equals $1/\gamma$. Then the confidence interval for the slope of the line was calculated using a confidence coefficient of 95%. The results for cells, where the half length of the confidence interval was larger than 50% of $1/\gamma$ were rejected.

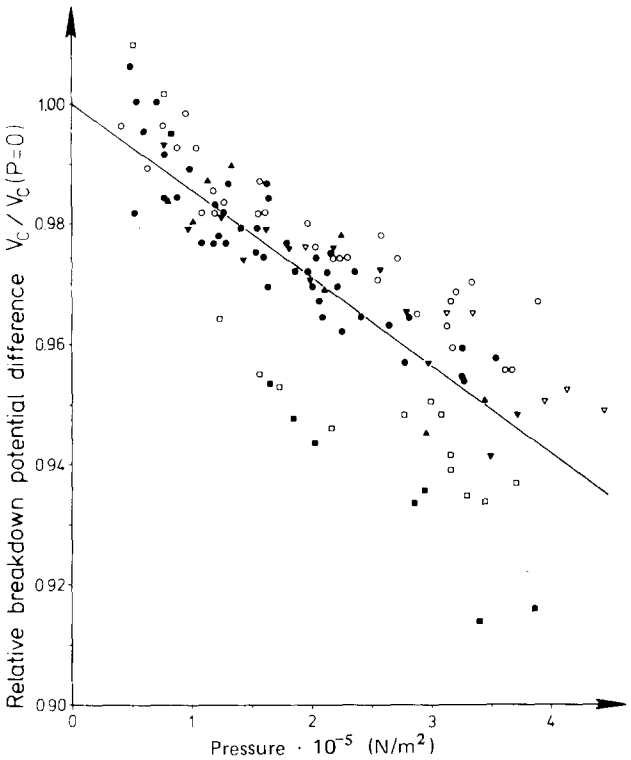

Fig. 3. Cumulative data on the changes in relative breakdown P.D. $V_0/V_c(P = 0)$ as a function of pressure for seven cells. The different symbols are referred to the seven different cells. It is obvious that for each cell the relative decrease in breakdown P.D. is a linear function of pressure. The data of all cells fitted to the straight line V_c/V_c $(P = 0) = 1 - (P/\bar{\gamma})$ (linear approximation of Eqn. 13) show a mean value of the slope $1/\bar{\gamma} = 1.45 \cdot 10^{-7}\,N^{-1} \cdot m^2$ (correlation coefficient 0.8).

The electrical breakdown to which the cells were subjected was, as pointed out previously [3,4], at constant pressure, quite reproducible in a given cell. Some long term changes in the resting membrane resistance (for small current pulses) did take place in the cells although this did not show up in the potentials required for breakdown. Two examples of the variation of the resting resistance, for 500-μs current pulses, with the number of breakdowns to which cells were subjected are shown in Fig. 4. One set of results (filled symbols) refer to the same cell to which the results in Fig. 2 refer, the other set (open symbols) to another cell. The resting membrane resistance at the beginning of the experiment agrees with the values reported previously for these cells measured with very long current pulses (20 s) [8].

It is clear from the results shown in Fig. 4 that the membrane resistance decreased markedly with increasing number of breakdowns to which the cells were subjected. It should be noted that the points shown represent measurements at various pressures and were obtained serially during the course of the measurements of the dependence of V_c on pressure. The resistance with such short pulses was apparently not dependent on the pressure (unlike the resistance measured with long (20 s) pulses) [8,12,15]. The gradual decrease in the

