PASSIVE ELECTRICAL PROPERTIES OF MICROORGANISMS

IV. STUDIES OF THE PROTOPLASTS OF *MICROCOCCUS LYSODEIKTICUS*

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ABSTRACT Observations of protoplasts of *Micrococcus lysodeikticus* show that removal of the cell wall of this organism decreases the dielectric constant by two orders of magnitude. The upper limit of the effective, homogeneous conductivity for the protoplast is 0.001 mho/m as compared with 0.045 mho/m for the intact cell. These results conclusively demonstrate the dominant effect of the cell wall on the low frequency dielectric properties of bacteria.

INTRODUCTION

It has been shown that bacteria have high, effective, homogeneous conductivities and dielectric constants at low frequencies and low environmental conductivities (Carstensen, 1965; Einolf, 1967). Existing evidence indicates that the high conductivities arise from the presence of the bacterial cell wall. This is supported by two recent studies. The first has shown that the resistance of the bacterial membrane is too great to account for the high conductivities at low frequencies (Carstensen, 1967 *a*). The second has shown that the conductivity of isolated bacterial cell walls is nearly high enough to explain the conductivities observed for intact cells (Carstensen, 1968).

Pauly (1962) demonstrated with protoplasts of *Micrococcus lysodeikticus* that the radio frequency dispersion of this organism arises from the presence of the cytoplasmic membrane rather than the cell wall. In the present study protoplasts are used to further confirm the importance of the bacterial cell wall in the low frequency, dielectric properties of bacteria.

It is shown here that the conductivity of the bacterial cell is very low if its cell wall is removed. Furthermore, removal of the cell wall from *M. lysodeikticus* reduces its low frequency, effective, homogeneous dielectric constant by more than two orders of magnitude. In this way, the use of protoplasts shows that the intact

cell wall is responsible, not only for the high conductivities, but also for the high dielectric constants of bacteria at low frequencies.

EXPERIMENTAL PROCEDURE

Bacteria, Growth Conditions, and Preparation

A complete series of dielectric measurements was carried out on intact cells and protoplasts of *M. lysodeikticus*. The organism was grown in a shaken broth medium (3% Oxoid tryptone, 1% glucose, $\frac{1}{10}$% yeast extract) at 33°C for 48 hr. After harvesting the cells were stored at 1°C.

1. *Bacteria.* The maximum storage time for bacteria used in this investigation was 5 days. Upon removal from storage the bacteria were washed thrice in 2 M sucrose.[1]

2. *Protoplasts.* The cells used for preparations of protoplasts were stored at 1°C no longer than 24 hr. Upon removal from storage they were added to a 2 M sucrose solution containing 0.35 mg/ml lysozyme. After wall removal was completed (judged by the lysis of cells when added to demineralized water) the protoplasts were harvested and washed in 2 M sucrose. Maximum storage time for protoplasts used in this investigation was 14 days.[2]

Before measurements were made, both intact cells and protoplasts were washed twice in the desired suspending solution. The supernatant from the second wash was saved and used to resuspend the particles to the desired volume fraction.

Size Determination

The radius of protoplasts of *M. lysodeikticus* was determined by use of a Nikon inverted, phase contrast microscope (Nikon, Inc., New York) with a 35 mm camera attachment. Photographs were made of protoplasts in 2 M sucrose which had been frozen and thawed before sizing. The protoplasts ranged in radius from 0.3 to 0.44 microns. From fifty measurements of photographic images the average radius was 0.36 μ with a standard deviation of 0.06 μ.

Electrical Conductivity and Dielectric Constant

If spherical particles with an effective, homogeneous, complex conductivity σ_2^+ are suspended in material of complex conductivity σ_1^+ at a volume concentration p, the complex conductivity σ^+ of the suspension is given by the equation (Fricke, 1955)

$$\frac{\sigma^+ - \sigma_1^+}{\sigma^+ + 2\sigma_1^+} = p\,\frac{\sigma_2^+ - \sigma_1^+}{\sigma_2^+ + 2\sigma_1^+}. \qquad (1)$$

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[1] M denotes molal; M denotes molar.

[2] The protoplast pellets were also frozen at −10°C. To test the effects of freezing and thawing, the protoplasts were allowed to thaw for 1 hr. The protoplasts were then prepared for measurement. Measurements by White[3] indicated that the dispersion in the frequency range 1–200 MHz is unaffected by freezing and thawing. Since the relaxation frequency was unaffected, it is reasonable to assume that the protoplast radius is also unaffected by freezing and thawing.

