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MEASUREMENT OF THE MEMBRANE POTENTIAL AND EVIDENCE FOR ACTIVE TRANSPORT OF IONS IN *CHLORELLA PYRENOIDOSA*

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SUMMARY

1. The internal concentrations of K^+, Na^+ and Cl^- have been determined for cells of *Chlorella pyrenoidosa* under fixed external conditions. The ratio of internal to external concentrations for light-treated cells gave equilibrium potentials of $E_{Na^+} = -3$ mV, $E_{K^+} = -71$ mV and $E_{Cl^-} = +8$ mV.

2. In the dark the internal Na^+ and Cl^- levels fell to about 30% of the light values but the K^+ content remained unchanged.

3. A method was devised by which microelectrodes were inserted into individual Chlorella cells held at the tip of a suction pipette.

4. A mean potential of -40 mV suggested that this organism accumulated K^+ and Cl^- and extruded Na^+ against the thermodynamic gradient.

5. Variation of the external ratio of K^+ to Na^+ resulted in changes of membrane potential. It was found that increasing the external K^+ concentration depolarised the potential and an estimate of the ratio of the permeability coefficients of Na^+ to K^+ gave values of about 0.1.

INTRODUCTION

The unicellular alga *Chlorella pyrenoidosa* is a non-vacuolated microorganism which has been studied intensively from the biochemical point of view, especially in connection with photosynthetic processes. Ion transport and membrane studies on this organism are rather less common. The work of HOPE[1], using a.c. measurements, suggested that Chlorella is bounded by a membrane having a similar value of capacitance as found for many animal cells. In addition, SCOTT[2] and SCHAEDLE AND JACOBSON[3,4] have investigated Na^+ and K^+ distribution and transportation in this alga and COHEN[5] has reported possible K^+ carrier sites on the cell surface.

Although these studies show that Chlorella is able to regulate its internal ionic composition, perhaps by specific carriers situated in a cell membrane, they do not clearly demonstrate which ions are actively transported. To do so would require the measurement of the electrical potential difference across the cell surface[6]. Obtaining such a parameter in this organism, as with other microorganisms, presents a serious

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difficulty because of the smallness of the cell dimensions. Nevertheless, it has been possible to devise a method of inserting microelectrodes into single Chlorella cells. The experiments were conducted under fixed external conditions such that the measured potential difference could be correlated with the distribution of K^+, Na^+ and Cl^- in deciding if one or more of these ions is actively transported. It was also possible to estimate the relative permeability of the cell membrane to K^+ and Na^+.

MATERIALS AND METHODS

The alga, *C. pyrenoidosa*, was an Emerson strain obtained from the Indiana University Alga Collection, stock 252. The cells were grown at 25° in liquid culture medium, gassed with 5% CO_2 + 95% air and illuminated at an intensity of 500 ft candles by a bank of fluorescent tubes. The composition of the liquid medium in mM was: 5.00 KNO_3, 0.50 $Na_2HPO_4 \cdot 2\,H_2O$, 0.50 KH_2PO_4, 2.00 $MgSO_4 \cdot 7\,H_2O$, 0.25 $Ca(NO_3)_2 \cdot 4\,H_2O$, 1.00 KCl and 2 ml/l of trace elements as given by HUTNER *et al.*[7]. Roux bottles containing 1 l of this inorganic medium were continuously shaken and the resulting cultures harvested 2 or 3 days after inoculation during their exponential phase.

The quantity of cells harvested was determined by centrifugation in calibrated tubes for 5 min at 2200 × *g* using an M.S.E. super minor centrifuge. The extracellular space of the packed cell pellets was measured using D-[1-^{14}C]mannitol and the water content was estimated by weighing the pellets before and after drying at about 95° for 24 h in an oven.

Ion analyses

Internal concentrations of K^+ and Na^+ were measured by emission flame photometry. Known volumes of cells were dry-ashed in a muffle furnace at 500° for 15 min and the residue dissolved in 0.1 M HNO_3. This solution was analysed on an EEL flame photometer by comparison with standard solutions of concentrations very close to those of the unknown.

Internal concentrations were also determined using the radioisotopes ^{42}K, ^{22}Na and ^{36}Cl. Cells were suspended in culture medium containing the required isotope until they were in isotopic equilibrium with this external medium. Aliquots of cells were then sedimented and washed on Millipore filters and assayed for internal radioactivity by means of a Nuclear Chicago gas flow counter or an I.D.L. scaler 1700, fitted to a Geiger–Müller tube (20th Century MX 152). Counting was carried out to an accuracy of ± 1 to ± 3%. The isotopes were obtained from the Radiochemical Centre, Amersham, except in some cases: ^{42}K came from the Atomic Energy Establishment, Harwell.

