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A METHOD FOR MEASURING THE KINETICS OF ENERGY-DEPENDENT CHANGES IN THE ELECTRICAL MEMBRANE RESISTANCE OF METABOLIZING PLANT CELLS

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SUMMARY

1. A method is described which enables the electrical potential across and the resistance of membranes of intact cells, and energy-dependent changes occurring therein, to be recorded simultaneously. Resistance changes are measured as changes in the amplitude of potential modulations brought about by stimulating square current pulses of low density.

2. Resistances of up to 120 $k\Omega \cdot cm^2$ of membranes with a capacitance of 1 $\mu F \cdot cm^{-2}$ can be recorded linearly with a response time of 3 s.

3. The method is illustrated with experimental results of light-induced changes in the potential across and resistance of the plasmalemma of *Nitella translucens*. The results are not inconsistent with the hypothesis that part of the membrane potential is maintained by an electrogenic ion pump. The potential generated by this pump decreases (membrane depolarisation) following a decrease in the membrane resistance, occurring in association with photosynthetic energy conversion.

INTRODUCTION

The energetic aspects of ion transport processes in plant cells and in energetically active cell organelles have received increasing experimental attention in recent years[1–6]. Among the several lines of experimental approach are electro-physiological studies on plant cells (*cf.* refs 1 and 7 for a review). There is renewed interest in measurements of electrical parameters of cellular and organellar membranes, notably the electrical potential difference and the electrical resistance, which are determining factors in the ion transport processes. The membrane potential is a component of the driving force for the passive movement of ions across the membranes. Ion fluxes on the other hand are dependent on the permeability characteristics of the membranes. The permeability coefficients of the several permeating anions and cations are constituting factors of the electrical conductance (and resistance) of the membrane. Electrical measurements in somatic cells and tissues of various origins (*cf.* refs 8–10

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; RC circuit, reactive resistance–capacitance circuit.

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for reviews) have indicated that at least part of the electrical potential across the membranes is maintained by an electrogenic ion pump. Changes in the membrane potential have been shown to occur in these cells upon alterations in energy supply to or conversion by the active pumps[11–14], and upon changes in the membrane conductance (resistance)[15]. Evidence is accumulating[16–18] that also in plant cells like *Nitella*, the membrane potential is controlled by an electrogenic pump.

Changes in the membrane potential have been observed to occur concomitantly with photosynthetic energy conversion in several plant cells[19–27]. Light-induced changes in the electrical resistance of the cytoplasmic membranes of *Nitella* and other algal cells have been reported[19,28,29]. These changes, which probably are also due to photosynthetic energy conversion in the chloroplast-localised photosynthetic apparatus, have been interpreted in terms of changes in the permeability coefficients of Na^+ and K^+[28]. So far light-induced changes in the membrane potential of plant cells and of *Nitella translucens* have been interpreted mainly in terms of changes in the passive membrane diffusion potential, due to primary ion translocations at the chloroplast membranes[21,22], although alternative interpretations have been discussed[25] and to some extent confirmed[30]. The fact that an electrogenic pump probably contributes to the membrane potential, and that light-induced changes in the membrane resistance occur, suggests the possibility that at least part of the potential changes observed is caused by changes in the potential generated by the electrogenic pump. This possibility has led us to measure the changes in electrical resistance and potential of the membranes simultaneously.

To our knowledge no suitable method is so far available, with which the kinetics of changes in potential and resistance can be recorded simultaneously as a function of time and of regulatory energy supply. This paper describes just such a method which has been found to be appropriate for this purpose. Preliminary results obtained with *Nitella* cells are presented. A short communication on the method was given at a recent meeting on photosynthesis research[31].

