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# PREPARATION OF ERYTHROCYTE GHOSTS BY DIELECTRIC BREAKDOWN OF THE CELL MEMBRANE

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## SUMMARY

Dielectric breakdown of membranes of red blood cells was observed in high electric fields (approx. $10^3$–$10^4$ V/cm) using an improved Coulter Counter with hydrodynamic focussing. In making measurements of the size distributions of red blood cells as a function of increasing electric field strength it was found that a sharp discontinuity occurred in the otherwise linear relation between the pulse heights in the Coulter Counter and the electric field strength due to dielectric breakdown of the membranes. Solution of Laplace's equation for the electric field generated at breakdown in the cell membranes yields a mean value of about 1.6 V for the membrane potential of red blood cells. Due to the dielectric break-down, release of hemoglobin occurred. Mechanical rupture of the red blood cells by the hydrodynamic forces in the orifice of the Coulter Counter or thermal rupture could be excluded as hemolysing mechanisms. The leaky ghost cells resealed at 37 °C as shown by incorporation of $^{131}$I-labeled albumin and repeated dielectric breakdown.

## INTRODUCTION

Erythrocyte ghosts are usually prepared by hemolysis of red blood cells in strong hypotonic solutions and resealing by restoration of isotonicity [1, 2]. The ghost populations so obtained are heterogeneous. Three types of ghosts can be distinguished: type I, ghosts which reseal immediately after hemolysis; type II, ghosts which reseal after reversal of hemolysis by addition of alkali ions; type III, ghosts which remain leaky under different experimental conditions. The ratio of the three fractions to each other depends on the temperature at which hemolysis is performed and on the time interval between hemolysis and restoration of isotonicity.

In this paper a new and rapid procedure for the preparation of ghosts in isotonic solutions is described. The method is based on the increase in membrane permeability in high, external, electric field strengths, first demonstrated with bacteria using an improved Coulter Counter [3–5]. The observed discontinuities in the current–voltage relationships previously reported were explained by dielectric breakdown of the cell

membrane. This assumption is supported for bovine red blood cells in this paper by ruling out thermal and mechanical effects and by a quantitative calculation of the critical membrane potential generated at which the increased permeability of the cell membrane was observed. If dielectric breakdown occurs the cell membrane should become leaky to large molecules. This was shown by checking the release of hemoglobin from red blood cells, and the resealing properties of the ghost cells investigated by incorporation of radioactively labeled albumin.

## MATERIALS

Bovine blood was withdrawn from apparently healthy animals and stored under sterile conditions in acid–citrate–dextrose buffer at 4 °C for not more than 2 days. The red blood cells were prepared in the usual way [2] and suspended for ghost preparation in the following solution (in mM): NaCl, 140; KCl, 16; $MgCl_2$, 4; $CaCl_2$, 2 and Tris, 5; the pH was 7.4. For sizing at different detector currents or voltages the cells were suspended in isotonic NaCl solution (0.9 %).

## METHODS

The electronic size measurement and dielectric breakdown were performed using the improved Coulter Counter of AEG-Telefunken, Ulm, G.F.R. [3, 5].

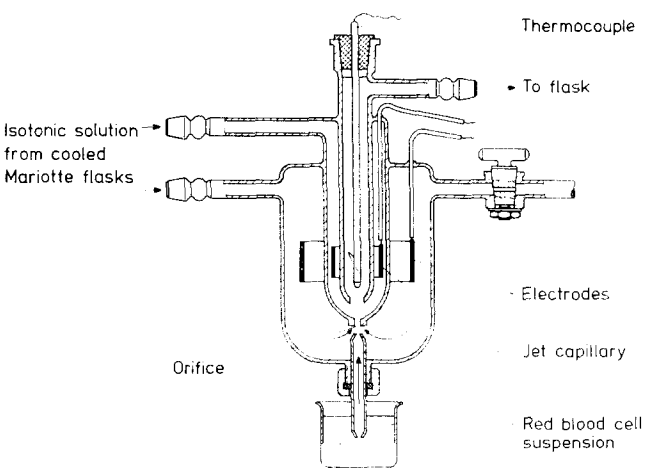



Fig. 1. A schematic diagram of the system used to produce ghost cells by dielectric breakdown.