Electrical potential measurements

Electrode insertions were conducted in a small perspex chamber mounted on the stage of a PZO microscope and viewed through a coverslip using a Cook AEI long working-distance reflecting objective of 40 × magnification and 10 × eye pieces.

By means of suction from a syringe, the cells were drawn onto the tip of a glass pipette as shown in Fig. 1. This pipette had an internal diameter of about 5 μ and cells greater than this could be fixed reasonably rigidly and removed simply by

releasing the suction. The microelectrodes were brought in horizontally, through the open end of the chamber, using a Prior micromanipulator. Good alignment of the electrode with respect to the cell was indicated by a visible dimpling of the wall. This was often followed by a good penetration when using fine electrodes. Because of KCl diffusion from the reference electrode the suspension was regularly changed and in some cases, when the cell was firmly impaled, it was possible to flow fresh solution through the chamber.

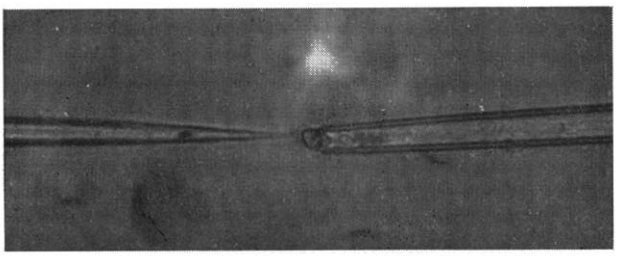

Fig. 1. A 400 × photomicrograph showing a Chlorella cell held on a suction pipette and an advancing microelectrode. The internal diameter of the suction pipette is about 5 μ.

The measuring and reference electrodes were connected to matched calomel half-cells by agar salt bridges and the potential changes measured on a Vibron electrometer coupled to a Bausch and Lomb chart recorder. To reduce interference from pick-up, the apparatus was shielded by a zinc mesh cage.

The glass microelectrodes, which probably had external diameters of less than 0.5 μ, were made with the help of Mr. E. TARR using a puller designed and manufactured by him. These were filled with 3 M KCl by diffusion replacement of methanol and had resistances of 20–50 MΩ. Only electrodes which had tip potentials of 5 mV or less in 100 mM KCl were used. The suction pipettes were pulled either on a Palmer or Narishige microelectrode puller. Tips with internal diameters of 7–8 μ were desired and slightly reduced by fire polishing with a laboratory-built microforge.

RESULTS

Extracellular space and cell water

Wet and dry weights were determined on packed cell pellets and using a dry weight conversion factor it was calculated that 1 ml of packed cells contains 0.958 $\pm$ 0.002 g of water (11 determinations). This value was independent of the size of the extruded pellet.

The extracellular space of the packed pellet as measured with [^{14}C]mannitol was 0.331 $\pm$ 0.041 ml/ml of packed cells (5 determinations). A value of 33% for the extracellular space suggests that the cells were not as tightly packed as would theoretically be possible since hexagonal packing of spheres would give 26% (ref. 8).

Using this value of extracellular space gives the internal water content of 1 ml of packed cells to be 0.627 $\pm$ 0.041 ml.

Intracellular concentrations

The internal concentrations of K^+, Na^+ and Cl^- are given in Table I.

The intracellular concentrations were also determined isotopically and, in

TABLE I

THE DISTRIBUTION OF IONS UNDER FIXED EXTERNAL CONDITIONS

The values listed show the internal levels of Na^+, K^+ and Cl^- as determined for illuminated cells at 25° in culture medium using chemical (flame photometry) and isotopic analyses. The figures given in parentheses represent the number of separate cultures analysed with 5–10 determinations being made on each culture to an agreement of 2–4%. The values are quoted with ± S.E. and the external concentrations of the 3 ions are also given.

Ion	*External concn.* (c_0) (*mM*)	*Internal conc.* (c_i) (*mM/l cell water*)	
		Chemical analyses	*Isotopic analyses*
Na^+	1.0	1.13 ± 0.28(8)	1.11 ± 0.35(14)
K^+	6.5	103 ± 8(29)	114 ± 8(12)
Cl^-	1.0	—	1.34 ± 0.11(18)

some cases checked by flame photometry, for cells which had been in the dark for about 2 h. It was found that the Na^+ and Cl^- levels fell in these dark-treated cells while there was no detectable change in K^+. Measurements with isotopes gave a dark Na^+ concentration of 0.30 ± 0.03 mM/l cell water (13 determinations) and a dark Cl^- level of 0.46 ± 0.10 mM/l cell water (15 determinations).

Electrical measurements

Potential differences under fixed external conditions

The mean potential obtained from impaling 63 different Chlorella cells and the S.E. is: potential difference = −40 ± 2 mV (range −28 to −58 mV).

