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EFFECTS OF HIGH ELECTRIC FIELDS ON MICRO-ORGANISMS

III. LYSIS OF ERYTHROCYTES AND PROTOPLASTS

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SUMMARY

The lysis of bacterial protoplasts, spheroplasts and erythrocytes by high electric fields has been examined. Fields up to 25 kV/cm were applied as a series of direct current pulses to suspensions of the cells; the degree of lysis of a population depended upon the field strength. The potential difference across the membrane of the spherical cell has been calculated; lysis occurred when this potential difference reached about 1 V (values ranged from 0.7 to 1.15 V according to the species). It is suggested that the potential may cause conformational changes in the membrane structure resulting in the observed loss of its semipermeable properties.

INTRODUCTION

A lethal effect of high electric fields, applied in direct current pulses to suspensions of vegetative bacteria and yeasts, has been demonstrated[1,2]. The death was not due to heating or electrolysis and was independent of current density and energy input. It was dependent on the field strength and the total time of treatment. Cell membrane damage was demonstrated in vegetative cells, protoplasts and erythrocytes, and the effects on its structure and function were examined. It was proposed that the electric field causes an irreversible loss of the membrane's function as the semipermeable barrier between the bacterial cell and its environment leading to the cell death.

Because of the association of the membrane damage with the electric field, we have considered the potential difference across the membrane. This has been done for protoplasts, spheroplasts and sphered erythrocytes, as these are spherical cells bounded only by the membrane and potentials may be calculated more easily for this shape.

MATERIALS AND METHODS

Protoplasts of *Micrococcus lysodeikticus, Sarcina ureae, Bacillus subtilis* and *Bacillus megaterium* were prepared as described previously[2]. Spheroplasts of *Escheri-*

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chia coli and the motile pseudomonad isolated as a culture contaminant[1] were also used. The spheroplasts were made by the Tris–EDTA–lysozyme method of REPASKE[3]. *E. coli* was grown in FRAZER AND JERREL'S[4] glycerol broth, washed twice in 0.03 M Tris buffer (pH 8) and resuspended in a medium containing 2 μg/ml lysozyme, 80 μg/ml EDTA and 10 % sucrose. Spheroplasts formed in about 30 min. NaCl was then added to a final concentration of 0.1 %. The pseudomonad was grown in a glucose–ammonium salts medium[5], washed twice in 0.067 M phosphate buffer and resuspended in a medium containing 0.03 M Tris buffer (pH 8), 20 μg/ml lysozyme, 500 μg/ml EDTA and 20 % sucrose; finally NaCl was added to a concentration of 0.1 %.

Dilute bovine erythrocyte suspensions were prepared from fresh blood by centrifugation and washing three times in 0.85 % NaCl, to allow the blood cells to assume the spherical form by removal of "anti-sphering substance" (BESSIS[6]). The cells were resuspended in 0.85 % NaCl. Equine erythrocyte suspensions were similarly prepared from oxalated horse blood. The formation of spherical cells was confirmed by microscopic examination.

Lysis of the cells was assessed by absorbance measurements with a Hilger absorptiometer at 580 mμ for protoplasts and 700 mμ for erythrocytes. Suspensions were sufficiently dilute for all the samples to have absorbances less than 0.6. As recommended by KAY[7], the linear relation between absorbance and the proportion of whole cells was checked; therefore the fall of absorbance was proportional to the number of lysed cells.

Details of the d.c. pulse treatment were the same as described previously[1]; 20-μsec pulses were applied at 1 pulse per sec.

THEORY

It was previously demonstrated[1] that vegetative bacteria were killed, not by heating of the suspension, but by damage to their membranes as a direct result of the electric field. Therefore it seemed pertinent to consider the potential difference developed across the membrane.

The bacterial protoplast, spheroplast and sphered erythrocyte may be considered as a core surrounded by a membrane (Fig. 1). The problem of such a two-phase sphere has been treated in connection with the electrical impedance of suspensions of spheres by MAXWELL[8], COLE[9] and others. If the thickness of the membrane is small compared to the radius of the cell, the two-phase sphere can be replaced by a homogeneous sphere of resistivity, r_0, given by

$$r_0 = r_2 + \frac{Z}{a}$$

where r_2 = resistivity of the core (Ω cm), a = radius of the cell (cm), Z = membrane impedance (Ω cm^2).

The membrane impedance results from its resistance and capacity; however, it will be shown that the capacity may be neglected for our experimental conditions. COLE[10] gives an expression for the time constant due to the membrane capacity, which, for a dilute suspension of spheres, becomes

$$\left[\frac{r_1}{2} + r_2\right] Ca$$

where r_1 = resistivity of the suspending medium (Ω cm), C = membrane capacity (F/cm^2).

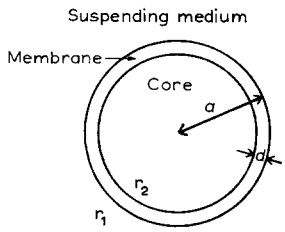


Fig. 1. Model cell for electrical theory.

