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Rotation of a single swollen thylakoid vesicle in a rotating electric field. Electrical properties of the photosynthetic membrane and their modification by ionophores, lipophilic ions and pH

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Rotation of single swollen thylakoid vesicles ('blebs') was induced by means of a rotating electric field of strength 104 $V \cdot cm^{-1}$, inducing a membrane voltage of 72 mV peak. Within the range of medium conductivities described (40–300 $\mu S \cdot cm^{-1}$), measurement of the field frequency (2–100 kHz) giving maximum rotation rate is equivalent to measuring the electrical time constant of the bleb membrane. Hence the membrane capacity (specific capacitance) was determined, and the value found at pH 8.1 (0.93 ± 0.07 $\mu F \cdot cm^{-2}$) is in agreement with values deduced from measurements using other techniques. However, the capacity was also found to decrease with pH: a minimum value of 0.77 ± 0.01 $\mu F \cdot cm^{-2}$ was measured at pH 4.4. The present study was extended to measurements of the effects of the lipid-soluble anion of dipicrylamine on the membrane capacity. At pH 7.2 and dipicrylamine concentration of 1.0 μM, a minimum estimate of the apparent membrane capacity was found to be 2.0 ± 0.2 $\mu F \cdot cm^{-2}$, with 2.6 ± 0.2 $\mu F \cdot cm^{-2}$ being observed at 5.0 μM concentration. In addition, it was found possible to measure the membrane resistivity (specific resistance) in the presence of either gramicidin (1.0 to 10 nM) or valinomycin (1.0 to 10 μM). In the case of gramicidin, it was possible to derive a maximum estimate of the mean channel conductance, and this agrees very well with the values for individual, single channels that may be deduced from artificial bilayer work. Unless the gramicidin channels in blebs are in fact substantially more conductive than in artificial bilayers, this indicates that a high percentage of the added gramicidin forms channels which are open for most of the time. In the case of valinomycin, a much greater amount had to be added to produce the same reduction of membrane resistivity as seen with a given concentration of gramicidin. However, calculations indicate that the majority of this effect is due to the difference in partioning behaviour of the two ionophores.

Introduction

When placed in strongly hypotonic solutions, chloroplasts swell greatly to produce relatively large spheres known as 'blebs' [1]. These blebs are 6–22 μm (10–18 μm in the work described here) in diameter and are bounded by what has been assumed to be a single bilayer [1,2] produced by unfolding of the thylakoid membrane system.

The dimensions of the blebs have two important consequences. The first is that their relatively large size means that application of a moderate field pulse to the medium causes a locally intense electric field to be induced in the mem-

brane. This has facilitated studies of effects such as electrophotoluminescence [3], a phenomenon related to the photosynthetic mechanism and to electrical phenomena at the membrane level [2–4]. The second consequence is that the wide size range of the blebs has complicated quantitative interpretation of this phenomenon. This is because the charging time of a vesicle in an externally applied electric field depends, amongst other things, on the size of the particles. Therefore any result obtained by the use of an optical membrane-voltage-sensitive probe will depend on the width of the size distribution, and so a natural approach to this problem would be to study a more homogeneous population. Indeed, this approach was adopted by Farkas et al. [5], using a size-selected population obtained using a fluorescence-activated cell-sorter.

On the other hand, observations of rotating-electrical-field-induced cell rotation [6–10] allow measurements on single cells or else on populations where the cell sizes can be individually measured. The torque responsible for cell rotation is dependent upon production of an induced dipole due to charge separation across the membrane. Hence values for membrane capacity and conceivably also resistivity may be derived from this technique. However, the presence of internal membrane systems in most cells means that assumptions regarding the contribution from each membrane must be made before the data can be interpreted [7,9,10]. Indeed, in the case of plant protoplasts the size-independent-scatter between cells (perhaps due to the presence of the very large vacuole) allowed only a lower limit of 30 $\Omega \cdot cm^2$ to be placed on the value of the effective membrane resistivity [9].

In view of the problems (associated with size-averaging) present in the studies of blebs discussed above, single cell rotation seemed to be a promising method with which to study this system. At the same time, these spherical vesicles (possessing no internal membranes) offered an almost ideal model system with which to explore the capabilities of the rotation technique. To this end, it was decided that the action of several membrane modifiers on blebs should be tested. These reagents (a pore former, a carrier molecule and a mobile ion) were all seen to give measurable effects consistent with their action in other systems.

Materials and Methods

Chloroplasts from lettuce (*Lactuca sativa*) or from spinach (*Spinacea oleracea*) leaves were prepared by the method of Avron [11] and stored at −180°C. The procedure used was that developed by Farkas and Malkin [12] to give optimal preservation of photosynthetic activities.

The chloroplasts were induced to form blebs by resuspending the thawed suspension at 1 : 1400 ratio in double-distilled water and adjusting the pH value to 8.0 ± 0.1 when necessary. After standing at room temperature for 15 min, a further 1 : 10 dilution was performed, using dilute KCl solution of the required conductivity. The final chlorophyll concentration of 1.2 $\mu g \cdot ml^{-1}$ reflects the very high dilution necessary to enable the study of single blebs whilst being able to eliminate interference from neighbouring ones.

Conductivity of the bleb suspensions was measured at 40 or 333 Hz using a digital conductometer (Knick GmbH, Berlin). Measurements taken before and after the rotation observations showed no change in conductivity during this period. Before rotation-maximum frequencies were measured, the rotation chamber was washed at least twice with the suspension.

The temperature within the chamber was measured by means of a miniature thermocouple probe (part of the 'Technoterm 9500', sold by Testoterm KG, 7825 Lenzkirch, F.R.G.). Unless otherwise stated, the initial temperature was in the range of 19°C to 22°C. In order to ensure that the temperature did not rise excessively during application of the rotating field, temperature readings were taken before and after the measurement of rotation frequencies. During the measurements reported here, the increase in temperature was found to be 3 deg. C or less.

