

# A Dielectric Dispersion Technique for Measuring the Ionic Permeability of Internal Membranes of Isolated Chloroplasts

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*Summary.* A method has been developed in which a chloroplast suspension is placed between electrodes to which a variable AC potential is applied. The dielectric constant of the suspension varies inversely as the square root of frequency within the range 0.5 to 50 MHz. Results are consistent with the view that this dielectric dispersion is due to ion movement across the chloroplast internal membranes, under the influence of the applied potential. The slope of the dispersion depends on the permeability of the membranes, and thus enables the mobility of externally added ions to be calculated. Results imply that the internal membranes of sugar cane chloroplasts are more permeable to  $K^+$  than  $Na^+$ , and more permeable to  $Cl^-$  than  $CH_3COO^-$ . Results also confirm the view that Triton X-100 increases the ionic permeability of membranes.

Information about the structure and ionic conductance of the membranes of organelles may be obtained from the dielectric dispersion of a suspension of the organelles. (The dielectric dispersion is the variation in dielectric constant or conductivity of the suspension, when placed in a suitable cell, as the frequency of the AC potential applied to the electrodes is varied.) Such techniques have been developed by Schwan (1963) and Cole (1968*a*) and applied to red blood cells (Fricke & Morse, 1925), mitochondria (Pauli, Packer & Schwan, 1960), and other biological materials. In this paper the method has been extended to include suspensions of chloroplasts.

## Materials and Methods

### *Equipment*

The cell shown in Fig. 1 was designed to hold a 50  $\mu$ liter sample which was confined to the center of the electrodes, thereby ensuring linear field lines through the sample. A Churchill Chiller Thermo-Circulator was used to pump fluid through the perspex

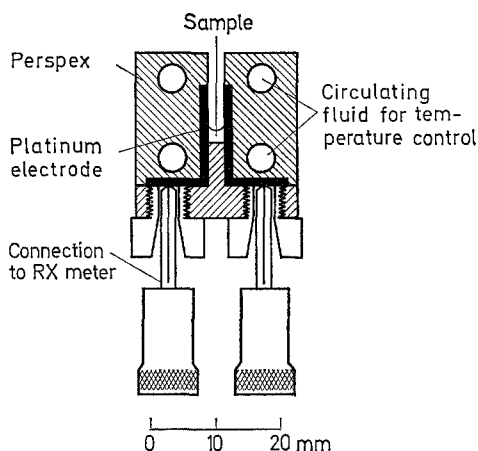


Fig. 1. Cell for dielectric measurements

jacket on either side of the electrodes, so that the sample was maintained at 10 °C. An AC potential of frequency between 0.5 and 50 MHz was applied to the electrodes by a Hewlett-Packard 250B RX meter, which also measured the parallel capacitance and conductance of the cell. The system was calibrated with standard fluids (formamide, water, aniline and air) to measure the dielectric constant, and with standard KCl solutions to measure conductivity. All calibrations were linear. The magnitude of inductive effects and electrode polarization was determined by replacing the sample by its supernatant, and results were corrected according to Mandel's Eq. 6b (Mandel, 1966). The dielectric constant of a single sample could be measured to within  $\pm 1\%$ , and its conductivity to within  $\pm 2\%$ .

### *Chloroplast Suspensions*

Sugar cane (*Saccharum sp.*, unknown variety) was grown in a greenhouse maintained at a minimum temperature of 10 °C. Chloroplasts were isolated by a modification of the method of Cockburn, Walker & Baldry (1968). We cut 20 g of deribbed leaf tissue into small strips and ground it in a domestic blender containing 200 ml of semi-frozen solution containing 0.33 M sorbitol, 5 mM MgCl<sub>2</sub>, 5 mM sodium iso-ascorbate and 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> · 10 H<sub>2</sub>O adjusted to about pH 6.5 at 4 °C with HCl. The macerate was squeezed through 2 layers and filtered through 8 layers of muslin, then centrifuged at 1,600 × *g* for 4 min at 4 °C. The supernatant was discarded and the pellet mixed and washed in 14 ml of 0.33 M sucrose solution and recentrifuged at 1,600 × *g* for 4 min. The supernatant was again discarded, and the pellet mixed into a small volume of 0.33 M sucrose solution to which an appropriate molarity of KCl or other compound was added.