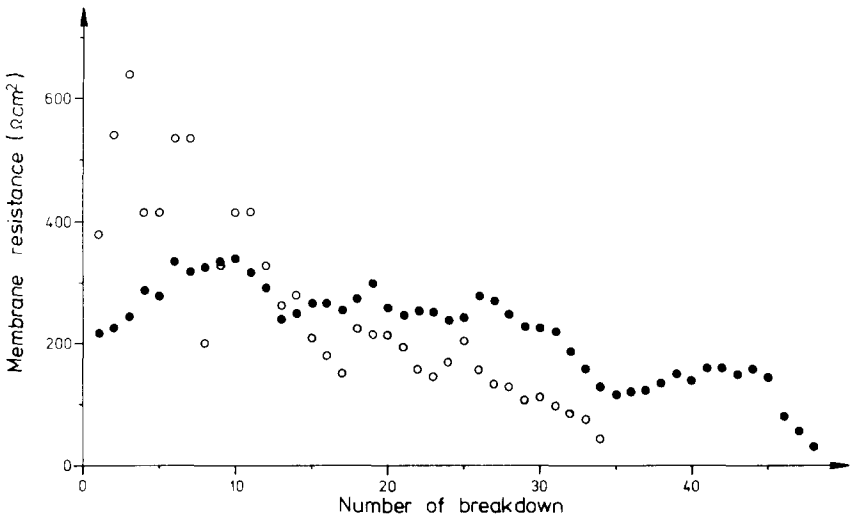

Fig. 4. The membrane resistance measured with small (subcritical) but short (500 μs) current pulses at different pressures, for two cells, indicated by the different symbols. The points (filled circles) refer to the cell to which the results in Fig. 2 are related.

resistance shown in Fig. 4 is probably not only due to damage by the electrical breakdown but is also most likely connected with increasing damage due to the changes in turgor to which the cell was subjected. At constant pressures such changes in resistance with increasing number of breakdowns were usually not observed.

The resting potential for these cells was usually +0 to +8 mV at the beginning of the experiments but sometimes the initial value was slightly negative. Following several (5—10) breakdowns the resting potential of the cells shifted to slightly more positive values (about 2 mV). Further breakdown then only led to small statistical fluctuations of the resting P.D. around this value.

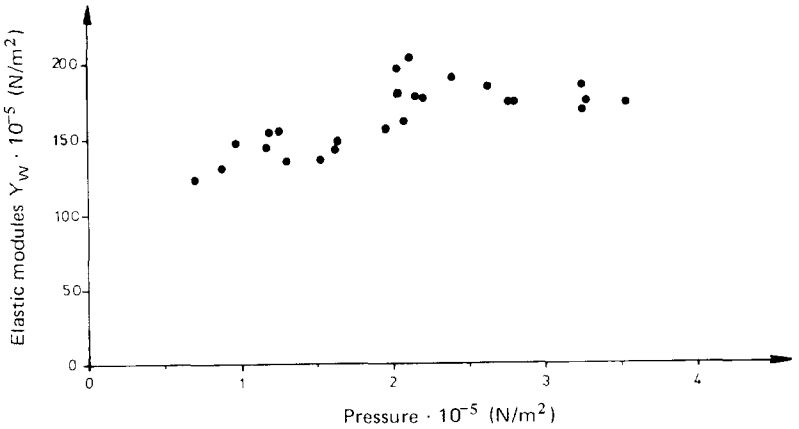

Fig. 5. The elastic modulus of the cell wall for a single cell of *V. utricularis* measured with the aid of the pressure probe. In each measurement the volume was increased stepwise with the micrometer plunger in the pressure probe which causes a small increase in pressure. The values obtained here are similar to those reported before [8,9,15] for cells of this species despite the fact that in this measurement an incompletely filled micropipette (used for the current electrode) was also in place in the cell.

As outlined in the theory the relative contribution of the elastic compressive modulus Y_m and the volumetric elastic modulus of the cell wall (Y_w) to γ can be separated since Y_w can be directly measured for a *Valonia* cell. This, however, was not done for each cell since such measurements introduce an increased risk of damaging the cell. One such result of Y_w as a function of pressure is shown in Fig. 5, for the same cell to which Fig. 2 is related. These measurements of Y_w, performed while the cell was impaled by the relatively large, partly filled micropipette (used for the current electrode), yielded values of the same order as those measured without such an additional micropipette present [15].

Discussion

As pointed out previously [3,5] the breakdown process is so rapid as to rule out any possibility that the sharp increase in conductance is due to the ionic punch-through phenomenon described by Coster [18,19]. Punch-through is associated with changes in ionic profiles in the membrane, the time constants for which are of the order of 1.0 s [19,20].

We have previously shown that the pulsed current-voltage characteristics and the breakdown phenomenon can be very adequately accounted for by considerations of electro-mechanical effects on the membrane [1,3]. The dependence of the critical breakdown voltage, V_c, on the turgor pressure is also in very good agreement with the experimental data. From this data we can obtain a measure of $1/\gamma = 2\alpha/3Y_w + 1/Y_m$, the average value of which from the data presented in Fig. 3 was $69 \pm 10 \cdot 10^5$ N/m^2.