The rotating field was produced by superimposition of two linear fields in a four-electrode chamber. The cube-shape chamber described earlier [7,10] is necessarily rather deep in order to permit the layering of two solutions of differing density. This allows particles of intermediate density to be trapped in a homogeneous and well-defined field. For the work described here, the smaller and more robust chamber shown in Fig. 1 was used. In order to allow observations from above or

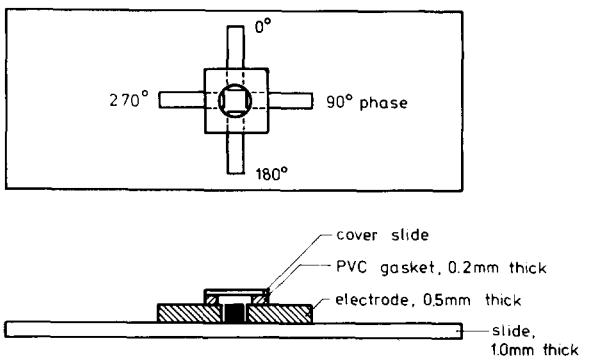

Fig. 1. Plan and side views of the four-electrode chamber used. The electrodes are of platinum, having a separation of 1.6 mm. The PVC (polyvinylchloride) and the electrodes are bonded to each other and to the microscope slide by use of epoxy resin. Colour-coded wires (not shown) connect the electrodes to a four-phase generator.

below, this chamber is shallower than the former type, and can carry a cover glass. Evaporation and the influence of air currents are thus eliminated. The reduced electrode separation results in a reduction of the voltage that must be available to produce a given rotation speed. The disadvantage of this small chamber, namely that solutions cannot readily be layered within it, is of no consequence in work with blebs. The density of the blebs is so close to that of water that vertical drift during measurements is not a problem as it is with cells, and so observations can be made on blebs in free suspension.

The four electrodes were supplied from a generator of four phases having the progressive 90° phase differences required to produce a rotating field. The generator was either of the type described earlier [7,10] or else of a novel design. The new generator manipulates the frequency and direction of the rotating field so that, when the frequency displayed matches the characteristic frequency of the cell under observation, a sharp null instead of a broad maximum in rotation rate is seen. The principles of this generator are described in Ref. 13. The production of the sharp null overcomes the problem of quickly estimating the central frequency of the rather broad maximum in rotation (which maximum was described in theory by Arnold and Zimmermann [7] and shown to be at least a good approximation by Glaser et al. [8]). Moreover, once the frequency is so close to the null value (between 2 and 100 kHz in this work) that rotation is slow, the applied voltage may be increased to allow a more precise measurement.

Due to this characteristic, the field strengths that are profitable to use with this direction-reversal technique are higher than those which give easily assessed rates of rotation in the simpler method [6–10]. In order to ensure that the experimental results are not artifacts due to the induction of unphysiologically high membrane voltages, the amplitude of the voltages that are induced must be estimated.

The peak membrane voltage (V_M volts) induced by a linear alternating field (peak amplitude E^0 volts $\cdot$ cm^{-1}) across the membrane of a cell decreases as the frequency of the field (f Hz) rises above the characteristic frequency of the cell [14]. The appropriate equation is [15,16]:

$$V_M = \frac{1.5 \cdot E^0 \cdot a \cdot \cos\theta}{\sqrt{1 + (2\pi f \cdot \tau)^2}} \quad (1)$$

where a is the cell radius, τ is the electrical time constant for charging of the membrane and the angle θ describes the orientation of the membrane patch under consideration with respect to the field lines. The medium and the cell membrane are assumed to behave as a pure resistance and a pure capacitance, respectively.

Using the rough estimate that the field throughout the four-electrode chamber is completely uniform, the highest field strength used in this work (20 volts peak across 1.6 mm) would be 125 V $\cdot$ cm^{-1}. However, field-mapping using a 400 $\times$ scaled-up-model chamber having a conductive-paper base shows that towards the corners and edges of the chamber, one or other linear component of the field is more intense than this. In the centre of the chamber (where observations on cells were carried out) the field is homogeneous and has orthogonal components which each have 0.83-times the amplitude given by the rough estimate above (i.e. 104 V $\cdot$ cm^{-1}, peak).

The highest membrane voltages will be induced when $f \ll (2\pi\tau)^{-1}$. In this case, Eqn. 1 predicts that a membrane voltage of 101 mV peak (72 mV r.m.s.) would be induced. On the other hand, as

discussed in association with Eqn. 4, rotation measurements are taken at the field frequency giving greatest torque, i.e. when $f=(2\pi\tau)^{-1}$, giving a reduction of the induced membrane voltage by a factor of $\sqrt{2}$. It can therefore be concluded that the measurements reported here were taken using induced membrane voltages of 72 mV peak (51 mV r.m.s.).

Observations were made using a microscope fitted with differential phase contrast optics. The diameter of the blebs was read against an eyepiece graticule or else against a scale on the screen of a video monitor. In both cases calibration was initially performed against an objective micrometer observed through the same thickness of liquid as present in the optical path in the experimental chamber. Determination of the direction of rotation was carried out with due regard to the possibility of left-right inversion at reflecting surfaces within the light path of the microscope.

Chemicals used were of 'Analar' or better grade; valinomycin was obtained from Sigma, gramicidin D from Calbiochem, and dipicrylamine (2,2',4,4', 6,6'-hexanitrodiphenylamine) from Fluka. Working solutions of these membrane modifiers were prepared in ethanol, with the addition of small amounts of KOH being necessary in the case of dipicrylamine.

Theoretical Considerations

The rotation of single cells in a planar homogeneous rotating electric field has been demonstrated previously [6–10]. The rotation appears to be due to the interaction of the rotating field with an induced cellular dipole. This dipole is due to charging of the membrane (a special case of interfacial or Maxwell-Wagner polarization, where the membrane can be considered as a double interface) and was originally observed as the rotation of adjacent cells in a linear applied field [17]. The charging time (τ) of a thin spherical membrane exposed to an external electric field has been given by Schwan [18] and by Jeltsch and Zimmermann [16]:

$$\tau = a \cdot C_M \frac{\sigma_i + 2\sigma_e}{2\sigma_i\sigma_e + \frac{a}{R_M}(\sigma_i + 2\sigma_e)} \tag{2}$$

where a is the radius (in cm), C_M and R_M are the specific membrane capacitance (in $\mu F \cdot cm^{-2}$) and resistance (in $\Omega \cdot cm^2$), respectively, and σ_i and σ_e (in $S \cdot cm^{-1}$) are the internal and external conductivities.

In the case of blebs, it has usually been assumed [5,19–21] that the internal and external conductivities are equal. When this is the case one may write $\sigma_i = \sigma_e = \sigma$ and therefore

$$\tau = a \cdot C_M \frac{3}{2\sigma + \frac{3a}{R_M}} \tag{3}$$

The case of unequal internal and external conductivities is considered in Appendix A. Earlier publications [17,7,9] have shown that, at least in the kHz region, the field frequency giving fastest rotation (the 'characteristic frequency', f_c) is that which matches the charging time (τ), the relationship being:

$$\tau = \frac{1}{2\pi f_c} \tag{4}$$

substituting the above for τ we obtain for Eqn. 3

$$f_c = \frac{1}{3\pi a C_M}\left(\sigma + \frac{3a}{2R_M}\right) \tag{5}$$

In practice, (see Refs. 7–9) it is desirable to include the radius of the blebs individually when the data are plotted and so the re-arranged form of Eqn. 5 is used

$$f_c \cdot a = \frac{\sigma}{3\pi C_M} + \frac{a}{2\pi R_M C_M} \tag{6}$$

Assuming R_M and C_M to be independent of frequency and conductivity, a plot of $f_c \cdot a$ against σ will give a straight line in which the gradient yields the membrane capacity and the intercept yields the membrane resistivity. (The case where R_M is not independent of conductivity is discussed in Appendix B).