[3] White, L. A. 1967. Unpublished report.

Here the complex conductivity is given by

$$\sigma^+ = \sigma + i \omega \epsilon_0 \kappa \quad (2)$$

where σ is the conductivity, ω is the angular frequency and κ is the relative dielectric constant, $\epsilon_0 = 8.854 \times 10^{-12}$ farad/m is the permittivity of free space. Whenever possible, the value of p was kept close to 0.3 to insure accurate determinations of σ_2^+ (Einolf, 1967).

1. *Low-Frequency Admittance Bridge.* A bridge and measurement procedure similar to those described by Schwan (1963) were used to measure dispersion in the conductance and capacitance in the frequency range from 20 Hz to 200 kHz. The diameter of the sample holder was 9.53 mm. For greatest accuracy all measurements were made by substitution. Electrode polarization was reduced by coating the platinum electrodes with platinum black. Electrode polarization was eliminated from the measurements by using two electrode distances and calculating the difference between the resultant complex impedances. With the low salt concentrations used and the high dielectric constants observed in this study, the error due to electrode polarization was 5 per cent at 20 Hz. Corrections for the stray field in the sample holder were also made. Drift in the sample admittance with time due to temperature changes or bacterial cell leakage was observed by repeating the measurement at 1 kHz. All measurements were then interpolated to one point in time. All dispersion measurements were made at 25°C.

2. *Wayne Kerr Bridge.* Routine conductivity measurements were made at a frequency of 1592 Hz with a Wayne Kerr Universal Bridge (Wayne Kerr Lab., Ltd., Chessington, Surrey, England). The bridge and conductivity cells have been previously described by Carstensen (1965). All measurements were made at 25°C.

3. *RX Meter.* A Boonton RX Meter (Boonton Radio Corporation, Rockaway Township, N. J.) was used to measure complex conductivities over the frequency range from 0.5 to 200 MHz. The sample holder and calibration procedures were similar to those described by Pauly and Schwan (1966).

Volume Fraction Determination

The volume fraction of protoplasts in the suspensions used for dielectric studies was determined initially from conductivity measurements. When the conductivity σ_2 of the suspended particle is much less than the conductivity σ_1 of the suspending medium Equation (1) reduces to

$$p = 2\frac{\sigma_1 - \sigma}{2\sigma_1 + \sigma}. \quad (3)$$

As discussed later the effective, homogeneous conductivity of the protoplasts is less than 0.001 mho/m. When the environmental conductivity σ_1 is 0.29 mho/m (2 M sucrose, 0.1 M NaCl), the error in p due to a nonzero value of σ_2 would be less than 2%. In this manner the volume fraction for protoplasts in 2 M sucrose, 0.1 M NaCl was determined. Since 2 M sucrose was used for the environment, it may be assumed that the protoplast volume is not significantly affected by changes in salt concentration over the range 0.01 to 0.1 M NaCl.

The volume fraction of intact cells was determined by the procedure described by Carstensen et al. (1965). This was a diluent technique using a 1% dextran (mol wt 150,000)

solution. Dextran concentrations were determined colorimetrically by the phenol-H_2SO_4 method (Dubois et al., 1956).

EXPERIMENTAL RESULTS

For an accurate determination of the effective conductivity σ_2 of a suspended particle from measurements of suspensions it is desirable to have the conductivity σ_1 of the suspending medium approach that of the particle (Carstensen, 1967 *b*). With protoplasts this was not possible. Although the cells were washed repeatedly in 2 M sucrose, leakage of ions set a lower limit for σ_1 at about 0.01 mho/m. Under these conditions the conductivities of suspensions of protoplasts were still significantly lower than σ_1. For this reason it has been possible to set only an upper limit for the value of the conductivity of the protoplast. As shown in Table I this upper limit is approximately 0.001 mho/m.