In order to calculate the elastic compressive modulus of the membrane we need to know both the elastic modulus of the cell wall, Y_w, and the membrane-wall coupling coefficient α. Whereas Y_w can be experimentally determined (see Results and Fig. 5) we do not have any information about the value of α. Two extreme cases $\alpha = 0$ (i.e. no coupling) and $\alpha = 1$ (complete coupling) should be considered. With $\gamma = 69 \pm 10 \cdot 10^5$ N/m^2 and α in the range 0—1 and taking $Y_w = 300 \cdot 10^5$ N/m^2, Y_m is calculated to be in the range $59 \cdot 10^5$—$96 \cdot 10^5$ N/m^2. From the critical breakdown P.D., the thickness δ_0 and the dielectric constant ϵ of the region where the breakdown occurs, it is also possible, on the basis of the electro-mechanical model of the breakdown process, to deduce appropriate values of Y_m for the breakdown region. Such calculated values of Y_m as a function of δ_0 for various values of ϵ are shown in Fig. 6, for the mean value of V_c ($P = 0$) 959 mV.

Also indicated in this figure is the range of values of Y_m deducted from the present experiments.

It is immediately clear from Fig. 6 that the choice of δ_0 and ϵ is limited. If breakdown occurs in units which span the entire membrane, or a substantial part thereof, and thus have a thickness 7—9 nm, the dielectric constant of these regions must be in excess 10 *. This would then rule out a lipid material and suggest that these regions might be a hydrated protein. If the breakdown region is $\approx$4—5 nm (corresponding to the thickness of the lipid bilayer) the dielectric constant, from Fig. 6, must be in the range $\epsilon = 3$—8, which would

* It should be noted, that this and the following conclusions are only valid for uniform material (see the theory section).

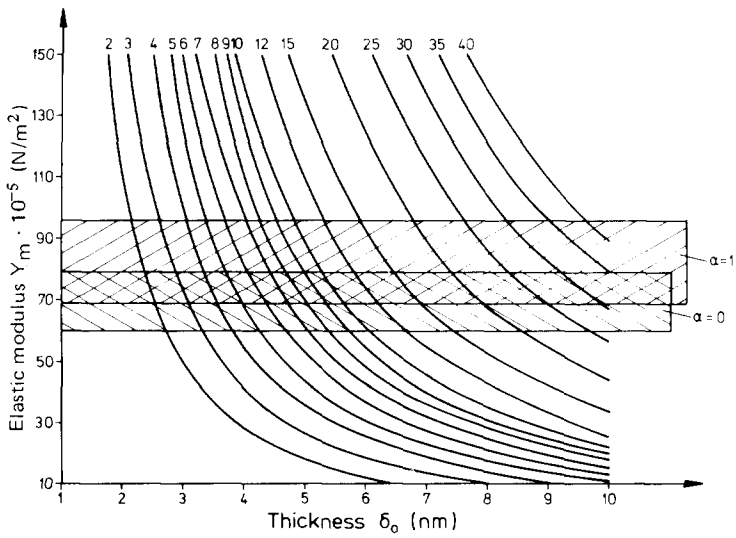

Fig. 6. The dependence of the theoretical value of the elastic modulus, Y_m, of the membrane on the thickness of the region where breakdown occurs. The curves were calculated from Eqn. 10 using the average value of 959 mV for the breakdown P.D. with various values of the dielectric constant, ϵ (indicated at top of curves). The shaded horizontal areas in the diagram indicate the range of Y_m determined from the dependence of V_c on the turgor pressure with either zero coupling ($\alpha = 0$) or complete coupling ($\alpha = 1$) between strains in the membrane and cell wall. For interpretation see Discussion.

suggest a lipo-protein material. On the other hand the breakdown region could conceivably be thinner. Thus in the fluid mosaic model [21], it is envisaged that some membrane modules penetrate only half of the bilayer. The possibility then exists that either half of the bilayer or the facing module could breakdown. If we allow that the breakdown region is of the order of 2 nm (see also ref. 22), the possible value of the dielectric constant deduced from Fig. 6 is then only 2—3. This immediately suggests that in that case it is the half bilayer which breaks down and not the facing (lipo)-proteinaceous module. It should be noted that the value of 959 mV is the upper value of the breakdown voltage of a single membrane. The breakdown event described here comprises the breakdown of both, the tonoplast and the plasmalemma. Continuing the line of argument presented in Materials and Methods, we assume the extreme case, that the breakdown P.D. of a single membrane is only half the value determined experimentally with the vacuolar electrodes. Since the critical breakdown P.D. is proportional to the square root of Y_m, a smaller breakdown P.D. value would affect the value of Y_m as derived from Eqn. 10. However, this is of no consequence if Y_m is calculated from the Eqn. 13 describing the pressure dependence of the breakdown P.D.