Volume concentrations of chloroplast suspensions were measured using the Hawksley Micro-Haematocrit Centrifuge, operating at 12,000 × *g* for 15 min. Chlorophyll concentrations were measured in solution in 80% acetone, by the method of Bruinsma (1961).

### *Terminology*

Throughout the paper the following symbols are used:

$\omega = 2\pi \times$  frequency of applied potential ( $s^{-1}$ )

$\epsilon'$  = dielectric constant of suspension

- $\epsilon_0$  = permittivity for free space ( $\text{F m}^{-1}$ )  
 $\sigma$  = conductivity of suspension ( $\Omega^{-1} \text{m}^{-1}$ )  
 $\rho$  = volume concentration of chloroplasts in suspension  
 $\epsilon'_s$  = dielectric constant of supernatant  
 $\sigma_s$  = conductivity of supernatant ( $\Omega^{-1} \text{m}^{-1}$ )  
 $\epsilon'_T$  = dielectric constant of chloroplast stroma  
 $\sigma_T$  = conductivity of stroma ( $\Omega^{-1} \text{m}^{-1}$ )

For electrodiffusion across a membrane:

- $\tau$  = time constant or relaxation time (s)  
 $V$  = mobility of ions moving across the membrane ( $\text{m s}^{-1}/\text{V m}^{-1}$ )  
 $e$  = ionic charge (coulombs)  
 $D$  = diffusion coefficient ( $\text{m}^2 \text{s}^{-1}$ )  
 $c_0$  = concentration of ions in region of membrane ( $\text{m}^{-3}$ )  
 $\delta$  = membrane thickness (m)  
 $A$  = total area of membranes per unit volume of suspension ( $\text{m}^{-1}$ )  
 $k$  = Boltzmann's constant ( $\text{J } ^\circ\text{K}^{-1}$ )  
 $T$  = temperature ( $^\circ\text{K}$ )  
 $C_M, G_M$  = parallel capacitance, conductance per unit area of membrane ( $\text{F m}^{-2}, \Omega^{-1} \text{m}^{-2}$ )  
 $Z_M$  = impedance per unit area of membrane ( $\Omega^{-1} \text{m}^{-2}$ )  
 $y, y_s, y_c$  = effective complex conductivities of the suspension, supernatant, chloroplasts.

## Results and Discussion

### Dispersion Curves

The dielectric dispersion for cane chloroplast suspensions between 0.5 and 50 MHz is shown in Figs. 2 and 3.  $\epsilon'$  was linear with  $1/\sqrt{\omega}$ , and the slope  $S_{\epsilon'}$  of the dispersion increased with increasing KCl concentration. Within the experimental error  $\sigma$  was linear with  $\sqrt{\omega}$ , and the slope  $S_\sigma$

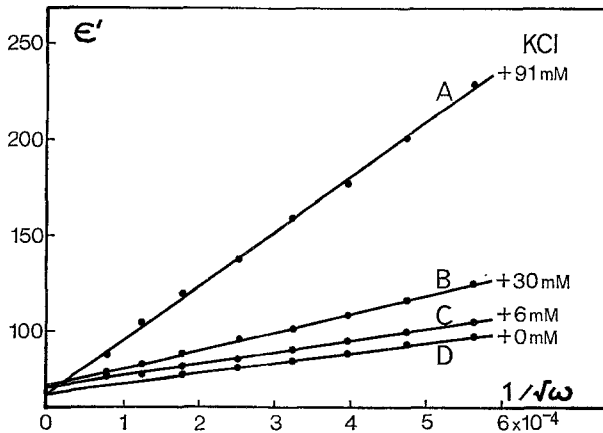


Fig. 2.  $\epsilon'$  dispersion for sugar cane chloroplast suspensions with added KCl. Suspension A contained 280  $\mu\text{g}$  chlorophyll per 0.1 ml; suspensions B and C 152  $\mu\text{g}/0.1$  ml; suspension D 150  $\mu\text{g}/0.1$  ml