METHODS, MATERIAL AND RESULTS

Experimental arrangement

A schematic diagram of the experimental arrangement is shown in Fig. 1. A 3-M KCl-filled glass capillary micro-electrode (a), in contact with an Ag–AgCl wire *via* an agar–KCl bridge, is inserted into the cell. The signal of the electrode, with reference to an external Ag–AgCl electrode (b), is fed after impedance matching by a unity gain (XI) amplifier (IL Picometric Model 181) into a differential amplifier (Tektronix 3A3) of the oscilloscope (Tektronix 564). The output of the amplifier is connected to Channel 1 of a multichannel galvanometric recorder. Periodic changes in the membrane potential, induced by stimulating square current pulses of a certain frequency in the range between 1.25 and 8.5 Hz are not, or at least with decreased amplitude, followed by the galvanometer of the recorder, due to the lower frequency cutoff (0.5 Hz) of the reactive resistance–capacitance circuit (RC circuit) in this potential recording part of the system. These periodic changes are however traced by the resistance-measuring part of the system (see below).

Current is passed across the cell membranes through an Ag wire in contact with the 3-M KCl-filled glass micro-capillary (c), inserted into the cell, towards an Ag wire

(d) placed parallel and near to the cell in the external medium. An adopted method for calculating the electrical resistance of the membrane is to measure the change in the membrane potential in the steady state (ΔV) in the absence and presence of a current (I) applied during a short period. Hogg *et al.*[32] have shown for *Nitella* cells

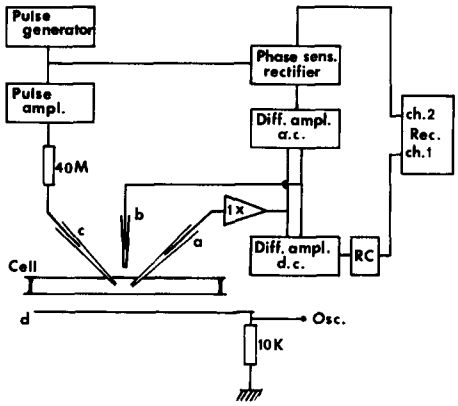

Fig. 1. Schematic diagram of the experimental arrangement for measuring the kinetics of energy-dependent changes in the electrical potential and resistance of the membrane of intact metabolizing (plant) cells. Explanations are in the text. ch. 1, channel 1; ch. 2, channel 2.

that when the current electrode is inserted into the cell at a distance of 0.42 L of the potential probing electrode, in which L is the half length of the cell, the electrical membrane resistance (R_m) is equal to $\Delta V/I$. In order to measure R_m as a function of the time during which the cell is energised or de-energised the current has to be applied intermittently over a certain time interval. This method, which would give individual points on the time curve, is rather time-consuming, especially when a time resolution better than 30 s is required. These shortcomings are circumvented with our method. Square current pulses of constant amplitude and frequency, usually in the range between 1.25 and 8.5 Hz, are injected into the cell. The output of a pulse generator (Tektronix 26G3), with reference to ground potential, is connected, after appropriate pulse amplification or attenuation, to the current circuit, consisting of a 40-MΩ resistor, internal electrode (c) and external electrode (d), which is grounded *via* a resistance of 10 kΩ. The amplitude of the current pulses is measured as the voltage drop across the 10-kΩ resistance and displayed on the oscilloscope. The pulses cause periodic changes in the membrane potential, probed by electrode (a). The modulated signal at the output of the unity gain amplifier, which can be monitored on the oscilloscope[31], is fed into an a.c.-coupled differential amplifier (Brookdeal Electronics, Type 432). The output of this amplifier is fed into a phase-sensitive rectifier (Brookdeal Electronics, Type 411), which is tuned to the frequency of the current pulses. The output of the rectifier is fed into a second channel of the recorder (ch. 2). This recorder signal is linearly proportional to the amplitude of the potential modulations of the membrane, brought about by the current pulses, and independent of the d.c. component of the potential probed by electrode (a), *i.e.* independent of the membrane potential. Thus, because current pulses of constant amplitude are applied, the recorder signal is proportional to the electrical membrane resistance. Changes in the membrane resistance, which may occur concomitantly with energy-dependent