In Fig. 7 the same plot is represented as in Fig. 6, but it is assumed that the breakdown P.D. is 480 mV. It is evident that in this extreme case only the reasonable combination of $\delta = 2$ nm and $\epsilon = 3$—8 is possible. This means that the (lipo)-proteinaceous module facing the half bilayer break through.

We cannot decide conclusively at this stage between these possible combinations of thickness and dielectric constants. However, that the breakdown does not occur in the lipid bilayer region of the cell membrane is consistent with the

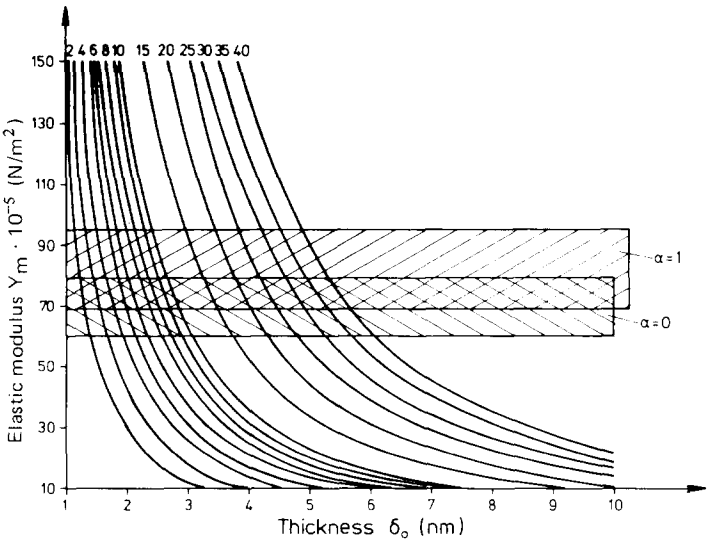

Fig. 7. Same plot as in Fig. 6 using an average value of 480 mV for the breakdown P.D. of a single membrane. It is assumed that the breakdown potential differences of the tonoplast and the plasmalemma are identical.

fact that artificial lipid bilayer membranes have breakdown voltages of about 200 mV or less [23]. The breakdown mechanism in these is probably different from that discussed here. There is now good evidence [24] from capacitance measurements that the solvent-free bilayer membranes made by the technique developed by Montal and Müller [25] have a thickness which is independent of membrane potential. The electro-mechanical breakdown mechanism under discussion here is thus not applicable to lipid bilayer membranes as was suggested by Crowley [26]. The decrease in the breakdown P.D. with benzyl alcohol in red blood cells [27] (which from NMR studies is thought to fluidize the membrane [28]) is consistent also with our present notion that the breakdown region is not the lipid layer matrix but probably a lipo-protein region or at a lipid-protein junction.

That some proteinaceous component is present in the breakdown region is further attested to by the inability of the membrane to reseal when the cell is exposed, prior to electrical breakdown, to a 0.1% glutaraldehyde solution. Glutaraldehyde even in high concentrations (1%) does not normally induce an increase in the hydraulic conductivity of the membrane (Zimmermann, U. and Lelkes, P.I., unpublished data). In electrical breakdown experiments in the presence of glutaraldehyde we found that one or two breakdowns could be obtained without any effect being evident. After that, however, the hydraulic conductivity increases sharply following subsequent breakdown. Increases in the hydrualic conductivity (which can be detected with our pressure probe) during and following electrical breakdown are not observed when glutaraldehyde is absent. It is interesting to note again here that the electrical resistance of the membrane (measured with the 500-μs pulses) does gradually decrease with increasing number of breakdowns (see Fig. 4). The breakdown P.D. nevertheless remains within the initial limits of the experimental reproducibility.