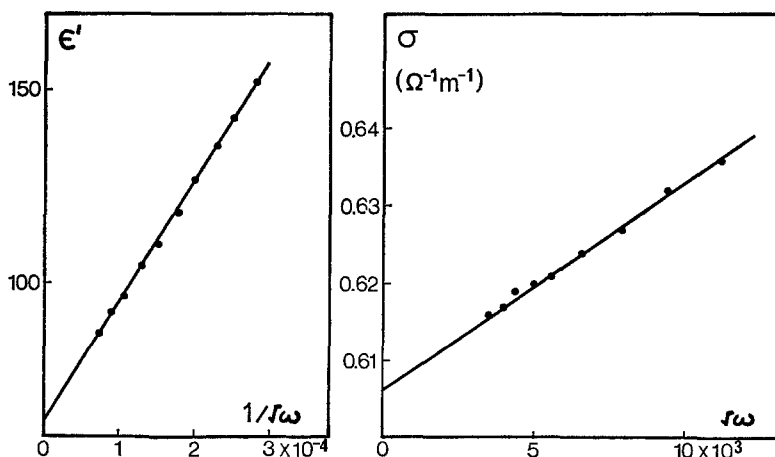


Fig. 3.  $\epsilon'$  and  $\sigma$  dispersions for cane chloroplasts, demonstrating a unity loss tangent for the dispersion. Suspension contained 219  $\mu\text{g}$  chlorophyll per 0.1 ml, and 96 mM KCl

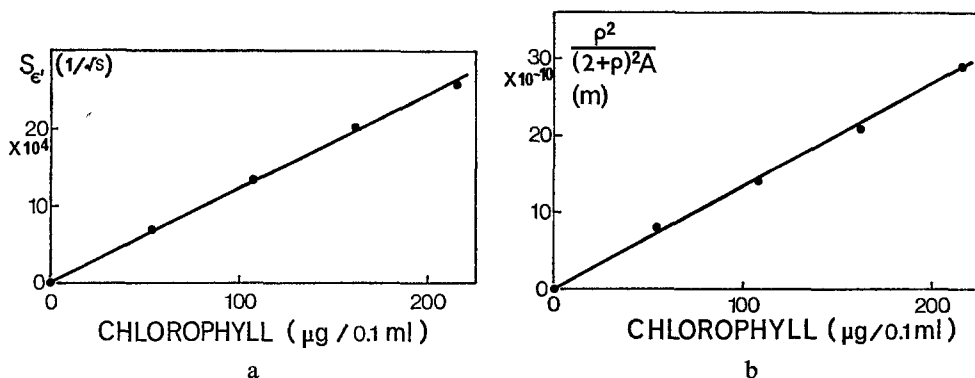


Fig. 4. (a) Effect of dilution on slope of  $\epsilon'$  dispersion for cane chloroplasts. Suspension contained 50 mM added KCl. (b)  $\rho^2/(2+\rho)^2A$  versus chlorophyll concentration during dilution experiment

for the conductivity dispersion was related to  $S_{\epsilon'}$  by

$$S_{\sigma} = \epsilon_0 S_{\epsilon'}$$

that is, the dispersion had a unity loss tangent within the error. However,  $S_{\sigma}$  could not be measured as accurately as  $S_{\epsilon'}$ , and therefore in further experiments the  $\epsilon'$  dispersion alone was measured.

In Fig. 4, a chloroplast suspension was diluted by adding more re-suspending medium at constant molarity of KCl.  $S_{\epsilon'}$  decreased approximately linearly with the suspension chlorophyll concentration. The dielectric constant of the supernatant did not vary between 0.5 and 50 MHz.

*Electrodiffusion Theory*

The observed linearity of  $\varepsilon'$  with  $1/\sqrt{\omega}$ , and the unity loss tangent for the dispersion, is reminiscent of the electrodiffusion theory for ions moving across a membrane under the influence of an applied potential. The impedance of such a membrane is given (Appendix I) by

$$Z_M = \frac{\sqrt{2kT}}{2c_0 e \sqrt{Ve}} \cdot \frac{1}{\sqrt{\omega}} \cdot (1-j). \quad (1)$$

Assuming that such electrodiffusion occurs mainly across the chloroplast internal membranes (evidence for this is given in the next section),  $\varepsilon'$  and  $\sigma$  for a chloroplast suspension would be (Appendix II)

$$\varepsilon' \approx \frac{2-2\rho}{2+\rho} \varepsilon'_s + \frac{6\rho \varepsilon'_T}{(2+\rho)^2} \left[ 1 - \frac{A\delta}{\rho} \right] + \frac{3\rho^2}{(2+\rho)^2} \cdot \frac{c_0 e \sqrt{Ve}}{\varepsilon_0 A \sqrt{2kT}} \cdot \frac{1}{\sqrt{\omega}}, \quad (2)$$

$$\sigma \approx \frac{2-2\rho}{2+\rho} \sigma_s + \frac{6\rho \sigma_T}{(2+\rho)^2} \left[ 1 - \frac{A\delta}{\rho} \right] + \frac{3\rho^2}{(2+\rho)^2} \cdot \frac{c_0 e \sqrt{Ve}}{A \sqrt{2kT}} \cdot \sqrt{\omega}. \quad (3)$$

This would therefore explain the linearity of  $\varepsilon'$  with  $1/\sqrt{\omega}$  at constant  $c_0$ , and the linearity of  $\sigma$  with  $\sqrt{\omega}$ .