metabolism in the cell, will be detected as proportional changes in the recorder deflection. As this part of the system only responds to signals of a frequency to which the rectifier is tuned, *i.e.* the frequency of the pulses, changes in the electrical membrane resistance are measured independently of energy-dependent changes in the membrane potential, provided that the latter occur with a rate constant which is lower than the frequency of the current pulses. It has been observed that the light-induced potential changes in the plant cells occur with half-times, which are not faster than 5 s (refs 22, 27, see also Figs 4–6 in this paper). However, it should be noted that the resistance measurement will be influenced by the change in the potential, when the latter is relatively high and occurs under conditions at which the current–voltage relationship of the membrane is not linear near the resting potential of the cell. As will be shown in a separate paper (W. J. Vredenberg, unpublished), this is the case when a cell is in a highly hyperpolarized state, due to an increased activity of an electrogenic pump which contributes to the membrane potential. Under these conditions a high dark resistance is measured.

An essential condition for a linear response of the resistance-measuring system is that the duration of the current pulses is longer than the membrane time constant. The membrane time constant is in a first approximation equal to R_mC_m, in which R_m and C_m are the membrane resistance and the membrane capacitance, respectively. The capacitance of the plasmamembranes of *Nitella* cells has been found to be about 1 μF/cm^2, a typical value for biological membranes[33]. The electrical resistance of *Nitella* cells has been reported to be in the range between 6 and 150 k$\Omega\cdot$cm^2 (refs. 34, 35). However, under certain conditions, dark values of R_m above 150 k$\Omega\cdot$cm^2 have been found in *Nitella translucens* (ref. 35; and W. J. Vredenberg, unpublished results). For cells with a resistance between 15 and 100 k$\Omega\cdot$cm^2, the membrane time constant will be in the range between 15 and 100 ms. A reasonable choice for the pulse duration at these values of the membrane time constant would be 45 and 300 ms, respectively, *i.e.* pulse frequencies of about 12 and 1.5 Hz, respectively. The lower limit (3 dB point) of the frequency range of the amplifier and phase sensitive rectifier used in our system, is 1 Hz. This limit presents an upper limit to the range of membrane resistances that can be recorded linearly by the detecting system. The response of the system has been measured and calibrated with an equivalent circuit for the membrane consisting of a 1-μF capacitance in parallel with a resistance that could be varied stepwise between 350 and 20 kΩ. The results are plotted in Fig. 2 which shows that with square current pulses of 400 ms duration (frequency 1.25 Hz) the response of the measuring system is linear for resistances of up to 120 kΩ. At higher frequencies of the current pulses, the resistance up to which the system responds linearly will of course be lower. At frequencies below 1.25 Hz the system was found to deviate from linearity in the low resistance range, probably due to a non-linearity of the amplifier and rectifier at their low frequency limits. The response time of the system, when 400-ms current pulses are used, is about 3 s, as shown in Fig. 3.

In our experiments with *Nitella* cells, 1.25-Hz depolarizing pulses with an amplitude of about 0.01 μA were applied. These caused potential modulations of about 0.5 to 3 mV amplitude, superimposed on the slowly changing membrane potential. In the potential measuring part of the system these modulations were attenuated in the RC circuit by a factor of about 3. Thus in cells with a high membrane resistance, the potential was measured with an amplitude modulation of about 1 mV, or less,

on the recorder. In the reproduction of the tracings this modulation has been omitted.