The arrangement is given in Fig. 1. A jet capillary is constricted at both ends and mounted centrally close to the orifice. The diameter of the top constriction outlet and the diameter of the orifice are approximately equal to the distance between the capillary outlet and the orifice input, i.e. 40 $\mu$m for sizing and counting red blood cells. Particle-free electrolyte surrounds the jet and also fills a container connected to the output of the orifice. If low pressure is applied to this container, particle-free electrolyte from a Mariotte flask and the particle suspension are drawn through the

orifice (streaming velocity about 2 m/s). Due to this arrangement a focussing cone is formed within the distance between the jet and the orifice, decreasing the diameter of the suspension stream by a factor of 10 or more. All particles then travel along the central axis of the orifice under the same conditions (orientation, electric field strength etc.). If a potential difference is applied to the electrodes a current flows through the orifice.

Due to the construction of the measuring device it is more convenient to preset the current rather than the voltage. Because of a linear current–voltage characteristic (Eqn of the straight line: $U(\mathrm{V}) = 29.4 \cdot I(\mathrm{mA}) + 2.1$, experimental conditions: orifice of 40 $\mu$m in diameter and length, 0.9 % NaCl solution as electrolyte) the corresponding voltage and electric field strength in the orifice can be calculated easily assuming that 95 % of the applied voltage drops along the orifice which is 40 $\mu$m both in diameter and length (see below). The current was varied between 0.15 mA and 1.2 mA, this corresponds to a voltage of 6.5 V and 37.4 V and therefore to an electric field strength of about 1.6 kV/cm and 9.1 kV/cm.

Due to the passage of a nonconducting particle through the orifice the electric resistance in the orifice is changed resulting in a current or voltage pulse which is, to a first approximation, proportional to the size (size = volume × shape factor) of the particle [3, 5]. Therefore, after linear electronic amplification and subsequent pulse-height analysis, the true size distribution of a particle suspension can be determined.

To obtain higher yields of cells which have lost their hemoglobin due to dielectric breakdown an orifice which was 500 $\mu$m in both diameter and length was used. The streaming velocity used was also 2 m/s. The applied voltage between the electrodes was about 300 V and therefore sufficiently high to reach the critical dielectric breakdown voltage, even for the smallest cells (see below).

After passage through the electric field the suspensions collected in a flask behind the orifice were centrifuged at $10\,000 \times g$ for 15 min. The content of hemoglobin (in the supernatant and sediment, respectively) was determined with a Zeiss spectrophotometer at 398 nm.

To investigate the resealing properties, the cells were reincubated under different incubation conditions in the solution described above supplemented with 1 mM ATP and $^{131}$I-labeled albumin.

After $^{131}$I-labeled albumin incorporation, the cells and medium were separated again by centrifugation and the cell sediment was washed twice with inactive isotonic solutions containing small amounts of inactive albumin to remove adsorbed radioactivity on the surface of the cells. After solving the samples in Insta-Gel the incorporated radioactivity was measured in a Tricarb scintillator (Packard Instrument).

## RESULTS AND DISCUSSION

### *I. Experimental and theoretical proof of dielectric breakdown*

Measuring the size distribution of Latex particles ($d = 1.857$ $\mu$m) as a function of the detector current in the Coulter Counter with a 40 $\mu$m orifice (Fig. 2) it was found that the pulse heights (channel numbers) of the mean size of the distributions increase linearly with increasing external electric field strength (Fig. 4). The same relation was found for pulse heights of other than mean sizes of the distributions of Latex particles. The correlation coefficients are 0.998. In Fig. 3 typical size distri-