The above implies that, given enough data, Eqn. 6 may be solved graphically or otherwise to yield values for R_M and C_M simultaneously. However, a linear plot is a pre-requisite for this procedure. Non-linear plots imply that one or more of R_M, C_M, or the inside : outside conductivity ratio

are changing over the plotted range. Further, unless it is absolutely certain that R_M is high enough to be ignored and that the conductivity ratio is known (see Appendix A), determinations at a single conductivity cannot yield unambiguous values for C_M: a plot should first be made over a sufficient conductivity range to derive a value for the slope.

If data from low enough conductivities are available, extrapolation to zero conductivity allows R_M to be estimated. The effect of a low membrane resistivity can be seen in the uppermost set of data of Fig. 2. The line fitted to the data should be compared with the lower-most line, which corresponds to the same membrane capacity but to a membrane resistivity which is too high to be measured.

Results

(a) Rotation of unmodified blebs

Application of rotating fields having frequencies in the kHz region readily induced rotation of blebs. The rotation was observed to be in the opposite direction to that of the field: this is consistent with earlier observations in this frequency region on cells, protoplasts and liposomes [7–10]. (In other frequency regions, rotation has been observed in the same direction as the field, see Ref. 10).

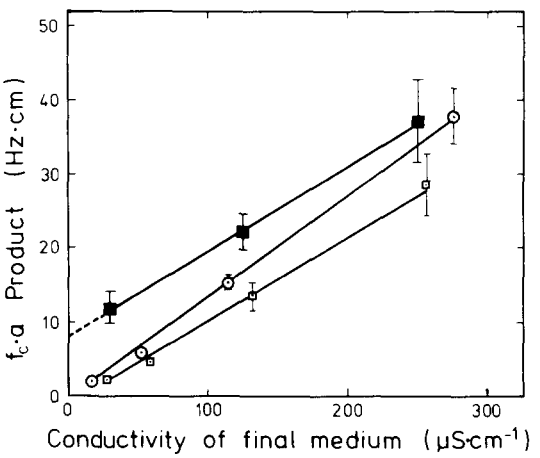

Fig. 2. Plots of the product of frequency giving optimum rotation (f_c, Hz) and of the bleb radius (a, cm) against the external conductivity. The conditions used were: ⊡, pH 8.2, no additions; ⊙, pH 4.4, no additions; ■, pH 8.2, in the presence of 5 nM gramicidin D. The increased slope (i.e., decreased membrane capacity) at pH 4.4 is readily apparent, as is the upwards shift and appreciable intercept due to the low membrane resistivity induced by gramicidin D. Each point is the mean and S.D. of 40 blebs, where no error bars are shown the S.D. is too small to display. Note that the percentage error remains approximately constant as the conductivity is raised: this indicates a random variability of the membrane capacity within the bleb population.

The frequencies giving optimum rotation of unmodified blebs were found to be a linear function of the conductivity of the medium (see Fig. 2). Plots such as the lower two in Fig. 2 (i.e. of unmodified blebs) intercepted the $f_c \cdot a$ axis, within the range of error, at the origin. The intercept was in fact more often below the origin than above it: the interpretation of this is as yet unclear. However, on the basis that an average intercept of 0.5 Hz · cm would be detectable, it may be calculated (Eqn. 6) that the unmodified bleb membrane resistivity must exceed 200 $\Omega \cdot cm^2$.

As can be seen from Fig. 2, the data points (each representing the mean values from 12 or more blebs) lay on accurately straight lines, even though individual blebs deviated from the mean by 10–15%. However, it should be noted that the scatter increased uniformly with the conductivity and therefore represented variation in membrane capacity within the bleb population. The sample size appears to have been adequate to give self-consistent mean values. The slopes of the plots (e.g. Fig. 2) varied with the pH of the medium. This reflects a pH-dependence of the membrane capacity, and this is shown in Fig. 3. Two differing methods were used to calculate this data; the first being that of taking the gradients of plots such that in Fig. 2, and then applying the first term in Eqn. 6. One set of points (⊙) in Fig. 3 was obtained in this way. As the calculation of each gradient requires the measurement of roughly 50 blebs, the taking of data over the full pH range took at least two days; this was thought to be undesirable because blebs prepared on successive days could not necessarily be relied upon to be identical.

However, once it had been established from these individual, linear plots that the membrane resistivity (at least at pH 4.4 and above) was too high to be significant, it became possible to derive meaningful capacity values from observations made at a single conductivity. Thus work over the

full pH range could be carried out in a single day. Two such runs are shown in Fig. 3 (△ and □) and it can be seen that the difference between the methods is small over most of the range.

However, in the case of unmodified blebs the use of the gradient method seems to give slightly lower estimates of the membrane capacity than does the use of measurements taken at a single conductivity.

On the other hand, at pH 3.3, the difference between the methods is relatively large, and it is certainly possible that at this pH the membrane resistivity is sufficiently lowered to invalidate the measurements taken at a single conductivity. Unfortunately, this point cannot be further investigated because the addition of sufficient HCl to bring the pH to 3.3 increases the medium conductivity to a level (170 $\mu S \cdot cm^{-1}$) at which the membrane resistivity cannot be estimated with the precision that is possible nearer to neutral pH.

The conclusion to be drawn from the above is that measurements, performed over a range of pH values within one day, confirm that the membrane capacity of blebs exhibits a well-defined minimum near pH 4.4, and that the membrane capacity rises with pH but is relatively pH-insensitive near neutrality. It is also evident that estimates of membrane capacity should be obtained by the use of the gradient procedure (in order to eliminate the possible influence of low membrane resistivity, see above).

The pH-dependence of membrane capacity is illustrated by the following results:

at pH 8.1 ± 0.1 $C_M = 0.93 \pm 0.07\ \mu F \cdot cm^{-2}$
(Mean and S.D. of six mean values obtained by the gradient procedure from six determinations involving over 300 blebs)

at pH 4.4 ± 0.1 $C_M = 0.77 \pm 0.01\ \mu F \cdot cm^{-2}$
(Mean and deviation of two mean values obtained by the gradient procedure from two determinations involving over 100 blebs).