All readings were determined at laboratory temperature, about 20°, and the cells were illuminated and bathed in culture medium. These values have been carefully selected from many others. They represent results recorded from successful punctures which gave sharp jumps to steady potentials ± 2 mV, usually for 10–60 sec, followed by the removal of the electrode and an immediate decrease to ± 3 mV of the original tip potential.

In general little variation of the recorded potentials occurred even over several minutes, although cells impaled for these longer periods of time were usually difficult to remove from the electrode so that no check could be made on the tip potential. Moreover, shorter measuring times were preferred simply because the vibration of the apparatus was often sufficient to cause the electrode to come out of the cell soon after penetration. In some cases the removal of the electrode from the organism did not result in a decrease of the reading to a low value and potentials of −10 to −20 mV persisted above the original tip potential. This was probably due to plugging of the electrode with cellular material which could, in many instances, be seen to adhere to the tip. In a few experiments it was found that a rapid hyperpolarization of the potential occurred if the electrode was disturbed after a cell had been punctured. This may have been due to diffusion of KCl from the electrode and the possibility of such a salt leakage is considered in more detail in the discussion.

Effect of changing the external ions on the potential difference

In some cases impaled cells could be moved clear of the suction pipette and a

steady potential difference recorded. Experiments were conducted on cells in this situation which involved varying the ratio of Na^+ to K^+ in the bathing medium and recording the potential difference changes. This type of measurement has been particularly successful when applied to nerve and muscle cells and has also been used on plant[9,10] and fungal cells[11]. In plant cells the wall can act as a leaky cation-exchange phase and in order to keep the resulting Donnan potential constant during the changes of Na^+ to K^+ ratio, the total NaCl *plus* KCl concentration was kept constant. This approach was adopted initially by HOPE AND WALKER[9] and shown by SPANSWICK, STOLAREK AND WILLIAMS[10] to be valid for the isolated cell walls of *Nitella translucens*.

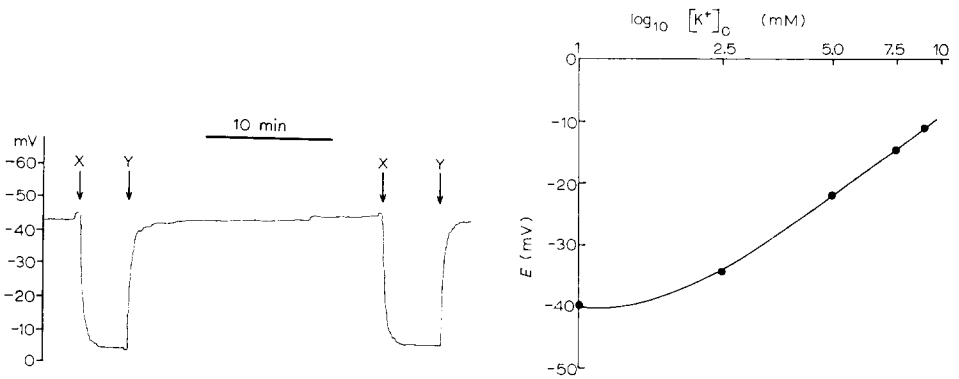

Fig. 2. A chart recording obtained for a punctured cell which was subjected to two different solutions, at x 10 mM KCl *plus* 1 mM NaCl and at y 1 mM KCl *plus* 10 mM NaCl.

Fig. 3. The results of an experiment on a single cell which involved 5 changes of external solution each varying in amount of KCl and NaCl but with the sum of the concentrations of these salts constant at 10 mM. The recorded potentials are plotted against $\log_{10}$ of the external K^+ concentration.

Fig. 2 shows a chart recording obtained for a punctured cell which was subjected to 2 different solutions which had a total concentration of 11 mM. The initial value of —44 mV was recorded when the external solution was 10 mM NaCl and 1 mM KCl. At point x the potential was depolarized by flowing 1 mM NaCl and 10 mM KCl passed the cell. The rapid change of solution was accomplished by means of a multiway tap incorporated into the experimental set up. This rapid 10-fold increase in external K^+ resulted in a change of the membrane potential by 38 mV. At y the external environment was changed back to the low K^+ solution and the membrane potential returned to -45 mV. This was repeated once again and it can be seen that the same sequence of reversible potential changes occurred.

Another similar experiment was conducted with solutions which had a total K^+ *plus* Na^+ concentration of 10 mM. Five different solutions were used which contained the following external concentrations of KCl, $[K^+]_0$, and NaCl, $[Na^+]_0$, in parentheses: 1.0 (9.0), 2.5 (7.5), 5.0 (5.0), 7.5 (2.5) and 9.0 (1.0) mM. Before the experiment, the cells were pretreated in 1 mM KCl *plus* 9 mM NaCl for 2 h. Fig. 3 shows the results obtained plotted as $\log_{10} [K^+]_0$ against the measured potential.