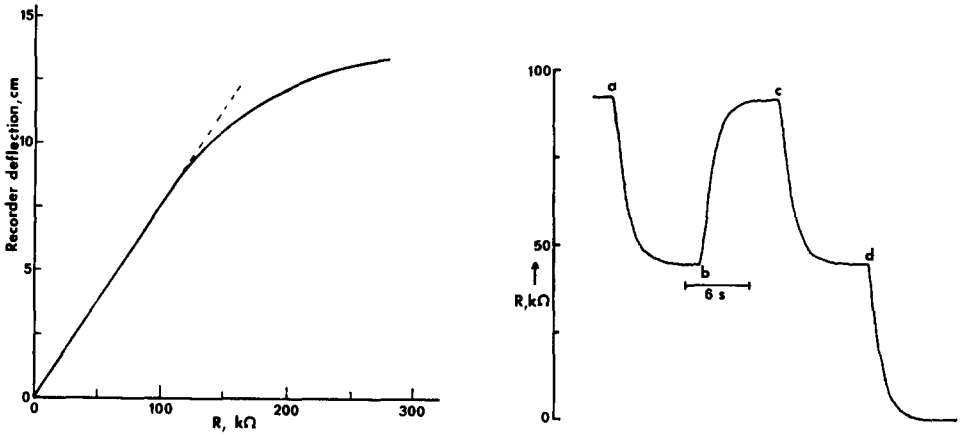

Fig. 2. A plot of the recorder response of the resistance-measuring part of the detecting device (Channel 2 in Fig. 1), for resistances in the range between 0 and 300 kΩ, as measured with square current pulses of 400 ms duration. The cell (Fig. 1) was replaced by an electrical analog of the membranes, consisting of a 1-μF capacitance, in parallel with a resistance that could be switched at discrete values in the range between 0 and 300 kΩ. The curve has been reproduced as a continuous one, drawn through a large set of measured points over the whole resistance range.

Fig. 3. Time recordings of the response of the resistance-measuring part of the detecting system (Channel 2 in Fig. 1) upon instantaneous changes in the resistance, as measured with 400-ms square current pulses. The cell was replaced by an electrical analog of the cell membrane, consisting of a 1-μF capacitance in parallel with a resistance that was switched from 94 to 46.8 kΩ (a and c), or *vice versa* (b), and from 46.8 to 0 kΩ (d).

Material

Freshly grown internodal cells of *Nitella translucens* of about 5 cm length and 0.06 cm diameter (*i.e.* with a surface area of about 1 cm^2), have been used. They were taken from day light-exposed aqueous laboratory cultures, consisting either of the original pond water or of a modified artificial pond water, which contained in addition to 1 mM NaCl, 0.1 mM KCl and 0.1 mM $CaCl_2$ (normal artificial pond water), 1 μM KH_2PO_4 and 0.1 mM $MgCl_2$. This phosphate-containing artificial pond water resembles fairly closely the chemical composition of the natural pond from which the cells originally were collected. Usually cells were presoaked a few days before the experiments in artificial pond water enriched with 0.9 mM $CaCl_2$ (denoted as Ca^{2+}–artificial pond water), and 0.1 mM $KHCO_3$. During this presoaking the cells were exposed to 12-h periods of white light illumination (intensity 12 kergs·cm^{-2}·s^{-1}) from fluorescent lamps separated by 12-h dark periods. This pretreatment was found to yield fairly reproducible conditions of the cells with respect to the light and dark responses of the electrical membrane parameters to be measured. Measurements were carried out in the bicarbonate-enriched Ca^{2+}–artificial pond water medium or in artificial pond water at room temperature.

The potential, resistance, and changes thereof have been measured across the cell wall, plasmalemma and tonoplast in series. However, it has been shown that the changes in potential are nearly exclusively due to changes at the plasmalemma[36].

This is certainly also true for the resistance changes. The resistance of the tonoplast has been shown to be about one tenth of that of the plasmalemma[34]. The observed resistance changes are as much as 30–50 % of the total resistance, and thus should be mainly, if not exclusively, due to changes at the plasmalemma.