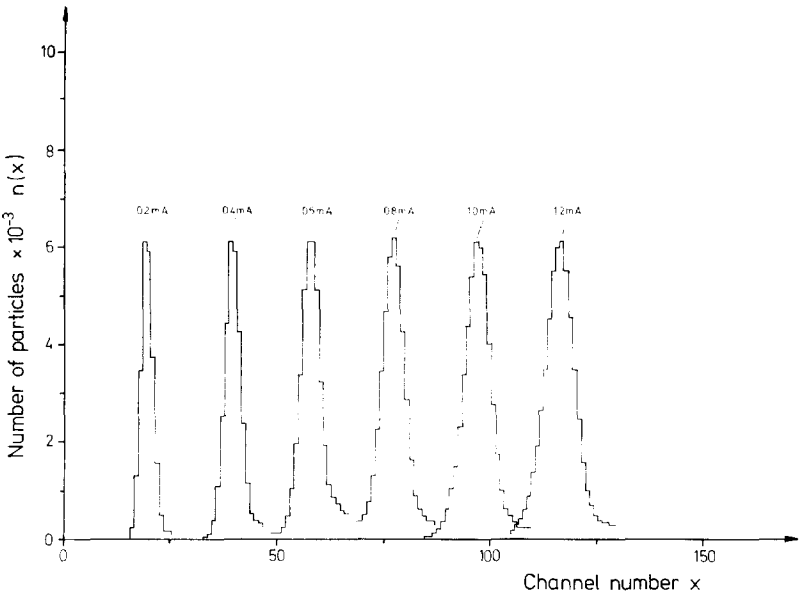



Fig. 2. Size distributions of Latex particles ($d = 1.857\,\mu$m) as a function of the detector current (0.2–1.2 mA, corresponding to 8–37.4 V detector voltage). The orifice was 40 $\mu$m in diameter and length. The electronic amplification was 320, the electrolyte 0.9 % NaCl solution.

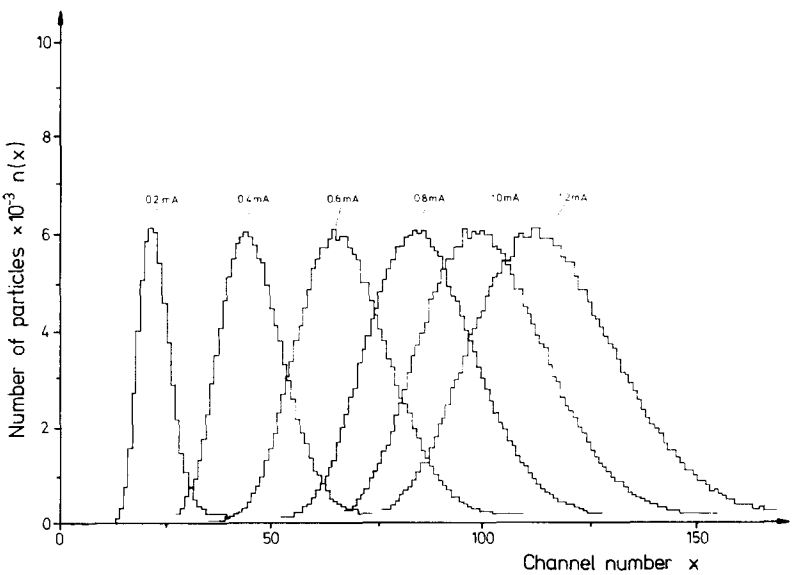



Fig. 3. Size distributions of bovine red blood cells as a function of the detector current. For experimental conditions see Fig. 2, the electronic amplification was 40.

butions of bovine blood cells at different detector currents are plotted. By calibration of the channel numbers with Latex particle size distribution, the modal volume could be calculated from

$$V_R = \frac{f_L}{f_R} \cdot V_L \tag{1}$$

where $f_L$ and $f_R$ are the shape factors of the Latex particles and of the red blood cells, respectively, and $V_L$ the volume of the Latex particles. The shape factor of the Latex particles is 1.5, the shape factor of bovine red blood cells 1.07 [5]. The modal volume is calculated therefore to 40.7 $\mu m^3$. Plotting the channel numbers of the modal sizes, or other sizes, against increasing external electric field strength a rather sharp bend in the expected linear curve is obtained as shown in Fig. 4. The correlation coefficients above and below the sharp bend are 0.998. These size-dependent discontinuities, which are comparable to those found with bacteria and algal cells [3, 6] can be explained by a dielectric breakdown of the cell membrane.