From Eq. (2)

$$S_{\varepsilon'} \approx \frac{3\rho^2}{(2+\rho)^2} \cdot \frac{c_0 e \sqrt{Ve}}{\varepsilon_0 A \sqrt{2kT}}. \quad (4)$$

From Eq. (3)

$$S_{\sigma} \approx \frac{3\rho^2}{(2+\rho)^2} \cdot \frac{c_0 e \sqrt{ve}}{A \sqrt{2kT}} \quad (5)$$

and therefore  $S_{\sigma} = \varepsilon_0 S_{\varepsilon'}$ , as shown experimentally. On diluting a chloroplast suspension,  $A$  and  $\rho$  are decreased, and since  $S_{\varepsilon'}$  is linear with chlorophyll concentration (Fig. 4a) Eq. (4) indicates that  $\rho^2/(2+\rho)^2 A$  should also be linear with chlorophyll concentration. This was demonstrated experimentally (Fig. 4b).

*Location of Electrodiffusion*

Electrodiffusion might occur at the chloroplast outer envelope, or within the internal lamellar system. However, there is good evidence that the observed dispersions were due to diffusion at the internal membranes:

(1) Cane chloroplasts as isolated here were considered entirely outer-membrane-free, since they did not have the highly refractive appearance of intact spinach or pea chloroplasts viewed in the phasecontrast microscope under light ground illumination (Walker, 1967).

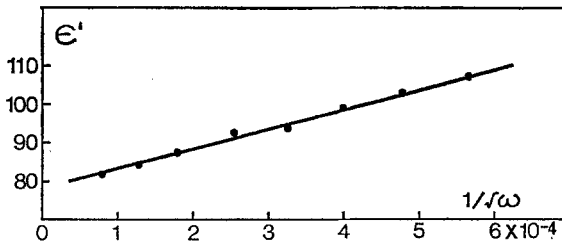


Fig. 5.  $\epsilon'$  dispersion for outer-membrane-free pea chloroplasts. Suspension contained 51  $\mu\text{g}$  chlorophyll per 0.1 ml, and 77 mM added KCl

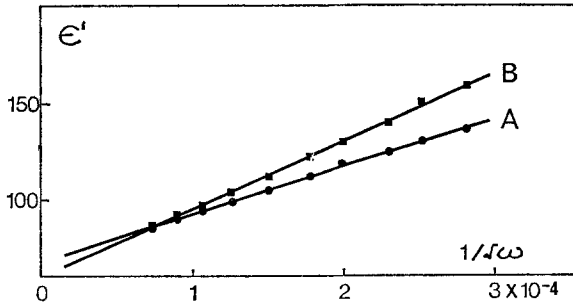


Fig. 6. Effect of Triton X-100 on  $\epsilon'$  dispersion for a cane chloroplast suspension containing 138  $\mu\text{g}$  chlorophyll per 0.1 ml. *A* has no added Triton X-100, and *B* has  $0.37 \times 10^{-6}$  ml Triton X-100 per  $\mu\text{g}$  chlorophyll;  $\rho_{\hat{A}} = .0863$ ;  $\rho_{\hat{B}} = .0975$

(2) Pea chloroplasts (*Pisum sativum* (L.) var. Laxton's superb) which were outer-membrane-free, showed a dispersion  $\epsilon'$  linear with  $1/\sqrt{\omega}$ , whose slope was comparable with that for cane suspensions of similar chlorophyll content and KCl concentration (Fig. 5). The pea chloroplasts were isolated as described in Materials and Methods except that they were washed in 14 ml deionized distilled water. The resulting osmotic shock caused the chloroplasts to lose their outer membranes.