The deviations quoted above mainly reflect variations between the means taken on different days. They also include errors due to the plotting procedure (which was done by eye). Each individual bleb measurement is subject to an additional uncertainty estimated at $\pm 7\%$ due to the radius ($\pm 5\%$) and conductivity ($\pm 2\%$) measurements. It can be concluded that a random variability of at least 8% must be present in the membrane capacity of the bleb population to account for the 15% variation from the mean values exhibited by individual bleb measurements.

(b) Rotation in the presence of gramicidin D

Gramicidin A, B and C are linear pentadecapeptide antibiotics secreted by *Bacillus brevis*. They form pores in membranes by selfassociation of two molecules, each spanning half the membrane. The pores are permeable to singly charged cations of 0.2 nm or less unhydrated radius (see for example, Refs. 22–24). The form used here (gramicidin D) is a mixture of the A, B and C forms, which differ only slightly from each other in properties or structure [25,26]. These molecules contain no hydrophilic groups, and have a solubility in water of less than 10^{-10} M [27]. Nevertheless, overall 'concentrations' much greater than this were used in suspensions of blebs. It must therefore be assumed that at least the majority of the gramicidin formed aggregates or became adsorbed to the bleb membranes: as will be shown in the Discussion, the latter seems to have been the case.

Gramicidin was found to increase the characteristic frequency of blebs (see Fig. 2). Initial work, using KCl as the only electrolyte, gave plots of $f_c \cdot a$ against σ which were not straight as in Fig. 2, but somewhat curved. Attempts at interpretation gave the impression that
(1) the specific capacitance seemed to be lower than in untreated blebs, and
(2) the specific resistance seemed to be altering with the KCl concentration.
It now seems that only the second effect was really occurring, and this was because the conductance of the gramicidin channel increases with the permeant ion concentrations, especially at low ionic concentrations [22,23,28], and this gives rise to artifacts as discussed in Appendix B.

In order to simplify the interpretation of the gramicidin results it was necessary to change the medium conductivity without alteration of the channel conductance. It is possible to do this because it is known from permeability studies on bilayers [28,29] that the gramicidin channel has a

very small permeability to the Me_4N^+ (tetramethylammonium) ion. In addition, the permability to chloride ions has also been shown to be small compared to the permeability to monovalent cations [29,27].

Therefore, the experimental approach adopted was to use a constant but low concentration (0.2 mM) of KCl in all work, the conductance of the medium being modified as required by the addition of Me_4NCl (up to a final concentration of 4 mM). It should be noted that although Ca^{2+} and other divalent ions are also unable to permeate the channel, they may nevertheless block it [30], and were therefore not as suitable as the Me_4N^+ ion.

Using the above technique, analysis of plots similar to Fig. 2 showed that the response to gramicidin was mainly due to a substantial drop in membrane resistivity, but that no significant change in membrane capacity could be seen. The membrane resistivity was found to decrease steadily as the gramicidin 'concentration' was raised over a wide range (see Fig. 4). Measurements using still higher calculated 'concentrations' than 10 nM were not possible because the rotation rate of the blebs became too slow to be useful. This is to be expected because extremely low values of membrane resistance short out the membrane voltage and prevent induction of the 'cellular' dipole.

The resistivity values shown in Fig. 4 were calculated either by application of the second term in Eqn. 6 to the intercept from plots such as that in Fig. 2, or else by taking the $f_c \cdot a$ values from just two conductivities and solving two simultaneous equations based on Eqn. 6. It can be seen that there is little difference in the results of the two procedures, although the intercept method seems to produce a smaller scatter between results taken on different days.

For reasons stated above, capacity values were

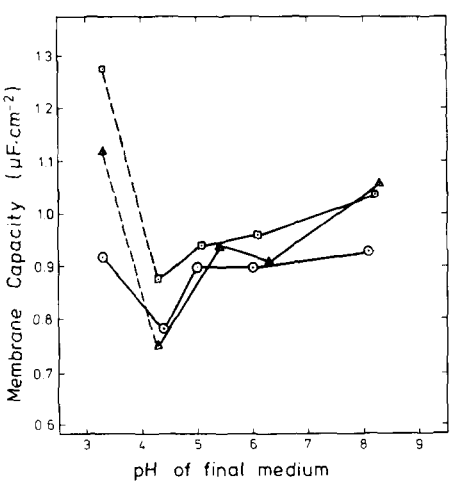

Fig. 3. The pH dependence of membrane capacity as revealed by data such as that shown in Fig. 2. The different curves display: ⊙, results determined by taking the gradient of lines such as the lower two in Fig. 2 (such a procedure could not be accomplished in a single day); ▲, values calculated from work at a single conductivity of 260 $\mu s \cdot cm^{-1}$; ⊡, results calculated from work at a single conductivity of 170 $\mu S \cdot cm^{-1}$. No error bars are shown in order to simplify the diagram, but are consistent with the results shown in Figs. 4 and 5. The salt used was KCl (0.2 to 4 mM).

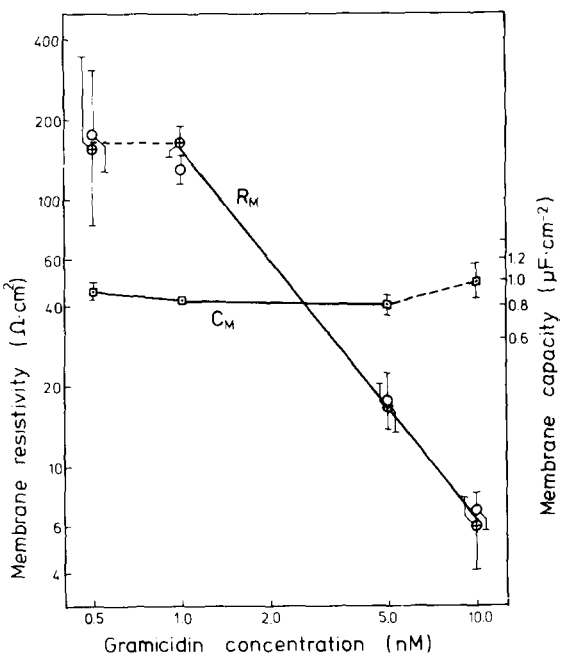

Fig. 4. Dependence of membrane resistivity and capacity on the calculated overall 'concentration' of gramicidin D. (Due to the extreme insolubility of gramicidin, it has to be assumed that effectively all the gramicidin partitions into the membranous material. For further details, see text.) The results for resistivity at 0.5 nM gramicidin 'concentration' are probably strongly affected by the intrinsic membrane resistivity, and are therefore considered too misleading to form part of the plot. Symbols: ○, R_M values taken from intercepts such as that shown by the upper-most curve in Fig. 2; ⊕, R_M values calculated from results obtained at just two different conductivities; ⊡, C_M values obtained from the slopes of plots such as in Fig. 2. Data points show the mean and S.D. (if significant) of the means from three determinations, each using 40–50 blebs. Conditions: pH 8.0–8.3; salts present were 0.2 mM KCl with from 0 to 4 mM tetramethylammonium chloride.