If this assumption is valid the current flows partially through the cell itself, so that its volume will be underestimated by the measuring device. Therefore, the observed shift in the size distribution towards smaller sizes can be explained.

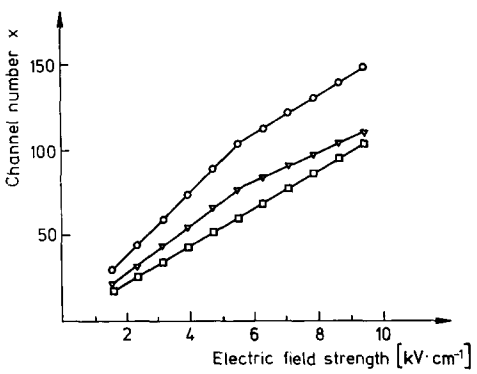
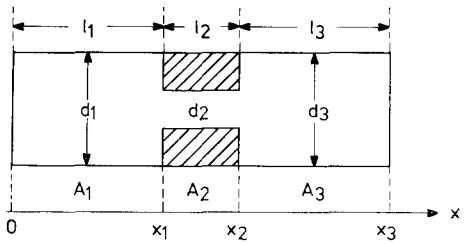



Fig. 4. Dielectric breakdown of the cell membrane of bovine red blood cells. Channel numbers (pulse heights) of the size distributions of Figs 2 and 3 are plotted against the external electric field strength. The field strength was calculated either from the detector current or voltage. □—□, latex particles with a volume of 3.35 $\mu m^3$; △—△, bovine red blood cells with a volume of 40.7 $\mu m^3$ (this volume corresponds to the modal size of the distribution shown in Fig. 3); ○—○, bovine red blood cells with a larger volume of 53.2 $\mu m^3$.

A theoretical estimation obtained by application of the Laplace equation shows that the potential difference $V_M$ generated across the membrane is large enough to cause the dielectric breakdown. An exact analytical solution of the Laplace equation cannot be obtained, as the boundary conditions are not known. A rough estimation was made by the following procedure: we assume the following simple model for the orifice of the Coulter Counter (see Scheme 1).

where $l_i$ are the lengths of the cylindrical containers and the orifice, respectively, $A_i$ are the cross-sectional areas, and $d_i$ are the diameters. ($l_1 + l_3 = 35$ mm, $l_2 = 0.04$ mm, $d_1 = 5$ mm and $d_2 = 0.04$ mm). The current density, $j$, is given by $j = \sigma \cdot E$




Scheme 1. Simple model for the orifice of the Coulter Counter.

and the current, $I$, by $I = \int_{A_i} j da$. Here $\sigma$ = electrical conductance.

It can be easily shown that with the above parameters 95 % of the potential difference between the electrodes drops over the distance $l_2$ [5].

We assume that this potential difference drops linearly along the length of the orifice if the inhomogeneities of the electric field are neglected in a first approximation. In this simplified case the Laplace equation can be solved in spherical coordinates $(r, \vartheta, \varphi)$ [7–17].

We consider a spherical particle with an interior radius $a$, an outer radius $b$ and a membrane thickness $d$, i.e. $d = (b-a)$. $\phi_1$, $\phi_2$, and $\phi_3$ are the potentials, $\sigma_1$, $\sigma_2$, and $\sigma_3$ the conductivities, the suffixes 1, 2, and 3 represent the interior of the cell, the membrane, and the external solution, respectively. Due to the fact that the particle is small compared to the dimensions of the orifice, the following boundary conditions must hold:

$$\phi \to -E_0 r \cos\vartheta \qquad \text{for } r \to \infty,$$

$$\phi \text{ must be finite} \qquad \text{for } r = 0,$$

$$\left.\begin{aligned} \phi_1(a) &= \phi_2(a) \\ \frac{\sigma_1}{a} \cdot \frac{d\phi_1}{dr} &= \frac{\sigma_2}{a} \cdot \frac{d\phi_2}{dr} \end{aligned}\right\} \quad \text{for } r = a,$$

$$\left.\begin{aligned} \phi_2(b) &= \phi_3(b) \\ \frac{\sigma_2}{b} \cdot \frac{d\phi_2}{dr} &= \frac{\sigma_3}{b} \cdot \frac{d\phi_3}{dr} \end{aligned}\right\} \quad \text{for } r = b.$$