TABLE I

ESTIMATE OF THE UPPER LIMIT FOR THE CONDUCTIVITY σ_2 OF THE PROTOPLAST OF *M. LYSODEIKTICUS**

Exp. No.	σ	Initial σ_1	Final σ_1	Estimated σ_1	p	Calculated σ_2
						mho/m
1	0.00846	0.00352	0.0171	0.0136	0.29	0.00092
2	0.00769	0.00389	0.0150	0.0120	0.29	0.00073
3	0.00755	0.00323	0.0143	0.0115	0.29	0.00102
4	0.00867	0.00335	0.0175	0.0140	0.31	0.00056

* Samples of the suspending medium taken before and after the measurement of σ indicate an increase in σ_1 due to leakage of ions from the protoplasts. The rate of change of σ_1 could be estimated from measurements of σ with respect to time. With this it was possible to interpolate between the initial and final values of σ_1 and thus to estimate the value of σ_1 corresponding to the value of σ given in the table. Volume fractions p were determined as indicated in the previous section.

Dielectric measurements of intact cells and protoplasts of *M. lysodeikticus* in 2 M sucrose were obtained with the low frequency bridge. As shown in Fig. 1, the bacteria have a dispersion in the relative dielectric constant κ_2 between the frequencies of 500 Hz and 500 kHz (α-dispersion)[4]. Standard errors for the data are indicated by vertical bars. From a circle plot (Cole, 1941) the low frequency limit of the effective, homogeneous dielectric constant of the intact cell κ_2 is found to be $3.2 \cdot 10^5$ (Fig. 3). When the cell wall is removed, κ_2 is reduced by at least two orders of magnitude at 1 kHz. Even if it is assumed that the relaxation frequency of the protoplast is somewhat lower than that of the intact cell, the low frequency limit of

[4] The nomenclature introduced by Schwan (1957) is used here. The term β-dispersion is used to identify the Maxwell-Wagner relaxation of the membrane-covered conducting particle. The term α-dispersion refers to a relaxation, regardless of its mechanism, which occurs below the frequency of the β-dispersion.

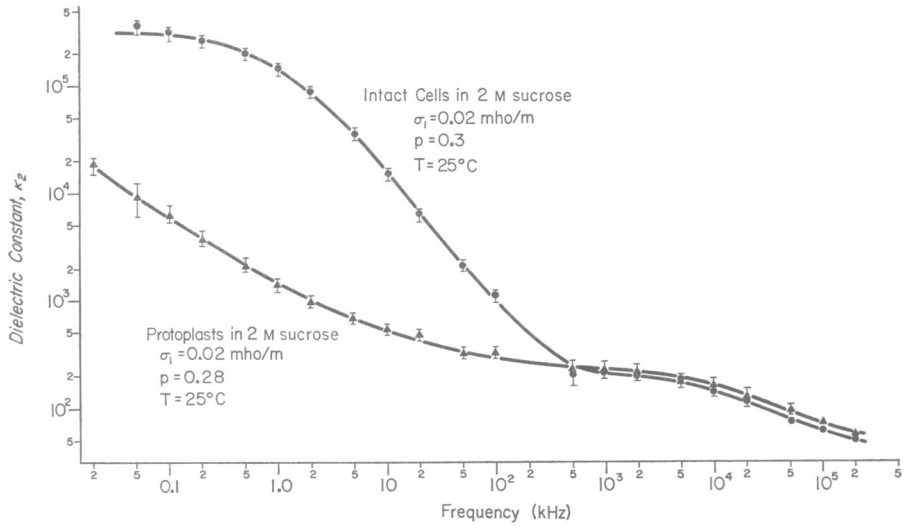

FIGURE 1 The effective, homogeneous dielectric constant κ_2 of intact cells and protoplasts of *M. lysodeikticus* as a function of frequency.