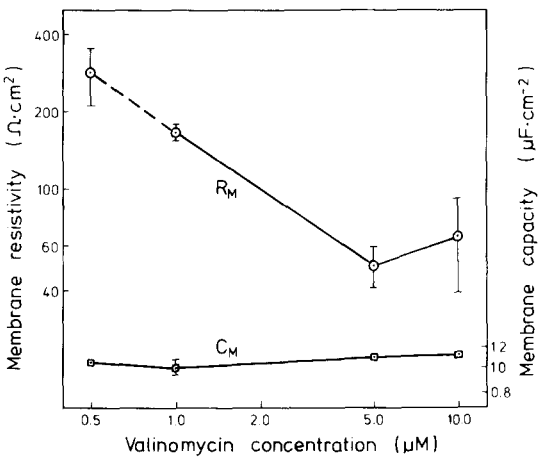

Fig. 5. Dependence of membrane resistivity and capacity on the concentration of valinomycin in the medium. Data points are the mean and S.D. of the means taken from three plots such as in Fig. 2, each plot using 40–50 blebs. Conditions: pH 8.0–8.3; salts present were 0.2 mM KCl with from 0 to 4 mM tetramethylammonium chloride.

always calculated from the gradients of plots such as that in Fig. 2. It can be seen from Fig. 4 that there is no significant change in membrane capacity as the gramicidin 'concentration' is raised.

(c) Rotation in the presence of valinomycin

The cyclic antibiotic valinomycin is known to make both artificial and natural membranes selectively permeable to the larger alkali cations. The action is that of a carrier molecule, both the neutral ionophore itself and its 1 : 1 complex with a cation being membrane soluble (for reviews, see Refs. 27, 31 and 32).

In order to eliminate the possibility that changing potassium ion concentrations could affect the valinomycin turnover rate, the conductivity of the medium was altered by the use of the non-permeable tetramethylammonium chloride (as in the case of gramicidin). When this was done, the plots of $f_c \cdot a$ versus conductivity for valinomycin-treated blebs were straight lines showing appreciable intercepts on the $f_c \cdot a$ axis. For reasons apparent from the above, the membrane capacity was obtained from the gradient, and the membrane resistivity from the intercept of these plots (Eqn. 6). It can be seen (Fig. 5) that valinomycin caused a similar drop in membrane resistivity to that seen with gramicidin D, but that the amount of ionophore which had to be added was 1000–2000-times higher. It is shown in the Discussion that the major part of this ratio was due to the fact that although valinomycin binds well to membranes, gramicidin binds much better.

(d) Rotation in the presence of dipicrylamine

Dipicrylamine is an acidic hydrophobic molecule present as the anion at neutral pH. The ion binds to membrane surfaces giving a layer of charge on either side of the membrane, as described by Ketterer et al. [33]. Application of membrane voltage causes a net movement of ions across the membrane: this gives (at least at low voltages) an apparent increase in the membrane capacity. Charge movements and capacity increases due to added mobile ions have been observed in artificial membranes [33–40] and in some cell membranes [41–43].

Rotation of blebs showed that the presence of 1.0–5.0 μM dipicrylamine caused a decrease in characteristic frequency, suggesting an increase in membrane capacity. This was confirmed by examination of $f_c \cdot a$ plots, which showed them to be linear up to field frequencies of 7–14 kHz, the gradient being considerably less than normal. (At higher frequencies the plots curved upwards, as if the mobile ions could not respond fast enough. As shown in the Discussion, this is consistent with measurements on other systems.) These $f_c \cdot a$ plots usually possessed a negligible intercept, therefore the membrane resistivity was not reduced below the detection limit of 200 $\Omega \cdot \mathrm{cm}^{-2}$.

Values of the total effective capacity, measured at frequencies less than 7 kHz, are as follows (conditions: pH 7.2 ± 0.3, temperature 22°C, blebs incubated with dipicrylamine for 30 min before measurement):

(1) At a dipicrylamine concentration of 1.0 μM,

$$C_M = 2.0 \pm 0.2\ \mu F \cdot \mathrm{cm}^{-2}.$$

(2) At a dipicrylamine concentration of 5.0 μM,

$$C_M = 2.6 \pm 0.2\ \mu F \cdot \mathrm{cm}^{-2}.$$

(The results are each the mean and S.D. of three mean values obtained by the gradient procedure

from six determinations. 40 blebs were measured in each determination.)

Discussion

(a) General

The specific capacitance of the photosynthetic membrane has been measured by several different methods based on the charging time. De Grooth et al. [19,20] measured the external-field-induced quadratic and linear electrochromic shifts in blebs and obtained capacity values of 2.0 and 1.7 $\mu F \cdot cm^{-2}$. By comparison, the value of 0.93 ± 0.07 $\mu F \cdot cm^{-2}$ (unmodified blebs at pH 8.1) derived here from cell rotation seems rather low. However, similar values (~ 1.2 and 1.2 ± 0.3 $\mu F \cdot cm^{-2}$) were found by Farkas et al. [21, 5] from observations of electrophotoluminescence, which serves as an intrinsic membrane-voltage-sensitive probe [1,3,4].

It is possible that the discrepancy between the results listed above may be due to the possibility of error (due to the assumption of equality of internal and external conductivities) inherent in all charging-time methods (see Appendix A). In this connection it may be worth noting that Packham et al. [44], using a method based upon knowledge of the absolute charge translocated across a bacterial chromatophore membrane, and of the voltage produced, found a value of 1.1 $\mu F \cdot cm^{-2}$ for the membrane capacity. This value is independent of the internal conductivity.

Nevertheless, it seemed worthwhile in the case of blebs to try to measure the internal conductivity directly. The method used was that found to be successful in the case of plant protoplasts, i.e. analysis of the signal from a modified Coulter counter under conditions of electrical breakdown of the membrane [45].