(3) Neumann & Jagendorf (1965) found that small volumes of Triton X-100 added to a chloroplast suspension uncoupled photophosphorylation, and they considered it probable that the Triton increased the ionic permeability of the internal membranes, thereby increasing  $V$ . If this is so, and if the chloroplast dispersion is due to electrodiffusion across the internal membranes, then from Eq. (4)  $S_{\epsilon'}$  should increase on adding Triton X-100 to a chloroplast suspension. This was confirmed experimentally (Fig. 6).

#### *Ionic Permeability Studies*

In Fig. 7 the KCl concentration of a chloroplast suspension was increased from 0 to 100 mM.  $S_{\epsilon'}$  increased in a non-linear fashion, and the

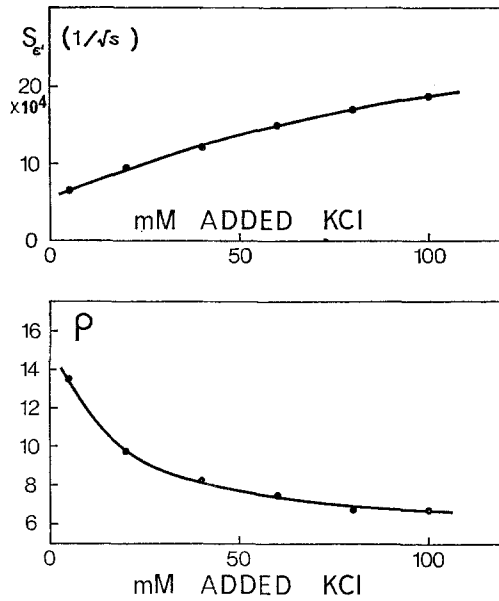


Fig. 7. Increase in  $S_e$  with added KCl, compared with decrease in  $\rho$  due to chloroplast shrinkage. Chlorophyll content of cane suspension: 127  $\mu\text{g}/0.1$  ml

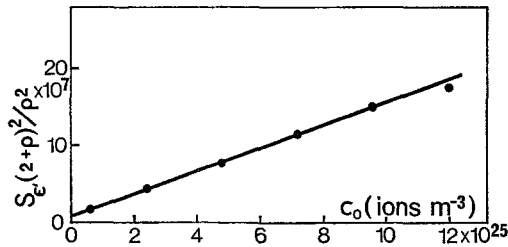


Fig. 8.  $S_e(2+\rho)^2/\rho^2$  versus concentration of added KCl. Chlorophyll content of suspension: 127  $\mu\text{g}/0.1$  ml as in Fig. 7

volume concentration  $\rho$  decreased, implying that the chloroplasts shrank due to the osmotic effect of added ions (Nishida & Koshii, 1964; Packer & Crofts, 1967). The dispersion observed in the absence of externally added KCl (Fig. 2) was probably due to undefined ions carried over from the isolation process (equivalent in conductivity to about 5 mM KCl).

Fig. 8 shows that between 0 and 80 mM KCl,  $S_e(2+\rho)^2/\rho^2$  was linear with  $c_0$ , which implies (Eq. (4)) that  $V$  was constant. Above 80 mM, the graph deviates from linearity, indicating a reduced ionic permeability, possibly due to saturation of the "pores" in the membranes (if these pores exist) or the membranes themselves, by the KCl ions.

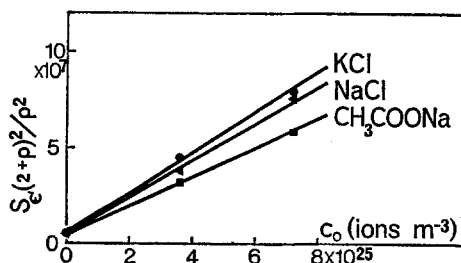


Fig. 9. Experiment to compare mobilities of KCl, NaCl and  $CH_3COONa$  ions, through the internal membranes of cane chloroplasts. Chlorophyll content of suspension:  $163 \mu g/0.1 ml$

Eq. (4) shows that the slope of the linear portion of the graph in Fig. 8 should be  $3e\sqrt{Ve}/\epsilon_0 A \sqrt{2kT}$ . Assuming the area of internal membranes to be  $160 m^2/g$  chloroplast protein (Mitchell, 1966) and since the protein to chlorophyll ratio is about 5 for cane chloroplasts, then knowing the chlorophyll content of a suspension,  $A$  can be calculated.  $T=283^\circ K$  and hence  $V$  may be determined. Three suspensions investigated in this way showed a mean mobility for KCl ions (anions+cations) of  $(3.1 \pm 0.4) \times 10^{-11} m s^{-1}/V m^{-1}$ , as compared with  $6.9 \times 10^{-8} m s^{-1}/V m^{-1}$  for 50 mM KCl in aqueous solution (from Kaye & Laby, 1966).