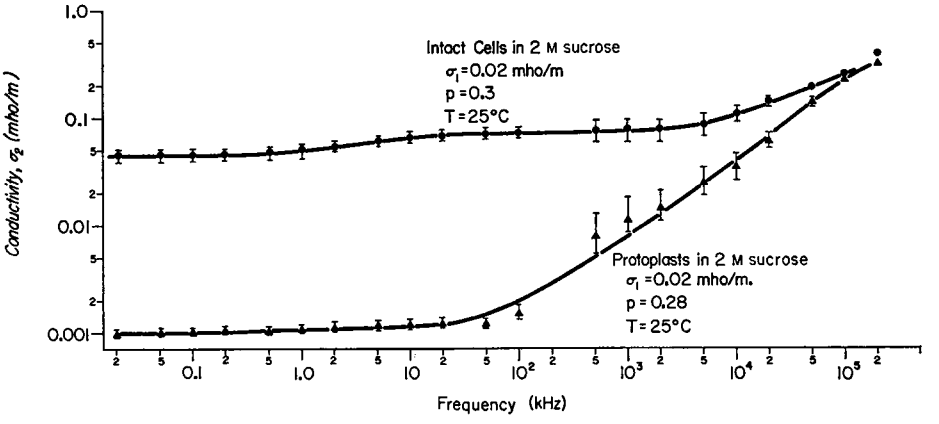

FIGURE 2 The effective, homogeneous conductivity σ_2 of intact cells and protoplasts of *M. lysodeikticus* as a function of frequency. The value of σ_2 for protoplasts was assumed to be 0.001 mho/m at 20 Hz.

the effective dielectric constant of the protoplasts is at least an order of magnitude lower than that of the intact cell (see Discussion). No significant change in κ_2 was noted for either protoplasts or intact bacteria when the environmental conductivity was increased about 10 times (2 M sucrose, 0.1 M NaCl).

The corresponding dispersion data for the conductivity of *M. lysodeikticus* and their protoplasts are given in Fig. 2. Although the low frequency bridge is very sensitive to changes in conductance with frequency, the accuracy of the absolute

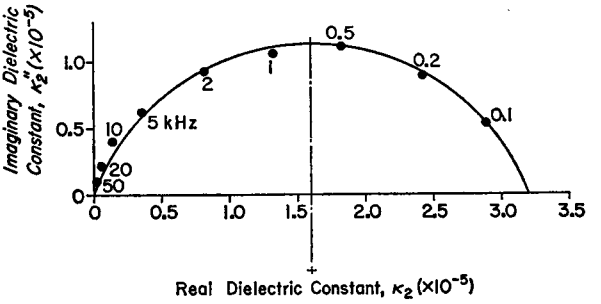

FIGURE 3 Cole-Cole circle plot of the real and imaginary, effective, homogeneous dielectric constant (κ_2 and κ_2'', respectively) of intact cells of *M. lysodeikticus* for the α-dispersion. The data presented in Figs. 1 and 2 are used here.

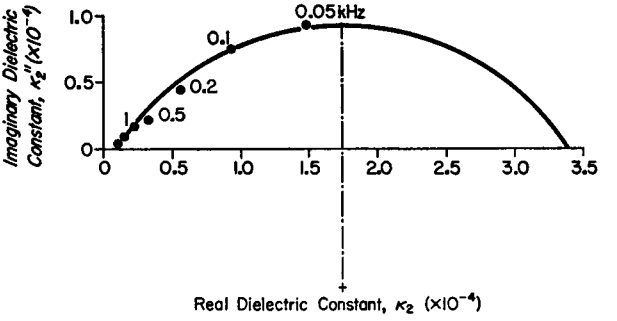

FIGURE 4 Cole-Cole circle plot of the real and imaginary, effective, homogeneous dielectric constant (κ_2 and κ_2'', respectively) of protoplasts of *M. lysodeikticus* for the α-dispersion. The data presented in Figs. 1 and 2, and the calculated value of $\Delta\kappa_2$ are used here.

values of the conductivities σ_2 calculated from these data depends upon a number of factors (Einolf, 1967). Since an upper limit of 0.001 mho/m has been obtained for σ_2 of the protoplasts, this value is simply assumed at 20 Hz in Fig. 2. The data show the pronounced drop in the low frequency, effective, homogeneous conductivity of *M. lysodeikticus* which accompanies removal of the cell wall.

A β-dispersion was also observed above 0.5 MHz for both protoplasts and intact cells. The results are in basic agreement with the data of Pauly (1962).

DISCUSSION

Although the evidence for the dielectric model of the bacterial cell which has been presented in earlier papers in this series (Carstensen, 1965, 1967 *a*, 1968) is reasonably convincing, the present study provides even stronger support for the model. Comparison of the dielectric data for intact bacteria and protoplasts clearly illustrates the dominant role of the cell wall in determining the low frequency dielectric properties of bacteria. Removal of the cell wall of *M. lysodeikticus* reduces the observed dielectric constant κ_2 by two orders of magnitude. Likewise, removal of

the wall reduces the effective, homogeneous conductivity σ_2 of this organism in 2 M sucrose from 0.045 mho/m to less than 0.001 mho/m. Previous studies with intact bacteria (Carstensen, 1967) set the lower limit of the resistance of the bacterial membrane at roughly 10^{-5} ohm·m². From the conductivity of protoplasts, however, it is now apparent that R_m must be somewhat greater than 10^{-4} ohm·m².