Unfortunately, the salt concentration (at least 75 mM) necessary for operation of this instrument caused drastic shrinkage or collapse of blebs. However, as shown in Appendix A, it is unlikely that the straight line plots such as in Fig. 2 would have been obtained unless the bleb internal conductivity was in fact equal to that of the medium.

When comparing the capacity values based upon the charging time, it may be significant to note that previously published values are based upon work at a single medium conductivity. On the other hand, the values presented here use results from a wide range of conductivities: this procedure allows identification and estimation of low membrane resistivities which can otherwise lead to errors in the capacity values.

A second issue that must be considered when comparing measurements performed by various methods is the possible voltage-dependence of the membrane capacity. This could be caused by electrostriction, i.e. field-induced membrane compression [46–48] or else by saturation of the orientation of high-dipole-moment membrane proteins [49]. However, during the course of this work (using external fields up to 104 $V \cdot cm^{-1}$, giving a membrane voltage of 72 mV peak for a typical-sized bleb) no change in the measured frequencies was apparent as the voltage was increased.

It is interesting to note that the other external-field-induced methods used to study the photosynthetic membrane have used considerably higher fields. In the electrochromic shift measurements, typically 1500 $V \cdot cm^{-1}$ [19] and 800 $V \cdot cm^{-1}$ [20] were used; in the electrophotoluminescence measurements, at least 1000–2000 $V \cdot cm^{-1}$ was applied [2–5], with a threshold membrane voltage of 240 mV being a pre-requisite for this phenomenon [5]. The agreement of the results reported here with those derived from measurements using much higher applied fields is evidence that no change in membrane capacity occurs over the range of membrane voltages from 70 to at least 600 mV.

(b) Effect of pH on membrane capacity

The cell rotation results presented above show a marked pH-dependence of the membrane capacity. Examination of publications on the electrophoretic mobility of chloroplasts highlights an interesting similarity. Studies conducted with chloroplast thylakoid membranes or with isolated intact chloroplasts have indicated the existence of an isoelectric point at pH 4.3 [50,51]. Furthermore it has recently been suggested that the membrane of the bleb originates from the stroma region of the thylakoid membrane [52,53]. The electrophoretic mobilities of vesicles enriched with stromal or granal lamellae are known to be the same [51], therefore we can assume a similar pH-dependent electrophoretic mobility profile for blebs and for

thylakoid membrane vesicles.

The coincidence of the minimum in bleb membrane capacity observed by cell rotation with the isoelectric point suggests the involvement of surface charge. The actual link between membrane capacity and surface charge is not clear, although it may be useful to note that a large change in the surface charge of thylakoid vesicles occurs between pH 2 and pH 6, and that this is due to the presence of aspartic and glutamic acid residues [50,51]. However, this does not necessarily mean that membrane proteins are the dielectric which is directly involved in the capacity change: the change in surface potential could act on the membrane as a whole.

(c) Effect of ionophores

By the use of ionophores the expected effects of a decreased membrane resistivity on cell rotation have been demonstrated. In the case of blebs in media of conductivity 40 $\mu S \cdot cm^{-1}$ or greater, the highest induced membrane resistivity that could be repeatably measured was 200 $\Omega \cdot cm^2$. Higher values should be measurable in media of lower conductivity. This is much less sensitivity than is possible in measurements on bilayers using external electrodes or on giant cells using an internal electrode, but was nevertheless sufficient to demonstrate the relative effects of two ionophores.

It has previously been shown by Barber [54] and by Farkas et al. [21,4] that gramicidin appears to be much more effective than valinomycin in lowering the resistivity of the thylakoid membrane. This was also seen in the work reported here, but the interpretation of this result in terms of ionophore concentration within the membrane is interesting.

In the case of gramicidin, an amount added which was sufficient to give an average concentration of 10 nM resulted in a membrane resistivity of 6 $\Omega \cdot cm^2$. As discussed in Results, this cannot be regarded as a true concentration because the solubility of gramicidin is so low that it is expected that all the ionophore binds to the available membrane surface. This is in agreement with the observation that gramicidin binds irreversibly to thylakoid membranes [55]. In the work presented here, the bleb concentration was $2 \cdot 10^8$ per litre, the mean radius of the blebs being 6.5 μm. The geometric area of the blebs contained in one litre of suspension was therefore 1060 cm^2. The total number of gramicidin molecules per litre is given by multiplying the Avogadro number ($6.0 \cdot 10^{23}$ mol^{-1}) by the molarity. At the quoted concentration of 10 nM, the number of molecules per litre is therefore $6 \cdot 10^{15}$, or approx. $6 \cdot 10^{12}$ per cm^2 of membrane. This implies a maximum density (assuming all molecules bind and form active channels) of active channels of $3 \cdot 10^{12}$ per cm^2 of membrane (two molecules per channel). It may therefore be seen that the observed membrane resistivity of 6 $\Omega \cdot cm^2$ (membrane conductivity 0.15 $S \cdot cm^{-2}$) is produced by a maximum of $3 \cdot 10^{12}$ channels per cm^2 of membrane. The minimum estimate of channel conductance is therefore $5 \cdot 10^{-14}$ S. If some gramicidin binds to other surfaces than bleb membrane, the active channels must have a still higher conductance.

Single-channel conductances in the concentration of KCl used here (0.2 mM) are probably too low to have been measured, however Urban et al. [28,23] give a linear extrapolation of results obtained from higher salt concentrations. From their data the unit channel conductance expected in 0.2 mM KCl is $6 \cdot 10^{-14}$ S. The agreement of this value obtained from d.c. measurements on artificial bilayers with the value obtained here by an a.c. method with thylakoid blebs is perhaps surprisingly good. However, it seems to be probable that a high proportion of the added gramicidin does become bound to blebs and gives rise to conducting channels across membrane in contact with the medium.

This is in contrast to measurements at low gramicidin concentrations on artificial bilayers which allow single channels to be observed. Under these conditions the channels have only a limited lifetime; however the channel lifetime is observed to increase rapidly as the solvent content and thickness decrease [27]. It may be reasonable to suppose that, at the relatively high concentrations of gramicidin used here in a natural (and therefore solvent-free) membrane, the percentage of time for which a given gramicidin molecule forms part of a conducting channel is very high.

In the case of valinomycin, the apparent sensitivity of the bleb membrane was one-to-two thousand times less than with gramicidin. However (as

discussed later in this section) valinomycin, unlike gramicidin, has a true partition coefficient between membranes and water: the value is approximately 10^4. The value given above for the area of bleb membrane per litre of suspension (1060 $cm^2 \cdot l^{-1}$) shows that one litre of suspension contains $2.65 \cdot 10^{-4}$ cm^3 of bleb membrane if the membrane hydrophobic core thickness is assumed to be 2.5 nm. This is a solvent : membrane volume ratio of $3.8 \cdot 10^6 : 1$. At this high dilution, even a partition coefficient as high as 10^4 will result in only a small fraction ($2.6 \cdot 10^{-3}$) of the added valinomycin binding to membranes.