The capacity of biological membranes is widely reported to be approx. 1 μF/cm^2 (*e.g.* COLE[11], SCHWAN[12]). The internal resistivity of the cell may be assumed to be of the order of 100 Ω cm (*e.g.* COLE[11], CARTENSEN *et al.*[13]) and the resistivities of the suspending media were 100–1000 Ω cm. With cells of about 10^{-4} cm radius, the time constant is under 10^{-7} sec, which is very much less than the 2×10^{-5} sec pulse length used in our experiments. Therefore the steady-state or d.c. condition can be used, which means that the membrane capacity can be ignored and the equivalent sphere resistivity becomes

$$r_0 = r_2 + \frac{R}{a}$$

where R is the membrane resistance (Ω cm^2).

If R lies between 10 and 1000 Ω cm^2 (*e.g.* JONHSON AND WOODBURY[14], COLE[11]), then $r_0 \gg r_1$, and the equivalent sphere is practically non-conducting. Under these conditions the potential at the surface of the sphere can be readily calculated by applying the method of potential theory (*e.g.* COULSON[15]) to the case of an insulating sphere in a conducting medium. The potential difference across the sphere is a maximum in the direction of the electric field (E) and is $3aE$. As the membrane is highly resistive and the core conductive the core is essentially at an equipotential and the maximum potential difference is shared by the membrane at the opposite poles. Therefore at these two places the potential difference (V) across the membrane is given by

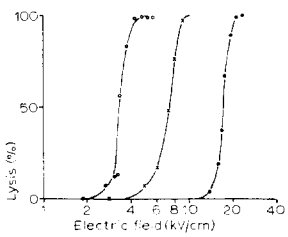
$$V = \frac{3}{2} aE$$

RESULTS

The various protoplasts, spheroplasts and erythrocytes listed under MATERIALS were subjected to d.c. pulse treatments of 10 pulses of 20 μsec at various electric fields, and the degree of lysis measured by absorbance. As an example of the results, Fig. 2 shows the lysis of bovine erythrocytes and protoplasts of *M. lysodeikticus* and *B. megaterium*; the spread of electric field strengths, over which the lysis of the population increased, was a characteristic of all the cells. Diameters of the cells were obtained by microscopic measurement and, for example, Fig. 3 shows the cumulative

size distributions of bovine erythrocytes, and protoplasts of *M. lysodeikticus* and *B. megaterium*.

The similarity of the shape of the curves of Figs. 2 and 3 suggests that the pread of electric field strengths causing lysis may be due to the spread of the sizes of



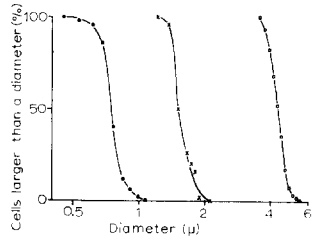


Fig. 2. Effect of electric field on lysis, assessed by absorbance measurements on cell suspensions. 10 pulses of 20 μsec. O—O, bovine erythrocytes; ×—×, *B. megaterium* protoplasts; ●—●, *M. lysodeikticus* protoplasts.

Fig. 3. Cumulative size distribution: the number of cells larger than a diameter *vs.* diameter. ●—●, *M. lysodeikticus* protoplasts; ×—×, *B. megaterium* protoplasts; O—O, bovine erythrocytes.

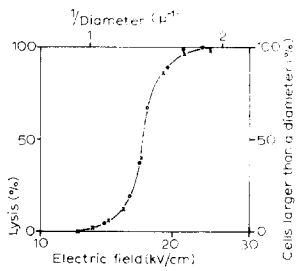


Fig. 4. *M. lysodeikticus* protoplasts; lysis by electric field and cumulative size distribution. O—O, lysis by electric field; 10 pulses of 20 μsec. ×—×, cumulative size distribution: the number of cells larger than a diameter *vs.* the reciprocal of the diameter.

the cells. The figures also suggest an inverse relationship between cell size and electric field. If we postulate that the membranes of all the cells in a population of one organism break down at the same value of the maximum potential difference (V), it follows from the expression for V that the field strength damaging a particular cell is inversely proportional to the diameter of that cell. The cumulative size distribution curve, relating the number of cells larger than a diameter, should therefore be the same shape as the curve of lysis *vs.* electric field. Taking for example the data for *M. lysodeikticus* protoplasts and plotting on the appropriate scales (Fig. 4) good agreement is demonstrated; this was also demonstrated for the other organisms. This supports the postulate that lysis by d.c. pulses results from the development of a critical potential difference (V_c) across the membrane.

The magnitude of V_c can be found from the expression for V, by putting a equal to the mean radius of the population and E equal to the electric field at which 50 % lysis occurs. The values of V_c are given in Table I and are considered to have a possible experimental error of $\pm$ 0.2 V. Admittedly the theory is a simplification and

the natural membrane potential of the cell has been ignored, but this seems justified as it is relatively small.

TABLE I

POTENTIAL DIFFERENCE ACROSS THE MEMBRANE AT WHICH BREAKDOWN OCCURRED FOR VARIOUS ORGANISMS

The critical potential difference (V_c) was calculated from the mean diameter of cells and the electric field at which 50 % lysis occurred. Treatment conditions were 10 pulses of 20 μsec at 1 pulse per sec.