Experimental results

Figs 4–6 show the results of some typical experiments with *Nitella translucens*, in which the response of the membrane potential and resistance upon illumination with light absorbed by the photosynthetic pigment systems, was recorded. A cell was illuminated by a homogeneous monochromatic light beam (wavelength band around 676 nm, half-width 10 nm) from a modified lamphouse assembly of a 24-V d.c. 250-W light projector. A homogeneously illuminated rectangular area near the condensor of the lamp system was focussed perpendicularly upon the bottom of the sample holder, at which the cell was positioned. Thus the upper surface of the cell was illuminated over its total length. Light intensities were measured with a YSI Radiometer, Model 65.

The experiment reproduced in Fig. 4 shows an initial depolarization of the membrane and a lowering of the membrane resistance in the light. The changes are reversed in the dark. Although variable for different cells, probably because of unknown effects of variations in critical conditions during growth, this type of kinetics, as shown in this figure, is preferentially observed in cells which have been preconditioned

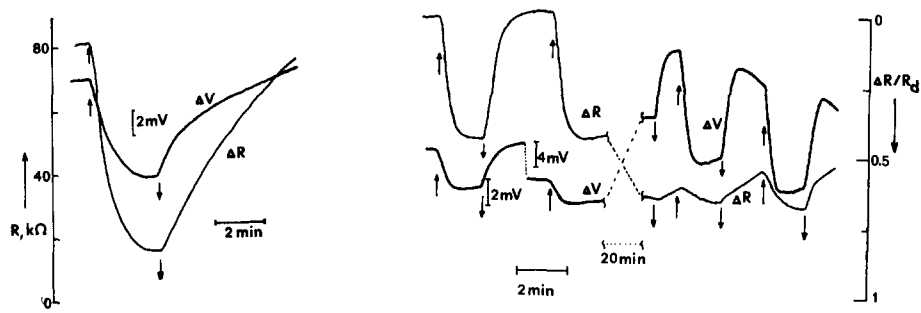

Fig. 4. Simultaneous recording of the changes in membrane potential (ΔV) and membrane resistance (ΔR), occurring in a single internodal cell of *Nitella translucens*, upon illumination and darkening. The cell, bathed in Ca^{2+}–artificial pond water enriched with 0.1 mM $KHCO_3$, was illuminated after a series of light–dark intervals of 2 and 20 min duration respectively. At the start of the experiment shown in the figure, the dark potential was −102 mV and the resistance in the dark was approximately 82 $k\Omega \cdot cm^2$. Upward and downward arrows mark the beginning and end respectively of the illumination period. A downward movement of the potential curve means an increase in the membrane potential (depolarization). The intensity of the monochromatic (676 nm) actinic light was 11 $kergs \cdot cm^{-2} \cdot s^{-1}$ (*i.e.* about 6.5 $neinsteins \cdot cm^{-2} \cdot s^{-1}$).

Fig. 5. Kinetics of the light-induced changes in the membrane potential (ΔV) and membrane resistance (ΔR), measured in *Nitella translucens*. The experiment was started after the cell was kept in darkness for more than 20 min. The cell was bathed in artificial pond water and was taken from a collection of cells that was bathed for a few days in Ca^{2+}–artificial pond water enriched with 0.1 mM $KHCO_3$. At the start of the experiment shown, the potential was −120 mV and the resistance approximately 60 $k\Omega \cdot cm^2$. For further details and explanations, see legend of Fig. 4. Note that after the second prolonged illumination during which the membrane has entered a hyperpolarized state (−140 mV) and the resistance has become low (approx. 18 $k\Omega \cdot cm^2$), the light-induced membrane depolarization is 4 times as large as in the dark-adapted state, and occurs with kinetics that are not comparable to those of the resistance changes, which are essentially different from those in the dark-adapted state.