The problem is now completely defined and its solution yields, for the potential difference $V_M$ across the membrane, the equation

$$V_M = \frac{\frac{3}{2+\beta} E_0 \left[a - b + \frac{1-\alpha}{2+\alpha} a^3 \left(\frac{1}{a^2} - \frac{1}{b^2}\right)\right] \cos\vartheta}{1 + 2\left(\frac{1-\beta}{2+\beta}\right)\left(\frac{1-\alpha}{2+\alpha}\right)\left(\frac{a}{b}\right)^3} \tag{3}$$

where $\alpha = \dfrac{\sigma_1}{\sigma_2}$ and $\beta = \dfrac{\sigma_2}{\sigma_3}$.

With $\sigma_1 \cong \sigma_3 \gg \sigma_2$ [18] one obtains the following expression

$$V_M = 1.5 \cdot b \cdot E_0 \cdot \cos\vartheta \tag{4}$$

For the modal sizes the radius of the corresponding spherical volume is $\approx$ 2.2 $\mu$m [5] and the critical external electrical field strength 5.3 kV/cm (Fig. 4). Using Eqn 4 the potential difference generated across the membrane is then calculated to be 1.8 V (mean value of 11 measurements: 1.6 V). A slightly higher value (2.9 V) is obtained using an ellipsoidal approximation as described elsewhere [5]. On comparing this mean value of 1.6 V to a bimolecular lipid leaflet arrangement for the membrane the electric field strength generated in the membrane would be about 4–5 · $10^6$ V/cm, i.e. of the order of the intrinsic membrane electric field strength which is between $10^5$ and $10^6$ [19]. The electric breakdown electric field strength of oil layers are also of the same order [20]. If we assume that the potential difference generated across the membrane for breakdown is an intrinsic membrane property, i.e. independent of volume, we can calculate the critical external electric field strength as a function of volume using the mean value of 1.6 V for the critical membrane potential difference. According to Fig. 5 the theoretically calculated curve is a good fit to the experimentally determined one.

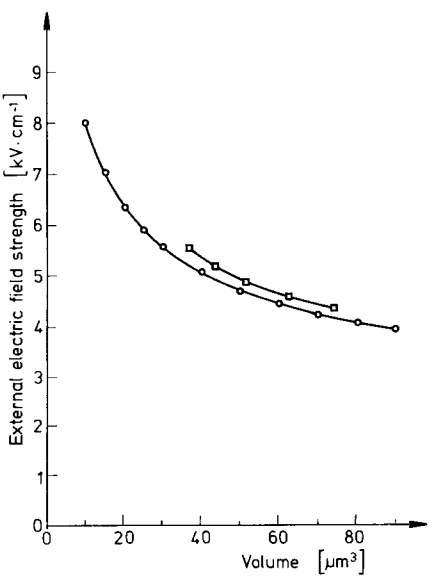



Fig. 5. The dependence on the external electric field strength (which leads to dielectric breakdown) of the particle volume. ○—○, external field strength calculated by Fig. 4 against the volume; □—□, external field strength, calculated either from the critical detector current or voltage, of the bovine red blood cell distribution from Fig. 3 or Fig. 4, respectively.

In spite of the good agreement between theory and experiment, mechanical and thermal effects must be taken into consideration as possible additional mechanisms for hemolysis. Since red blood cells lose hemoglobin above the critical electric field strength (see below) a possible influence of the hydrodynamic forces on the red blood cells by mechanical deformation and rupture [21, 22] was investigated in the absence of an electric field. After passage through the orifice the cells were collected in a flask and centrifuged at 10 000 $\times g$ for 15 min. The supernatant was free of hemo-

globin in the absence of an electric field indicating that mechanical effects can be excluded.