On the basis of the above, it can be said that the apparent 2000 : 1 molar advantage of gramicidin over valinomycin seen at these high dilutions is reduced to roughly 5 : 1 if the concentrations of ionophore within the membrane are compared.

Although the measurements discussed above seem to agree with other work, it should perhaps be mentioned again that rotating field measurements use alternating current, whereas most measurements of the effects of ionophores have used direct current. This difference should be of little significance in the case of a pore-former such as gramicidin, where ion conduction involves no kinetically limiting steps once the pore is open. However, in the case of carriers not only must translocation across the membrane of the ion-ionophore complex occur, but the carrier must first bind and later release the ion. Investigations using the charge pulse technique on artificial bilayers have shown that these ion-binding steps are kinetically important [56,57] and that, in the case of valinomycin, they occur at the membrane-to-solution interfaces (Ref. 57, compare Ref. 58). This may be important if the field cycle time becomes shorter than the time-constant for ion dissociation, in which case the valinomycin complex may be expected to act less as a carrier and more as a mobile charge (compare dipicrylamine below). Under these conditions an effect on membrane capacity, not on membrane resistivity, might be predominant.

According to the data on artificial bilayers [57], the time-constants observed due to the individual steps involved in the valinomycin-mediated transport are in the range of 1–50 μs. The rotating field measurements used here applied alternating frequencies of period 10–200 μs. It was therefore expected that the conductance increment seen at d.c. should be measurable in full at the lower frequencies, but possibly not at the higher frequencies. In practice, no such effect was apparent: the plots of $f_c \cdot a$ against medium conductivity displayed the straight lines expected of a frequency-independent membrane resistivity.

A further difference between the ionophores was noted when they were present at relatively high concentrations. In the case of gramicidin, membrane resistivities of 6 $\Omega \cdot cm^2$ were easily and reproducibly achieved, and there was no significant change in membrane capacity (Fig. 4). On the other hand, the lowest membrane resistivity that could be reproducibly attained with valinomycin was approx. 50 $\Omega \cdot cm^2$. Further addition of valinomycin gave only an increase in variation between experiments. It is interesting to note that in this case a small but definite increase in capacity can be seen.

Possible causes of the capacity increase are significant changes in either the average dielectric constant of the membrane or else of the membrane thickness. The concentration of valinomycin required to cause such changes may at first sight seem to be beyond the range of this work, but it must be remembered that the concentration within the membrane depends critically on the membrane: solution partition coefficient of valinomycin. For thylakoid membranes, the value of this coefficient has been given as approximately 10^4 [55], which is supported by the value of $1.6 \cdot 10^4$ obtained for artificial bilayers [59]. Use of the round value at an added concentration of 5 μM leads to an expected concentration within the membrane of 50 mM, or roughly 5% (the molecular weight of valinomycin is 1111).

At such a concentration, an influence on membrane thickness certainly seems to be possible. On the other hand, the dieletric constant of valinomycin within the membrane needs only to be twice that of the unmodified membrane components in order to give a capacity increase of 5%, also sufficient to account for the observed results. It seems reasonable to assume a relatively high dielectric constant for valinomycin, as the ion-binding groups can be expected to be hydrated. Hydration of proteins leads to a rapid increase in

dielectric constant [49], and valinomycin, having a polypeptide-like structure, can be expected to act similarly. It must also be said that the valinomycin-cation complex may to some extent act as a mobile ion within the membrane. However, in the absence of kinetic data for valinomycin at or near saturating concentrations, it is at present difficult to say if this effect could give the observed capacity increase.

(d) Effect of lipid-soluble ions

It has only been possible to quantitatively analyse the data on the effect of dipicrylamine up to field frequencies of 7 to 14 kHz. However, up to this frequency region the results show that the thylakoid membrane can allow the translocation of a lipid-soluble ion, and that the resulting effective capacity can be well in excess of the value of nearly 1.0 $\mu F \cdot cm^{-2}$ which is typical of many biological membranes (Refs. 40, 49; but as noted below some membranes have considerably higher values).

The curvature in the plots of $f_c \cdot a$ versus medium conductivity beyond 7 to 14 kHz amounts to a decrease in membrane capacity at higher frequencies. This is expected from the theory of Ketterer et al. [33] if the period of the applied field becomes shorter than the time required for equilibration between the two layers of mobile charges. The characteristic frequencies of the blebs at a conductivity of 150 $\mu S \cdot cm^{-1}$ were between 7 and 14 kHz, which correspond to time constants of 11 to 23 μs. This is of the same order as the dipicrylamine-induced time constants (38 to 53 μs) of the solvent-free artificial bilayers used by Benz and Läuger [38].

The large capacity values measured here with dipicrylamine can be viewed as capacity increments above that of the unmodified membrane. That is, dipicrylamine concentrations of 1.0 and 5.0 μM produce capacity increments of 1.1 and 1.7 $\mu F \cdot cm^{-2}$, respectively. It is interesting to note the good agreement with the increment of 1.3 $\mu F \cdot cm^{-2}$ predicted by Ketterer et al. [33] from field-jump measurements on solvent-containing artificial bilayers. However, higher values have been measured in artificial bilayer systems. Thus an increment of over 10 $\mu F \cdot cm^{-2}$ (in 1 μM dipicrylamine) can be deduced from the results of Bruner [34], and an increment of 3.5 $\mu F \cdot cm^{-2}$ (in 0.1 μM dipicrylamine) was measured by Benz and Zimmermann [60].

The measurements reported here show no detectable change in membrane resistivity even in the presence of 5.0 μM dipicrylamine. However, dielectric dispersion theory (see, for example, Refs. 18, 49) predicts that the decrease in capacity with frequency seen here should be accompanied by an increment in membrane conductivity. This increment would only become fully effective at frequencies above 14 kHz and so the extrapolation of the low frequency data carried out here would not be expected to show it.

It is interesting to note that Bruner [34], working with artificial bilayers, showed that membrane voltages much above 50 mV caused some desorption of dipicrylamine anions and saturation of charge transport. The former might be expected to decrease the membrane resistivity for alternating current, whilst the latter gives a decrease in apparent capacity. Although no decrease in membrane resistivity was apparent in this work, it may be that the membrane capacities measured here are slightly lower than the maximum values, because the calculated peak membrane voltage of 72 mV may have slightly exceeded the linear region for charge transport.