Even if the membrane resistance of the protoplast is infinite, it is likely that surface conductance arising from the presence of counterions near the surface of the cell would give it an effective, homogeneous conductivity of the order of 0.001 mho/m. A rough estimate of the magnitude of this effect can be obtained from measurements by Few, Gilby, and Seaman (1960) of the electrophoretic mobility of protoplasts of *M. lysodeikticus*. As shown in the Appendix, this approach leads to a surface charge density of roughly 0.02 coul/m² and, with some assumptions regarding the mobility of the counterions, to an effective, homogeneous conductivity for the protoplast which may be as high as 0.002 mho/m.

An interesting phenomenon is the increase in the effective, homogeneous dielectric constant κ_2 of protoplasts as frequency decreases. At low frequencies (20–500 Hz) this change in κ_2 appears to be linear on the log-log plot of Fig. 1. Schwan (1957) discusses mechanisms which may lead to this behavior.

One possibility is that the membrane capacitance itself may be frequency dependent (Cole, 1934). If the membrane capacitance is proportional to f^{-m} (where f is the frequency and m is a constant) in a manner similar to the polarization capacitance of metal electrodes in electrolytes (Schwan, 1966), the low frequency, effective, homogeneous dielectric constant of the cell would have a slope of $-m$ on a log κ_2 vs. log f plot. Observed values of electrode polarization capacitance can be represented over several decades of frequency with a value of m which is nearly constant. For protoplasts, however, Fig. 1 shows that the dielectric constant κ_2 levels off (slope $m = 0$) at approximately 330 for at least one decade in frequency around 0.5 MHz between the α-dispersion and the β-dispersion. This plateau shows that a frequency-dependent membrane capacitance is not a suitable explanation unless the slope m itself is frequency dependent. Such a model would give little insight into the mechanism of the α-dispersion.

Another possible explanation of the α-dispersion in protoplasts is related to the relaxation of the counter-ion layer near the charged surface of the cell. Schwarz (1962) was able to explain the dispersion in the dielectric properties of charged polystyrene spheres in terms of such a mechanism. Protoplasts are electrically similar to polystyrene spheres at low frequencies, i.e. the two particles are nearly equivalent in size, in fixed surface charge density, and in effective, low frequency conductivity.

For the Schwarz mechanism the magnitude of the dispersion in the dielectric constant $\Delta\kappa_2$ is given as

$$\Delta\kappa_2 = \frac{ae_0\Sigma}{\epsilon_0 kT} \qquad (4)$$

where $\epsilon_0 = 8.854 \times 10^{-12}\, f/m$ is the permittivity of free space, e_0 is the electronic charge, k is the Boltzmann constant, and T is the absolute temperature. Using the value of the surface ion density Σ calculated from electrophoretic mobility data (see Appendix) the low frequency limit of the dielectric constant κ_2 was calculated to be 3.2×10^4 for protoplasts. This value may be reasonable since the highest measured value for κ_2 was 1.9×10^4.

Schwarz's expression for the relaxation frequency f_0 is

$$f_0 = \frac{ukT}{\pi a^2}. \tag{5}$$

Using a counterion mobility u of 1.1×10^{11} mks units (Na ion in 2 M sucrose) leads to a relaxation frequency of 1.1 kHz. In contrast, the half value frequency for the dielectric constant from Fig. 1 is about 30 Hz (assuming $\Delta\kappa_2 = 3.2 \times 10^4$). The size of the protoplast was 0.36 μ with a standard deviation in the population of 0.06 μ. Thus, the distribution in size of the protoplast is too small to have a significant effect upon the relaxation frequency. Electrostatic interaction of the counterion with the charged surface of the cell can cause a reduction in the mobility. Schwarz (1962) discusses this effect which results in a distribution in the activation energies of the counterions at the protoplast surface. When the distribution in activation energies is considered, a mean relaxation frequency f_m results. This is somewhat lower than the relaxation frequency f_0. The mean relaxation frequency f_m was calculated from the protoplast data using the following equation given by Schwarz (1962)

$$f_m = \sqrt{\xi_0}\, f_0\, e^{-\bar{\alpha}/kT} \tag{6}$$

where $\bar{\alpha}$ is the minimum activation energy and ξ_0 is obtained from

$$\frac{2\Delta\kappa_2''}{\Delta\kappa_2} = \frac{2 \tan^{-1}\left(\dfrac{1 - \xi_0}{\sqrt{\xi_0}}\right)}{\ln \xi_0^{-1}}. \tag{7}$$