by keeping them in the dark for a couple of hours in a Ca^{2+}-artificial pond water (pH 6.5–7) medium after a normal light–dark regime of 12 h each at room temperature. Under these conditions the kinetics of the changes in potential and resistance are nearly if not completely identical when actinic light of moderate intensity is given for 1 to 2 min and dark periods between successive illuminations are not shorter than 10 min. Fig. 5 shows an illustrative example of an experiment which has been performed with a dark-adapted cell that, after one short illumination period, was illuminated with monochromatic light, also of relatively low intensity, for several minutes. During this prolonged illumination period, the initial membrane depolarization is followed by a hyperpolarization which in general is completed after 5 to 15 min. The hyperpolarization, which in this case is about 20 mV, can be as large as 40 mV. During the long illumination period the resistance has been changed too, *via* a rather complicated kinetic pattern, which will be omitted from the discussion in this paper. At the end of the illumination period, when the cell has reached a steady hyperpolarized state, the membrane resistance in general is low and about the same as or even lower than the resistance value reached after one or two minutes of illumination. After shutting off the light the membrane becomes more hyperpolarized and the change in resistance is small. As the figure shows, illumination now causes an intense depolarization of the membrane which is completed within 1 or 2 min, and is reversed in the dark. The changes in the membrane resistance are relatively small and occur at a low rate, both in the light and in the dark. It is evident from the figure that, when the cell is in the hyperpolarized state, the kinetics of the potential and resistance changes induced by light are completely different. The short light and dark periods, as applied in the experiment shown in the figure, will cause the cell to return slowly to the more depolarized state in which it originally was and usually is after a long dark period. During this reversion phase, which may take more than 20 min for completion, the amplitude of the changes in the potential in light and darkness was found to decrease and that of the changes in resistance was found to increase. In the

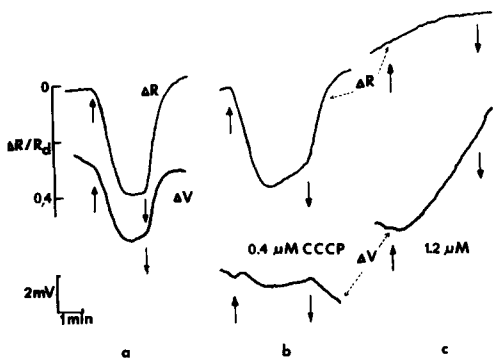

Fig. 6. Kinetics of the light-induced changes in the membrane potential and membrane resistance of a single cell of *Nitella translucens*, (bathed in artificial pond water), in the absence (a), and presence of 0.4 μM (b) and 1.2 μM CCCP (c). Illumination was given after the cell was kept in the dark for several minutes. The potential and resistance in the dark, before adding CCCP were −120 mV and 30 $k\Omega \cdot cm^2$, respectively. Addition of 0.4 μM CCCP in the dark caused an 8-mV depolarization of the membrane, and no change in the dark resistance. Extra addition of 0.8 μM CCCP in the following dark period caused a slight increase in the dark potential and dark resistance. Further explanations and details are in the legend of Fig. 4.

final more depolarized state the original, mutually comparable kinetics are observed again.

Fig. 6 shows the effect of addition of low concentrations of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on the light-induced changes in potential and resistance in a dark-adapted cell. These and other control experiments (W. J. Vredenberg, unpublished results) indicate that those potential changes which occur in association with the changes in resistance are inhibited by the uncoupler at concentrations at which the resistance change is not yet inhibited. More detailed characteristics and interpretations of these changes in potential and resistance and of the apparently associated shifts in the energy state of the membranes will be discussed in a forthcoming paper.

DISCUSSION

The method described in this paper permits simultaneous measurements of the kinetics of changes in the electrical membrane potential and resistance of intact metabolizing cells. This makes possible comparative studies on the physical parameters that determine ion transport across the membranes. A study of energy-dependent changes of these parameters will contribute to knowledge about the connection between active ion transport processes across cellular membranes and energy-generating cellular reactions. The method has been developed primarily for measurements with intact plant cells, in which the ion transport processes across the cytoplasmic membranes have been shown to be under control of energy delivered by, or coupled to, respiration- and photosynthesis-linked primary reactions in the mitochondria and chloroplasts respectively[1,3,4,6]. The effect of regulation and modulation of the energy supply either by illumination and darkening or by using specific inhibitors of electron transfer reactions in the respiratory and photosynthetic electron-transport chains, and by chemical compounds that interfere with energy-coupling reactions at the organellar and cellular membranes, can be recorded simultaneously.