The mobilities of the ions of KCl, NaCl and  $CH_3COONa$  were compared using the dispersion technique (Fig. 9) and found to be in the ratio 1:0.8:0.5, respectively [compared with 1:0.83:0.58 in aqueous solution (Kaye & Laby, 1966)]. The concentration of each compound did not exceed 60 mM. This result indicated that the chloroplast internal membranes were more permeable to  $K^+$  than  $Na^+$ , and more permeable to  $Cl^-$  than  $CH_3COO^-$ .

Thus the dispersion technique proved to be an effective means of investigating the ionic permeability of chloroplast internal membranes.

## Appendix I

### Derivation of Eq. (1)

Electrodiffusion theory assumes that charge carriers or ions move between two parallel sites (e.g., across a membrane of thickness  $\delta$  (Racker, 1970)) under the opposing influences of an electric field and a charge concentration gradient. Theory predicts that for such a membrane

$$Z_M = \frac{\delta}{2ve^2c_0} \cdot \frac{\tanh\sqrt{j\omega\tau}}{\sqrt{j\omega\tau}}$$



where  $\tau = \delta^2/4D$ ,  $v = V/e$  and  $D = vkT$  (Cole, 1968*b*). If  $\tanh \sqrt{j\omega\tau} \rightarrow 1$  at high frequencies, then

$$Z_M = \frac{\sqrt{2kT}}{2c_0 e \sqrt{V} e} \cdot \frac{1}{\sqrt{\omega}} \cdot (1-j) \quad (\text{I-1})$$

$1/Z_M = G_M + j\omega C_M$  and, therefore,

$$C_M = \frac{c_0 e \sqrt{V} e}{\sqrt{2kT}} \cdot \frac{1}{\sqrt{\omega}}$$

$$G_M = \frac{c_0 e \sqrt{V} e}{\sqrt{2kT}} \cdot \sqrt{\omega}.$$

Thus the constant value for  $C_M$  [typically 1  $\mu\text{F}/\text{cm}^2$  for some biological membranes (Cole, 1968*a*)] is not encountered in this type of membrane, where  $C_M$  varies with frequency, ionic concentration and permeability.

## Appendix II

### *Derivation of Eqs. (2) and (3)*

For a suspension of chloroplasts, Maxwell's equation (Cole, 1968*b*) shows that

$$\frac{1-y/y_s}{2+y/y_s} = \rho \frac{1-y_c/y_s}{2+y_c/y_s} \quad \text{for small values of } \rho$$

where

$$y = \sigma + j\omega \epsilon' \epsilon_0$$

$$y_s = \sigma_s + j\omega \epsilon'_s \epsilon_0$$

$$y_c = \sigma_c + j\omega \epsilon'_c \epsilon_0.$$

Maxwell's equation would strictly apply only for spherical chloroplasts, but it is a good approximation for non-spherical particles, provided the eccentricity is not too large (Fricke, 1924).

Solving Maxwell's equation for  $y$ ,

$$\begin{aligned} y &= y_s \frac{2y_s(1-\rho) + y_c(1+2\rho)}{(2+\rho)y_s + (1-\rho)y_c} \\ &= \frac{2(1-\rho)}{(2+\rho)} y_s + \frac{9\rho y_c}{(2+\rho)^2} + \text{terms of higher power in } y_c. \end{aligned}$$

Assuming  $y_c \ll y_s$ , the terms of higher power in  $y_c$  may be ignored, and

$$y \approx \frac{2(1-\rho)}{(2+\rho)} y_s + \frac{9\rho y_c}{(2+\rho)^2}.$$

A chloroplast contains membrane lamellae lying parallel to each other, and surrounded by stroma. On the assumption that the stroma is highly conducting compared with the membranes, chloroplasts which lie with their membranes perpendicular to the applied field [effectively one-third of the total population (Pauli *et al.*, 1960)] will offer greater resistance to ionic movement than those which lie with their membranes parallel to the field.