From the foregoing discussion it is clear that the effect of turgor on the breakdown process is consistent with the notion that electro-mechanical forces play a decisive role in the breakdown.

A more crucial test, however, would be the effect of absolute pressure on the breakdown potential *. A null result for this would positively mitegate against the possibility that pressure leads directly to a decrease in membrane thickness. The effect reported here must then also be ascribed to the indirect effect of stretching due to strains in cell wall or we must eliminate the electro-mechanical model of the breakdown phenomenon. It would not rule out the possibility that electro-mechanical forces play a significant role in determining the membrane structure (e.g. see Coster [29] following Coster and Kaplin [30]). Electro-mechanical compression could still occur and might be important as turgor pressure sensing and regulation mechanism as recently suggested [11,31,32]. A null result for the proposed experiment, however, would rule out an electro-mechanical catastrophic collapse of the membrane.

Finally, we must reiterate our previous remarks [3], that the results obtained do not allow us to eliminate the possibility that breakdown may result, not from a catastrophic collapse of the membrane, but due to another critical compression phenomenon. Electro-mechanical compression of the membrane material which, when sufficiently large, could, for instance, expose transmembrane conduction modules which by virtue of their relative size to the membrane are normally buried in the membrane matrix. When this occurs the electrical conductance through the membrane would increase sharply and breakdown could then result.

Appendix A

Effect of longitudinal stresses in the cell wall on membrane thickness

Changes in turgor pressure give rise to changes in cell volume. The relationship between pressure and volume can be obtained by integration of the Philip equation (Eqn. 1). Here we must also take into account that Y_w is pressure dependent [9,13,15] (it is also volume dependent but this is of no consequence here since only a small range of volumes are involved). It has been found experimentally (Steudle, E. and Zimmerman, U., unpublished) that the elastic modulus varies with pressure according to the relation

$$Y_w = Y_w^0 + (Y_w^\infty - Y_w^0)(1 - \exp(-\beta P)) \tag{A1}$$

where Y_w^0, the elastic modulus at $P = 0$; Y_w^∞, the limiting high pressure modulus; β, is a constant.

With Eqn. A1, Eqn. 1 may be integrated to yield

$$v = v_0 \exp(P/Y_w^\infty)\left[\frac{Y_w^\infty - (Y_w^\infty - Y_w^0)\exp(-\beta P)}{Y_w^0}\right]^{1/\beta Y_w^\infty} \tag{A2}$$

For cells of *V. utricularis* β will be of the order of $1 \cdot 10^5$ N/m^2, Y_w^0 and Y_w^∞ are of the order of $30 \cdot 10^5$—$50 \cdot 10^5$ N/m^2 and $200 \cdot 10^5$—$300 \cdot 10^5$ N/m^2, respectively. Thus for our considerations it is readily shown that the term in the large

* There are many technical difficulties in doing such experiments. Equipment for this purpose is at present under development.

bracket is essentially constant and equal to 1 for pressure greater than $0.5 \cdot 10^5$ N/m^2. The Eqn. A2 then reduces to

$$v = v_0 \exp(P/Y_w^\infty)$$

This equation can also be written in terms of the radius of the cell. Thus for a spherical cell

$$r = r_0 \exp\left(\frac{P}{3Y_w^\infty}\right) \tag{A3}$$

where r is the radius of the cell, r_0 is the radius when $P = 0$. It should be noted, however, that the relevant cell wall modulus relating the volumes to pressure is Y_w^∞ and not the general value Y_w. However, for simplicity of nomenclature we will continue to use the symbol Y_w but it is understood to be the value Y_w^∞ at $P \to \infty$. The relation between cell volume and area of the cell wall depends in a complicated way on the shape of the cell. For a homogeneous, spherical, cell the relationship is simple and the area of the cell wall, A_w, as a function of pressure, is then given by (using Eqn. A3)

$$A_w = A_{w_0} \exp\left(\frac{2P}{3Y_w}\right) \tag{A4}$$

where A_{w_0} is the wall area at $P = 0$.

When the membrane is coupled to the cell wall, changes in area, A_w, of the latter induces strains in the membrane (area A_m).