A small global temperature increase of the solution passing the orifice is unavoidable. An increase of 5 °C was measured by a thermocouple introduced behind the 500 μm orifice. A rough calculation confirmed this value. Since the streaming velocity is about 2 m/s, 0.4 ml/s of suspension passes the orifice. In the orifice an energy of approx. 7.5 W is released per s, i.e. the suspension is heated by 4.5 °C. As described by Sale and Hamilton [23, 24] this global temperature increase cannot cause a release of hemoglobin. This was also verified by injecting red blood cells previously cooled to 4 °C into an isotonic solution of 37 °C, where no hemolysis occurred. Possible localised heating in the membrane, leading to thermal damage, is potentially of greater importance than global heating. This possibility has now also been excluded in experiments with cells of *Valonia utricularis* in which direct measurements of the dielectric breakdown could be made [25]. The results obtained with these cells are, in other aspects, very similar to those reported here and we conclude that localised thermal damage in the membrane was also not significant in producing the dielectric breakdown reported here.

*II. Preparation of ghost cells*

A suspension of bovine red blood cells (1 : 1) was sucked through the 500 μm orifice while applying a voltage of 300 V. The temperature of the suspension was kept at about 1 °C. After passing through the electric field the suspension was collected in a flask at 0 °C as indicated in Fig. 1. The density of the cell suspension in this flask decreased by a factor of 70 or more since a cell-free isotonic solution from the Mariotte flasks with the suspension was being drawn simultaneously through the orifice. The cells collected in the flask were separated from the medium by centrifugation for suitable time intervals. The sedimented cells were nearly colourless, suggesting the release of hemoglobin from the red blood cells. The measurement

TABLE I

INCORPORATION OF $^{131}$I-LABELED ALBUMIN INTO BOVINE GHOST CELLS UNDER DIFFERENT EXPERIMENTAL CONDITIONS AFTER DIELECTRIC BREAKDOWN OF THE CELL MEMBRANE

The cpm of the ghosts refers to the ghost sediment obtained by centrifugation after the last washing. No correction due to the trapped extracellular fluid was made.

| Experiment No. | Resealing time without $^{131}$I-labeled albumin (h)* | | Resealing time with $^{131}$I-labeled albumin (h) | | Incorporation of $^{131}$I-labeled albumin (cpm of 1 ml ghosts) / (cpm of 1 ml supernatant) |
|---|---|---|---|---|---|
| | 0 °C | 37 °C | 0 °C | 37 °C | |
| 1 | 0 | 0 | 1 | 2 | 0.31 |
| 2 | 1 | 0 | 0 | 2 | 0.08 |
| 3 | 1 | 1 | 0 | 1 | 0.02 |
| 4 | 1 | 2 | 0 | 0.1 | 0.01 |

* The time of preparation and concentration of the ghost suspension (about 30 min) was not taken into account.

of the hemoglobin content in the cells with the spectrophotometer at 398 nm yielded values of less than 1 % bound to the membranes. In order to obtain insight in the rate of the resealing process after dielectric breakdown, aliquots of the nearly hemoglobin-free ghost sediment were first preincubated for several time intervals at 0 °C and 37 °C in isotonic solutions supplemented with 1 mM ATP and then $^{131}$I-labeled albumin was added to the solutions. Other aliquots were immediately suspended in isotonic solutions containing 1 mM ATP and $^{131}$I-labeled albumin for 1 h at 0 °C and then the temperature was raised to 37 °C. The various experiments are listed in Table I. According to Table I, $^{131}$I-labeled albumin is indeed trapped in the cells, which demonstrates that the dielectric breakdown of the membrane is a reversible process to some extent. Furthermore, the results of experiments No. 3 and No. 4 indicate that the resealing process is completed after 1 h at 37 °C, since no radioactivity was then incorporated into the cells. Comparison of experiments No. 1 and No. 2 show that resealing can also occur at 0 °C. Only 25 % of the radioactivity incorporated in the cells in experiment No. 1 was trapped in the cells subjected to the incubation conditions of experiment No. 2. The amount of incorporated $^{131}$I-labeled albumin (referred to as the activity of the supernatant) is very high in cells of experiment No. 1, compared to the results obtained by the common method of osmotic lysis [2].