For the protoplasts, a value of 0.55 was obtained for the ratio, $2\Delta\kappa_2''/\Delta\kappa_2$, using the circle plot shown in Fig. 4. With the assumption that $\bar{\alpha}=0$, as used by Schwarz for polystyrene particles the mean relaxation frequency becomes 82 Hz. However, by setting $\bar{\alpha} = kT$, f_m can be made consistent with the dielectric constant data for protoplasts. Thus, the Schwarz mechanism is a possible explanation for the α-dispersion of protoplasts.

SUMMARY

Through a study of protoplasts this paper has illustrated that the bacterial cell wall is responsible for the high dielectric constant and conductivity observed for *Micrococcus lysodeikticus* at low frequencies. The effective, homogeneous conductivity

of the protoplast is at least an order of magnitude lower than that of the intact cell. The dielectric constant of the protoplast is nearly two orders of magnitude lower than that of the intact cell over most of the frequency range below 100 kHz. The protoplast itself has an α-dispersion which may possibly be explained by the theory of Schwarz for the low frequency dielectric dispersion of colloidal particles.

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APPENDIX

From electrophoretic mobility data it is possible to estimate the surface charge density $e_0\Sigma$ and the effective, homogeneous conductivity for particles in suspension. First the zeta-potential is computed from Henry's equation (Henry, 1931).

$$\zeta = \left(1 + \frac{\sigma_2}{2\sigma_1}\right)\frac{\eta}{\epsilon} U \text{ volts} \qquad (A1)$$

where ζ is the potential at the surface of shear of the bacteria relative to the potential of the bulk medium (zeta-potential), η is the viscosity, and ϵ is the permittivity. The surface charge density is then computed from (Abramson, 1931).

$$e_0\Sigma = \frac{2\sqrt{2N_0\epsilon kT}}{1 + \frac{a_i}{L_D}}\sqrt{c}\sinh\frac{ze_0\zeta}{2kT} \text{ coul/m}^2 \qquad (A2)$$

where

c = salt concentration
N_0 = Avogadro's number
L_D = thickness of the diffuse double layer (Debye length)
z = valence of counter- and co-ions
a_i = radius of the counterions = 2.67×10^{-10}m (sodium ion)

In order to calculate surface charge density from the electrophoretic mobility, the ratio $\sigma_2:\sigma_1$ must be known in Equation A1. However, if σ_1 is sufficiently high then the ratio $\sigma_2:\sigma_1 \ll 1$. The ratio $\sigma_2:\sigma_1$ for protoplasts in 0.05 M NaCl will be less than 0.01. The electrophoretic mobility, U, of protoplast membranes in 0.05 M NaCl was shown by Few, Gilby, and Seaman (1960) to be -2.8×10^{-8} m²/v-sec. The electrophoretic mobility of protoplasts and protoplast membranes was shown to be the same in 1 M sucrose at pH 7 (approximately -0.7×10^{-8} m²/v-sec). Assuming that if protoplasts could exist in 0.05 M NaCl, the mobility U for protoplasts would also be -2.8×10^{-8} m²/v-sec. From this mobility and Equations A1 and A2 the surface charge density of *M. lysodeikticus* protoplasts was calculated to be -0.02 coul/m².

The contribution of this surface charge density to the effective, homogeneous conductivity σ_2 of the protoplast would then be (Fricke, 1936)

$$\sigma_2 = \frac{2e_0^2\Sigma u}{a} \qquad (A3)$$

where u is the counterion mobility and a is the radius of the particle. With $e_0\Sigma = +0.02$ coul/m^2, $u = 1.1 \times 10^{11}$ mks units (the mobility of the Na ion in 2 M sucrose) and $a = 0.36$ μ, the effective, homogeneous conductivity σ_2 of the protoplast becomes 0.002 mho/m. As indicated in the Discussion, electrostatic interaction of the counterion with charged groups on the surface of the cell may cause a further reduction in the counterion mobility. Hence, 0.002 mho/m is probably an upper limit for the contribution of surface charge density to the effect tive, homogeneous conductivity of the protoplast.

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