The alteration of the capacity of natural membranes by added hydrophobic ions has seldom been observed, indeed no effect of dipicrylamine could be observed in work on cells of *Valonia utricularis* (Zimmermann and Benz, unpublished results) or on mesophyll protoplasts of *Avena sativa* (Zimmermann, Wendt, Salhani, Küppers and Arnold, unpublished results). However, Benz and Conti [41] were able to show a marked dipicrylamine-induced voltage relaxation in squid axon membrane. The time constant of 8–70 μs agrees with that of 11–23 μs observed in blebs. In addition experiments with frog nerve showed a dipicrylamine anion translocation rate constant even higher than that obtained with the thinnest of artificial lipid bilayers (the rate constant in such bilayers increases as the membrane thickness decreases [38]). These observations on nerve membranes, and the results presented here on the thylakoid membrane, show that at least part of these membranes acts similarly to a thin (2.5 nm or less

hydrocarbon thickness) lipid membrane, a result supported by studies of the effects of pressure and temperature on the dipicrylamine-doped squid axon [43].

Natural mobile charges seem to be present in several systems. Thus work using the charge-pulse method has already demonstrated the striking similarity between the membrane voltage decays of dipicrylamine-doped artificial bilayers and of the untreated membrane of the giant alga *Valonia utricularis* [60]. The apparent capacity in the giant algal system is roughly 10-times the value calculated for a lipid membrane, but the expected value is reversibly obtained by treatment with medium of pH 4.

These observations led to the conclusion [60] that the *Valonia* membranes contain natural mobile charges, which may form part of transport or turgor-pressure-sensing systems [61,62]. In addition, a membrane capacity of 5 $\mu F \cdot cm^{-2}$ has been found in investigations of the chloride pump of *Acetabularia mediterrania* [63,64]. The similarity of the effects of lipophilic ions in artificial bilayers to observations of nerve 'gating currents' and of muscle 'charge movements' has also been noted [40].

The detection of mobile charges and the measurement of membrane resistivity in natural membranes has previously required the use of large or giant cells so that an intracellular electrode may be used. It has been shown that the technique of single cell rotation can detect mobile charges and measure membrane resistivity changes in cell analogues of normal size. It therefore seems likely that the effect of substances which perturb the cell membrane (e.g., some types of ecotoxins) can be assessed by this method.

Appendix A

The derivation of the bleb membrane charging time and hence of the characteristic frequency f_c (Eqn. 5) assume equality of inner and outer conductivities. It is instructive to consider the consequences if the inner conductivity is in fact less than the outer. This would occur if ions do not cross the membrane as fast as water, and if insufficient time is allowed for equilibration during swelling of the blebs. The result will be that

$$\sigma_i = k \cdot \sigma_e \tag{A1}$$

where $k < 1$. Eqn. 6 then becomes

$$f_c \cdot a = \frac{k\sigma_e}{\pi C_M (2+k)} + \frac{a}{2\pi R_M C_M} \tag{A2}$$

Hence the values of C_M derived from work at single medium conductivities will be a strong function of k. For example, it is easy to calculate that when:

(1) $k = 0.5$, then $C_{M(real)} = 3/5\ C_{M(measured)}$ or

(2) $k = 0.1$, then $C_{M(real)} = 1/7\ C_{M(measured)}$.

In practice, when attempting to work over a range of conductivities, as σ is changed, the value of k can also be expected to change (except in the unlikely case that the membrane ion-permeability decreases in proportion to the rise in σ). This would not lead to a straight line when $f_c \cdot a$ is plotted against σ.

Hence the demonstration of straight lines over the range of conductivities used in this work (see Fig. 2) is itself evidence that the internal and external conductivities can be considered equal.

It is interesting to note that when $k \gg 1$, which has already been excluded here, the derived capacity becomes independent of k. This is the case originally considered in rotation work [5–7], namely that of a cell with a highly conductive interior, suspended in a relatively non-conductive medium.

Appendix B

It has been shown above (Eqn. 6 and Fig. 2) that plots of $f_c \cdot a$ product against medium conductivity are normally straight lines. The line corresponding to infinite membrane resistivity passes through the origin, whilst the effect of a low, medium-conductivity-independent membrane resistivity is to give a line parallel to, but above, the high resistivity case. A family of such lines is shown in Fig. 6. The parallelism implies that all lines will give the same C_M on application of Eqn. 6.

However, when the membrane resistivity decreases as the medium conductivity increases, which is likely in work with ionophores, the data acquired at increasing medium conductivities will shift to lines corresponding to decreasing mem-

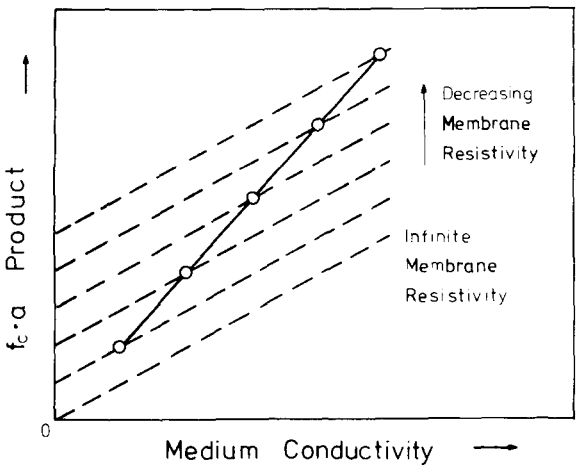

Fig. 6. Theoretical exercise to show the effect of a membrane resistivity which varies with the medium conductivity. The broken lines show 'ideal' cells or blebs the membrane resistivity of which is independent of medium conductivity. The solid line shows a worst case of membrane resistivity changing in such a manner that a straight line can be plotted which leads to a false estimate of the membrane capacity.

brane resistivities. In general, a curve rather than a straight line will result and so the experimenter is warned of the presence of behaviour not in accordance with Eqn. 6. If the conductivity-dependence is such that a straight line can be plotted through the data points, a false interpretation may be made based on a line such as that shown as solid in Fig. 6. Such a line will have a greater slope than the lines corresponding to constant membrane resistivity, and so the calculated membrane capacity will be less than the actual value.

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A forthcoming theory [65] analyses from first principles the response of a cell to a rotating field, and predicts both the observed directions of rotation, i.e. both with and against the field. The equations derived differ from the relatively simple ones used here especially at high frequencies and high medium conductivities. At the low frequencies and low conductivities used here, the difference between the membrane properties derived by analysis using one or other interpretation is within the accuracy of the data given here. Therefore, no alteration of the results of this work is called for in the light of the theory of Fuhr et al. [65].

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