These findings suggest the formation of ghosts. This conclusion is also supported by further dielectric breakdown measurements of ghost cells prepared under the experimental conditions No. 1. Typical size distributions of such ghost cells plotted against increasing detector current (or electric field strength) are shown in Fig. 6. The ghost cells were prepared from bovine red blood cells the size distributions of which are plotted in Fig. 3. The modal volume of the ghost distribution is 47.9 $\mu m^3$

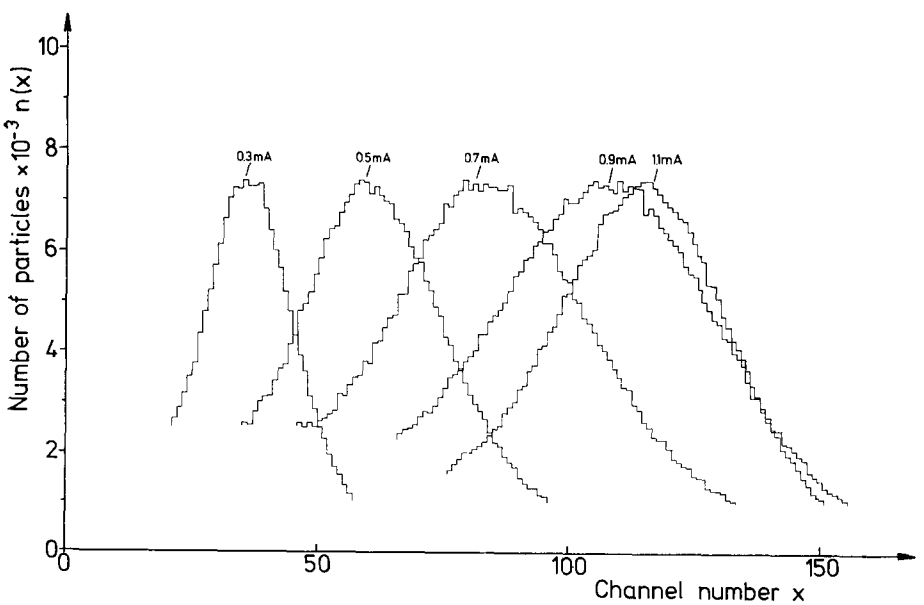



Fig. 6. Size distributions of ghost cells from bovine red blood cells. For experimental conditions see Fig. 2, the electronic amplification was 34.

and therefore larger than the modal volume for the corresponding bovine red blood cells (40.7 $\mu m^3$). This finding is in agreement with the results obtained for ghosts prepared by osmotic lysis from human red blood cells [26]. Plotting the channel numbers of a size against increasing electric field strength, size (or volume) dependent dielectric breakdown is revealed. (Small sizes to the left of the distribution cannot be evaluated since the resolution was not high enough for separation of the pulses produced by cell fragments (Fig. 6).) It seems plausible that the dielectric breakdown may only occur if the membrane is resealed again*. After breakdown, the curve in Fig. 7 becomes parallel to the abscissa. This is expected if the conductivity of the cell interior is the same as that of the external medium [5].

Density gradient centrifugation after resealing yielded two bands corresponding to type II and type III of Bodemann and Passow [2]. Since the separation by sucrose density gradient centrifugation includes several problems and sources of errors, the barrier centrifugation method was used which results in the sedimentation of nearly all of the leaky ghosts. We therefore used the latter method, and the heterogeneous ghost suspension was layered on the top of a sucrose cushion. After centrifugation at 34 800 $\times g$ for 1 h, approx. 80 % of the ghosts were found on the top of the sucrose cushion. If we go along with the conclusions of Bodemann and Passow, this result seems to point to a more homogeneous ghost preparation than the one obtained by osmotic lysis, although, at present, the functional behaviour of the ghost cells prepared by the electrical method are unknown.