We shall assume here that the ratio of the strains in the cell membrane and cell wall is pressure independent and equal to α, a parameter which we will refer to as the coupling coefficient. (The value of α is thus between 0 and 1). Hence

$$\frac{dA_m}{A_m} = \alpha \frac{dA_w}{A_w}$$

and thus

$$\frac{A_m}{A_{m_0}} = \left(\frac{A_w}{A_{w_0}}\right)^\alpha \tag{A5}$$

where A_{m_0} refers to the area of the membrane when $P = 0$.

Changes in the area of the cell membrane could also lead to changes in its thickness. Again we lack detailed information about the mechanical structure of the cell membrane which would allow us to calculate exactly such changes in thickness.

To proceed we will follow the considerations of White [33] and assume, for illustrative purposes, that the membrane density will remain constant. Such considerations have also been invoked by Fettiplace et al. [17] to explain differences in the apparent thickness of the hydrocarbon component of cell membranes as compared with artificial bilayers. Thus when no transverse mechanical stress and no electric stress is present,

$$A_m \delta_{c=0,\, t=0} = A_{m_0} \delta_0 \tag{A6}$$

Hence using Eqn. A6 and A5 with Eqn. A4 the changes in membrane thickness

with turgor pressure due to longitudinal stresses is given by,

$$\delta_{e=0,\, t=0} = \delta_0 \exp\left(-\frac{2\alpha P}{3Y_w}\right) \tag{A7}$$

Eqn. A7 with Eqn. 11 then yields the breakdown P.D. as a function of pressure (Eqn. 13).

Conclusions

Our present studies allow us to conclude that

(1) Electrical breakdown potential in cells of *V. utricularis* decreases with increasing turgor pressure.

(2) The decrease in the critical breakdown P.D. with increasing turgor pressure is consistent with the notion that breakdown occurs as a result of an electro-mechanical instability as the critical breakdown P.D. is reached.

(3) On the basis of the electro-mechanical model of the breakdown process we estimate the compressive elastic modulus of the membrane to be in the range of $59 \cdot 10^5$—$96 \cdot 10^5$ N/m^2.

(4) Assuming that the breakdown is due to an electro-mechanical instability the present results of the effect of turgor pressure on the breakdown P.D. allows us to deduce under the condition that the critical membrane P.D. is 959 mV that (i) if the thickness of the breakdown region is 7—9 nm, (i.e. the thickness of the membrane) the dielectric constant $\epsilon > 10$, or (ii) if the thickness is 4—5 nm (i.e. the thickness of the bilayer), $\epsilon = 3$—8, or (iii) if the thickness is ≈ 2 nm (i.e. half of the bilayer) $\epsilon = 2$—3. If we assume that the breakdown P.D. for a single membrane is only half the value of 959 mV, as experimentally measured with vacuolar electrodes then it turns out that only one reasonable combination of membrane thickness with the dielectric constant is possible: $\delta = 2$ nm, $\epsilon = 3$—8 (that is a (lipo)-proteinaceous module facing half of a bilayer).

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Glossary of symbols

A_m	Area of the cell membrane.
A_{m_0}	Area of the cell membrane at zero turgor pressure.
A_w	Area of the cell wall.
A_{w_0}	Area of the cell wall at zero turgor pressure.
P	Pressure (e.g. turgor pressure).
P_e	Compressive pressure due to the electric field.
P_m	Elastic restoring pressure to strains present in the membrane.

r Radius of the (spherical) cell.
r_0 Radius of the (spherical) cell at zero turgor pressure.
v Volume of the cell.
V Potential difference across the cell membrane.
$V_c(P=0)$ Critical potential for electrical breakdown when the turgor pressure, P, is zero.
$V_c(t=0)$ Critical breakdown P.D. when longitudinal strains are present in the membrane due to stretching of the cell wall.
Y_m Compressive modulus of the membrane for stresses normal to the surface.
Y_{w_0} Cell-volumetric elastic modulus of the cell wall.
Y_w Volumetric elastic modulus of the cell wall at zero pressure.
Y_w^∞ Limiting value of the volumetric elastic modulus of the cell wall for high pressures (i.e. as $P \rightarrow \infty$).