DISCUSSION

This work represents the first experiments reported which measure the membrane potential of *C. pyrenoidosa* and correlate it with the ionic distribution in these cells. Such electrical measurements are not common for similarly sized microorganisms, therefore, during this discussion some attempt to justify the results will be made.

One obvious factor which must be considered when dealing with cells of dimensions of about 10 μ or less is the effect of salt leakage from the microelectrode. An estimate of the maximum rate of diffusion can be obtained by considering a theoretical model of the tip of a microelectrode as presented by KRNJEVIC *et al.*[12]. After making some assumptions the rate of K^+ diffusion in moles/sec is given by $Q_D = c_1 D\pi \tan \Theta\, r$, where c_1 is the bulk concentration in the microelectrode in moles K^+ per cm^3, and D is the diffusion coefficient in cm^2/sec. If certain values are assumed for the half angle of taper $\Theta = 1°$ and the internal radius of the tip $r = 0.1$ μ, then Q_D approximately equals 10^{-14} mole/sec. Considering a cell of radius 3 μ and an internal concentration of approx. 100 mM K^+, the cellular K^+ should double in about 1 sec. If this were so, and the membrane potential is controlled by K^+ diffusion, then the potential difference would be expected to hyperpolarize by about 15 mV during the first second. A further 10-mV hyperpolarization would occur after 2 sec. This gradual change in potential was not generally observed and suggests that the rate of KCl diffusion was much lower than the calculation predicts. Alternatively it must be assumed that salt leakage into the cell did not affect the measured potential.

Although there is no evidence that the potential is sensitive to changes of internal K^+ it is clear that the measured potential difference was sensitive to external changes of this cation. The results are similar to those obtained for *Chara australis*[9] and *N. translucens*[10] which were explained in these cells by using an equation derived by HODGKIN AND KATZ[13] from the constant-field theory of GOLDMAN[14]. It was assumed that these algae had a low passive permeability to Cl^- such that the membrane potential, E, is given by:

$$E = \frac{RT}{F} \ln \frac{[K^+]_0 + \alpha [Na^+]_0}{[K^+]_i + \alpha [Na^+]_i} \qquad (1)$$

where $\alpha = P_{Na^+}/P_{K^+}$, the ratio of the permeability coefficients, and the other symbols have their usual meanings.

Application of Eqn. 1 to the results obtained with Chlorella, assuming the internal concentration parameter (denoted by i) remains constant, gives a mean permeability ratio of 0.11. This value is quite plausible and falls in the same range as those found for Chara[9] and Nitella[10].

The selective sensitivity of the measured potential difference to K^+ strongly suggests that the measurements presented in this paper were in fact transmembrane potentials. The mean value of −40 mV is quite acceptable if compared with the limited amount of knowledge of membrane potentials in other non-vacuolated microorganisms[11,15]. Errors in measured values could arise, however, from development of large tip potentials within the cells[16].

Some speculation about the occurrence and direction of active transport processes in Chlorella can be drawn from the knowledge of the membrane potential

TABLE II

THE EQUILIBRIUM POTENTIALS AS CALCULATED FROM THE NERNST EQUATION

In calculating the values for Na^+ and K^+ the mean of the chemically and isotopically determined internal concentrations, as given in Table I, were used.

Ion	$\frac{c_0}{c_i}$	$E = \frac{58}{Z} log \frac{c_0}{c_i} (mV)$*
Na^+	0.900	− 3
K^+	0.060	−71
Cl^-	0.746	+ 8

* Is the Nernst equation where E is the equilibrium potential, Z is the algebraic valency and c_i and c_0 are the internal and external concentrations.

together with values of ionic concentrations. The equilibrium potentials for each ion have been calculated, using the Nernst equation, and are given in Table II. It can be seen that all 3 values do not agree with the measured cytoplasmic potential and it appears that both K^+ and Cl^- are transported into the cells against their electrochemical potential gradients while Na^+ seems to be actively extruded. These conclusions have assumed the cells to be in flux equilibrium and, indeed, measurement of tracer influx and efflux for the three ions have shown this to be the case[17]. It has also been assumed that the ions are in free solution and although this has not been shown for Chlorella, measurements of the intracellular osmolarity in other microorganisms suggest that the bulk of the low molecular weight solutes are not cytoplasmically bound[18,19].

Similarly directed pumps have been found in several other algae as listed by RAVEN[20], although only the active cation mechanisms have been suggested[15,21] in the microorganisms studied to date. It has been found in these microorganisms that Cl^- is either passively distributed[15] or else excluded[8]. These are non-photosynthetic systems and suggest perhaps that the active Cl^- uptake by Chlorella may be a consequence of its photosynthetic nature.

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