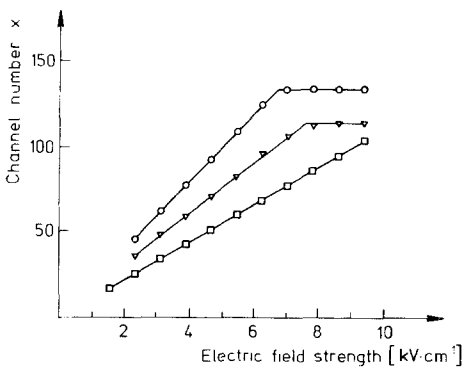



Fig. 7. Dielectric breakdown of the cell membrane of bovine ghost cells. Data taken from Fig. 6. □—□, latex particles with a volume of 3.35 $\mu m^3$; △—△, bovine ghost cells with a volume of 47.9 $\mu m^3$ (this volume corresponds to the modal size of the distribution shown in Fig. 6); ○—○, bovine ghost cells with a larger volume of 62.6 $\mu m^3$.

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* In recent experiments using intracellular electrodes with *Valonia utricularis* it has been shown that after a short resealing time of ≈ 5 s following dielectric breakdown the membrane resistance returns to its high value and the breakdown process could be repeated. This is consistent with our present findings [25].

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REFERENCES

1 Hoffmann, J. F. (1962) J. Gen. Physiol. 45, 837–859
2 Bodemann, H. and Passow, H. (1972) J. Membrane Biol. 8, 1–26
3 Zimmermann, U., Schultz, J. and Pilwat, G. (1973) Biophys. J. 13, 1005–1013
4 Zimmermann, U. and Pilwat, G. (1973) Proc. 9th Int. Congr. Biochem., Stockholm, 1–7 July, p. 260
5 Zimmermann, U., Pilwat, G. and Riemann, F. (1974) Biophys. J., in the press
6 Zimmermann, U., Pilwat, G. and Riemann, F. (1974) Membrane Transport of Plants (Zimmermann, U. and Dainty, J., eds) pp. 146–153 Springer-Verlag, Berlin
7 Jackson, J. D. (1962) Classical Electrodynamics, J. Wiley & Sons, Inc., New York
8 Eder, G. (1967) Elektrodynamik, BI-Hochschultaschenbuch Nr. 233/233a, Bibilographisches Institut, Mannheim
9 Stratton, J. A. (1941) Electromagnetic Theory, McGraw-Hill, New York
10 Fricke, H. (1924) Phys. Rev. 24, 575–587
11 Fricke, H. (1931) Physics 1, 106–115
12 Fricke, H. (1953) J. Appl. Phys. 24, 644–646
13 Velick, S. and Gorin, M. (1940) J. Gen. Physiol. 23, 753–771
14 Morse, P. M. and Feshbach, H. (1953) Methods of Theoretical Physics (Int. Ser. Pure Appl. Phys., Parts I and II), McGraw-Hill, New York
15 Bernhardt, J. and Pauly, H. (1973) Biophysik 10, 89–98
16 Turnbull, R. J. (1973) J. Membrane Biol. 14, 193
17 Neumann, E. and Rosenheck, K. (1973) J. Membrane Biol. 14, 194–196
18 Johnson, S. L. and Woodbury, J. W. (1964) J. Gen. Physiol. 47, 827–837
19 Coster, H. G. L. (1973) Biophys. J. 13, 1119–1123
20 Crowley, J. M. (1973) Biophys. J. 13, 711–724
21 Rand, R. P. (1964) Biophys. J. 4, 303–316
22 Rand, R. P. and Burton, A. C. (1964) Biophys. J. 4, 115–135
23 Sale, A. J. H. and Hamilton, W. A. (1967) Biochim. Biophys. Acta 148, 781–788
24 Hamilton, W. A. and Sale, A. J. H. (1967) Biochim. Biophys. Acta 148, 789–800
25 Coster, H. G. L. and Zimmermann, U. (1974) Biochim. Biophys. Acta, in the press
26 Seeman, P. (1970) Permeability and Function of Biological Membranes (Bolis, L., Katchalsky, A., Keynes, R. D., Loewenstein, W. R. and Pethica, B. A., eds), pp. 40–56, North-Holland Publishing Company, Amsterdam