Greek symbols

α Membrane cell wall coupling coefficient (= ratio of strains in surface area in the membrane and cell wall).
δ Thickness of the membrane.
δ_0 Membrane thickness when no transverse stresses at all, or longitudinal strains, are present.
$\delta_{e=0}$ Membrane thickness when the electric field stress is zero (i.e. when $V = 0$).
$\delta_{e=0,t=0}$ Membrane thickness when the electric field stress is zero and longitudinal strains (due to stretching of the cell wall) are present.
γ Effective compressive modulus of the cell membrane for the effect of turgor pressure

$$\left(\frac{1}{\gamma} = \frac{2\alpha}{3Y_w} + \frac{1}{Y_m}\right) .$$

References

1 Zimmermann, U., Pilwat, G. and Riemann, F. (1974) Biophys. J. 14, 881
2 Zimmermann, U., Pilwat, G., Beckers, F. and Riemann, F. (1976) Bioelectrochem. Bioenerg. 3, 58
3 Coster, H.G.L. and Zimmermann, U. (1975) J. Membrane Biol. 22, 73
4 Coster, H.G.L. and Zimmermann, U. (1975) Z. Naturforsch. 30c, 77
5 Coster, H.G.L. and Zimmermann, U. (1975) Biochim. Biophys. Acta 382, 410
6 Zimmermann, U., Schulz, J. and Pilwat, G. (1973) Biophys. J. 13, 1005
7 Riemann, F., Zimmermann, U. and Pilwat, G. (1975) Biochim. Biophys. Acta 394, 449
8 Zimmermann, U. and Steudle, E. (1974) J. Membrane Biol. 16, 331
9 Zimmermann, U. and Steudle, E. (1974) in Membrane Transport in Plants (Zimmermann, U. and Dainty, J., eds.), p. 64, Springer-Verlag, Berlin
10 Steudle, E. and Zimmermann, U. (1971) Z. Naturforsch. 266, 1302
11 Zimmermann, U., Beckers, F. and Steudle, E. (1976) in Transmembrane Ionic Exchange in Plants (Thellier, M., ed.), C.N.R.S., Paris, in the press
12 Steudle, E. and Zimmermann, U. (1974) in Membrane Transport in Plants (Zimmermann, U. and Dainty, J., eds.), p. 72, Springer-Verlag, Berlin
13 Zimmermann, U. and Steudle, E. (1975) Aust. J. Plant Physiol. 2, 1
14 Philip, J.R. (1958) Plant Physiol. 33, 264
15 Zimmermann, U., Steudle, E. and Lelkes, P.I. (1976) Plant Physiol., in the press
16 Evans, E. and Simons, S. (1975) J. Colloid Sci. 51, 266

17 Fettiplace, R., Andrews, D.M. and Haydon, D.A. (1971) J. Membrane Biol. 5, 277
18 Coster, H.G.L. (1965) Biophys. J. 5, 669
19 Coster, H.G.L. (1969) Aust. J. Biol. Sci. 22, 365
20 Coster, H.G.L. and Smith, J.R. (1974) in Membrane Transport in Plants (Zimmermann, U. and Dainty, J., eds.), p. 154, Springer-Verlag, Berlin
21 Singer, S.J. and Nicholson, J.L. (1972) Science 175, 720
22 Kreutz, W. (1972) Angew. Chemie 84, 597
23 Tien, N.T. and Diana, A.L. (1967) J. Colloid Interface Sci. 24, 287
24 Benz, R., Fröhlich, L., Läuger, P. and Montal, M. (1975) Biochim. Biophys. Acta 394, 323
25 Montal, M. and Müller, P. (1972) Proc. Natl. Acad. Sci. U.S. 69, 3561
26 Crowley, J.M. (1973) Biophys. J. 13, 711
27 Pilwat, G., Zimmermann, U. and Riemann, F. (1975) Biochim. Biophys. Acta 406, 424
28 Metcalfe, J.C. (1970) in Permeability and Function of Biological Membranes (Bolis, L., Katchalsky, A., Keynes, R.D., Loewenstein, W.R. and Pethica, B.A., eds.), p. 222, North-Holland Publ. Co., Amsterdam
29 Coster, H.G.L. (1975) Biochim. Biophys. Acta 382, 142
30 Coster, H.G.L. and Kaplin, I.J. (1973) Biochim. Biophys. Acta 330, 141
31 Coster, H.G.L. and Zimmermann, U. (1976) Z. Naturforsch. 31c, 461
32 Coster, H.G.L., Steudle, E. and Zimmermann, U. (1976) Plant Physiol., in the press
33 White, S.K. (1974) Biophys. J. 14, 155