Organism	*Mean diameter* (μ)	*Electric field* (*kV/cm*)	V_c (*V*)
Protoplasts			
B. megaterium	1.63	7.3	0.9
S. ureae	0.97	16	1.15
M. lysodeikticus	0.80	17	1.0
B. subtilis	0.81	14.2	0.9
Spheroplasts			
E. coli	0.87	12.9	0.85
Pseudomonad	0.78	11.7	0.7
Erythrocytes			
Equine	4.2	3.1	1.0
Bovine	4.2	3.5	1.1

DISCUSSION

The damage to the membrane occurs when there is about 1 V across it. Before ascribing the damage to electrical breakdown, the possibility that it is due to electrical heating of the membrane itself must be examined, although the heating of the cell suspension as a whole is negligible[1].

When heat is being generated uniformly in a membrane the steady-state temperature rise at its centre, according to CARSLAW AND JAEGER[16], is

$$\frac{Qd^2}{8k}$$

where Q = rate of generation of heat in unit volume of the membrane (cal·cm^{-3}·sec^{-1}), d = membrane thickness (cm), k = thermal conductivity of the membrane (cal·cm^{-1}·sec^{-1}·degree^{-1}).

The temperature rise reaches 90 % of the steady-state value in a time

$$t = \frac{d^2 s\rho}{4k}$$

where ρ = density of the membrane (g/cm^3), s = specific heat of the membrane (cal/g).

The membrane is mainly composed of protein and lipid; lipids have a very low thermal conductivity, so if we assume that the thermal resistance of the membrane is due to a lipid layer, the value of t will tend to err on the high side. Taking typical values of $k = 0.0005$, $d = 10^{-6}$, $\rho = 0.9$, $s = 0.5$, then t is approx. 10^{-10} sec; even if this estimate is somewhat in error, t is so much less than the pulse length of $2 \cdot 10^{-5}$

sec used in the experiments, that the steady-state expression applies. It remains to estimate Q. The power dissipated in a small section of the membrane of surface area A, which is subjected to a potential difference of V, is

$$\frac{V^2 A}{R},$$

and the power density,

$$Q = \frac{V^2}{Rd}.$$

The maximum value of V is about 1 V and typical values of R and d are 100 Ω cm^2 and 10^{-6} cm. Substitution of these values gives $Q = 10^4$ W/cm^3, which corresponds to a maximum steady-state temperature rise at the centre of the membrane of the order of 10^{-6} °. Even if this estimate is somewhat in error, the temperature rise is obviously negligible. Therefore we can conclude that the membrane damage does not arise from uniform heating of the membrane itself, but is an electrical breakdown at a potential difference corresponding to an electric field of 10^6 V/cm in a uniform membrane 100 Å thick.

There are two principal theories of the structure of biological membranes. In the model of DANIELLI AND DAVSON[17] the membrane is visualised as having a bimolecular layer of lipid as its core, with protein deposited on either side and bound to the lipid by electrostatic forces. ROBERTSON[18] has put forward the unit membrane hypothesis in which he states that all biological membranes have basically the same structure, as described in the Danielli-Davson model. GREEN AND PERDUE[19] have recently proposed that the membrane is composed of an aggregation of lipoprotein sub-units to form the essential structure of the membrane continuum; in these sub-units the lipoprotein bonds are covalent rather than electrostatic.

Although it is not possible to choose between the models on the basis of present knowledge, both suggest that the membrane has an ordered structure. However, support for the Danielli-Davson model is given by considerable evidence that synthetic phospholipid bilayers mirror many of the transport and electrical properties of natural membranes. Of particular relevance to our studies is the demonstration by HUANG, WHEELDON AND THOMPSON[20] and MIYAMOTO AND THOMPSON[21] that a phospholipid bilayer prepared from lecithin and *n*-tetradecane was shattered by a steady potential difference of about 200 mV. At potential differences between 150 and 300 mV, irreversible changes of a number of natural membranes have been reported. Examples are the squid giant axon (COLE AND MOORE[22]), the lobster giant axon (JULIAN, MOORE AND GOLDMAN[23]) and *Chara australis* and NITELLA sp. (COSTER[24]).

The explanation of the dissimilarity between the potential differences causing the above effects and our results of about 1 V for the lysis of erythrocytes, bacterial protoplasts and spheroplasts may be in the nature of the membranes involved, or in the particular criteria of breakdown, or in the comparatively short time scale of our pulse experiments.

PETHICA[25] has discussed the changes in dipolar orientation which can occur in phospholipid monolayers when quite modest potentials are applied. He argues that such polarisation distortion may well be a triggering process leading to conformational changes in the membrane structure related to the alteration in permeability during

the action potential in nerve cells. On the basis of this idea one can suggest that the application of excessive potential differences across the membrane may lead to irreversible changes in the ordered structure to give rise to the observed forms of membrane breakdown. In micro-organisms the structural changes due to a potential difference of about 1 V could give rise to the irreversible loss of the membrane's function as the semipermeable barrier between the cell and its environment and so result in the observed lysis of erythrocytes and protoplasts and to bacterial cell death.

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