Since the volume of membranes per unit volume of suspension is  $A\delta$ , the density of membranes within a chloroplast is  $A\delta/\rho$ . It can be shown that the effective conductivity of the chloroplasts lying perpendicular to the applied field approximates to  $G_M\delta/(A\delta/\rho)$  and the effective conductivity of the remaining two-thirds of the chloroplast population approximates to  $\sigma_T(1 - A\delta/\rho)$ . Thus, the mean chloroplast conductivity

$$\sigma_c \approx \frac{1}{3}(G_M\rho/A) + \frac{2}{3}\sigma_T(1 - A\delta/\rho).$$

Similarly,

$$\varepsilon'_c \approx \frac{1}{3}(C_M\rho/\varepsilon_0 A) + \frac{2}{3}\varepsilon'_T(1 - A\delta/\rho).$$

Expressions for  $C_M$  and  $G_M$  were derived in Appendix I.

Substituting for  $y$ ,  $y_s$  and  $y_c$  in the equation for  $y$ , and equating real and imaginary parts of the equation

$$\varepsilon'_c \approx \frac{2-2\rho}{2+\rho} \varepsilon'_s + \frac{6\rho\varepsilon'_T}{(2+\rho)^2} \left[ 1 - \frac{A\delta}{\rho} \right] + \frac{3\rho^2}{(2+\rho)^2} \cdot \frac{c_0 e\sqrt{Ve}}{\varepsilon_0 A\sqrt{2kT}} \cdot \frac{1}{\sqrt{\omega}}, \quad (\text{II-1})$$

$$\sigma_c \approx \frac{2-2\rho}{2+\rho} \sigma_s + \frac{6\rho\sigma_T}{(2+\rho)^2} \left[ 1 - \frac{A\delta}{\rho} \right] + \frac{3\rho^2}{(2+\rho)^2} \cdot \frac{c_0 e\sqrt{Ve}}{A\sqrt{2kT}} \cdot \sqrt{\omega}. \quad (\text{II-2})$$

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## References

- Bruinsma, J. 1961. A comment on the spectrophotometric determination of chlorophyll. *Biochem. Biophys. Acta* **52**:576.
- Cockburn, W., Walker, D. A., Baldry, C. W. 1968. The isolation of spinach chloroplasts in pyrophosphate media. *Plant Physiol.* **43**, no. 9:1415.
- Cole, K. S. 1968a. Membranes, Ions and Impulses. University of California Press, Berkeley and Los Angeles.
- Cole, K. S. 1968b. Membranes, Ions, and Impulses. University of California Press, Berkeley and Los Angeles. pp. 22, 174, 182, 186.
- Fricke, H. 1924. A mathematical treatment of the electrical conductivity of colloids and cell suspensions. *J. Gen. Physiol.* **6**:375.

- Fricke, H., Morse, S. 1925. The electric resistance and capacity of blood for frequencies between 800 and  $4\frac{1}{2}$  million cycles. *J. Gen. Physiol.* **9**:153.
- Kaye, G. W. C., Laby, T. H. 1966. Tables of Physical and Chemical Constants. Longmans, Green and Co. Ltd., London. p. 162.
- Neumann, J., Jagendorf, A. T. 1965. Uncoupling photophosphorylation by detergents. *Biochem. Biophys. Acta* **109**:382.
- Nishida, K., Koshii, K. 1964. Volume changes of isolated spinach chloroplasts caused by electrolytes and sugars. *Physiol. Pl.* **17**:846.
- Packer, L., Crofts, A. R. 1967. The energised movement of ions and water by chloroplasts. *In: Current Topics in Bioenergetics*. Vol. 2. D. R. Sanadi, editor. p. 23. Academic Press Inc., New York, London.
- Pauli, H., Packer, L., Schwan, H. P. 1960. Electrical properties of mitochondrial membranes. *J. Biophys. Biochem. Cytol.* **7**, no. 4:589.
- Racker, E. 1970. Membranes of Mitochondria and Chloroplasts. Van Nostrand Reinhold Co., New York. p. 26.
- Schwan, H. P. 1963. Determination of biological impedances. *In: Physical Techniques in Biological Research*. W. L. Nastuk, editor. p. 323. Academic Press Inc., New York.
- Walker, D. 1967. Photosynthetic activity of isolated pea chloroplasts. *In: The Biochemistry of Chloroplasts II*. T. W. Goodwin, editor. p. 53. Academic Press Inc., New York and London.