As shown in Figs 4 and 5, illumination with photosynthetic light causes changes in the potential across and the resistance of the plasmalemma. The kinetics of the changes are not comparable under all conditions. In many cases the kinetics of the potential changes during illumination periods of about 2 min are biphasic, *i.e.* an initial depolarization is followed by a hyperpolarization. Under certain conditions, the kinetics of the potential change (depolarization in the light), are monophasic and comparable, if not identical, to those of the monophasic resistance changes, at least during short illumination periods. Rather than discussing these results thoroughly, we will briefly comment on a part of them, to illustrate the usefulness of the method. Identical kinetics of the initial potential and resistance changes brought about by illumination suggest that both are caused by or are associated with the same energy conversion reaction. They would be self-evident if the potential change is due to an energy-dependent change in the membrane resistance. This would be the case when a component of the membrane potential is maintained by an active membrane current. A change in the membrane resistance in the light and in the dark would then be accompanied by a proportional change in the potential, provided that the active membrane current is not altered initially during short illumination. Evidence has been presented that an electrogenic pump, notably a proton-extrusion

pump[16], contributes to the potential of the plasmalemma of *Nitella* species[18]. Thus the identical kinetics of the initial potential and resistance change observed in dark-adapted *Nitella* cells in short illumination and long dark periods would *a priori* suggest the possibility that they are caused by an electrogenic membrane pump, the generated potential of which changes due to a light-induced decrease in the membrane resistance. If this is true, then a second conclusion would be that under these conditions the products or intermediates of the photochemical reactions initially interact mainly with the membrane transport mechanisms at the plasmalemma, causing changes in the ionic permeabilities, *i.e.* resistance, of the membrane, presumably through chemical or ionic interference with membrane constituents. Supporting evidence for these conclusions comes from the results given in Fig. 6. At relatively low concentrations of CCCP (0.4 μM), the photochemical reaction causing the decrease in membrane resistance is scarcely, if at all, affected. However, the change in potential is totally inhibited. This indicates that the chemical agent at this concentration has not affected the energy-coupling reactions at the chloroplast membranes appreciably, if at all, but apparently has uncoupled the electrogenic pump at the plasmalemma from its energy source, or alternatively has caused a rapid dissipation of the energy needed for its operation. The finding that the addition of the uncoupler in the dark has caused a decrease in the (dark)potential without causing an appreciable change in the resistance is consistent with this conclusion about the inhibition of the electrogenic pump. Finally, the steady state differences between light and darkness of the potential (ΔV) and resistance (ΔR) as measured in the experiments of Figs 4 and 6 would indicate that the electrogenic pump has activated a membrane current i ($= \Delta V/\Delta R$) of 0.12 and 0.25 μA/cm^2, respectively, equivalent to ion fluxes (i/F) of 1.3 and 2.8 pmoles$\cdot$cm$^{-2}\cdot$s^{-1}, respectively. These values are of the same order of magnitude as those calculated by others for the fluxes generated by the electrogenic pump[16-18]. However, it will be shown in a subsequent paper (W. J. Vredenberg, unpublished), that in cells in a higher energy (hyperpolarized) state, the active flux in the dark can be as high as 7 to 10 pmoles$\cdot$cm$^{-2}\cdot$s^{-1}, due to a stimulated activity of the electrogenic pump caused by photosynthetic high-energy products or intermediates.

The functioning of the measuring system and the preliminary results obtained with it justify the conclusion that its application will contribute to an experimental approach that is directed to the analysis of the complex linkages between the active ion transport processes and the energy-delivering cellular reactions.

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