# The Membrane Potential

# of *Acetabularia mediterranea*

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ABSTRACT  The cytoplasm of an *Acetabularia* cell is normally at a potential of about $-170$ mv relative to the external solution; the vacuole is also at this potential. Although there is strict flux equilibrium for all ions, the potential is more negative than the Nernst potentials of any of the permeating ions. Darkness, CCCP, low temperature, and reducing $[Cl^-]_o$ by a factor of 25 all rapidly depolarize the membrane and inhibit $Cl^-$ influx. Some of these treatments do not inhibit the effluxes of $K^+$ and $Na^+$. Increasing $[K^+]_o$ also depolarizes the membrane both under normal conditions and at low temperature; in the latter case the membrane is partially depolarized in normal seawater (low $[K^+]_o$) and in high $[K^+]_o$ positive potentials of up to $+15$ mv are attained. It is concluded that the membrane potential is controlled by the electrogenic influx of $Cl^-$, and also, at least in some circumstances, by the diffusion of $K^+$. In addition, it is suggested that electrogenic efflux of $H^+$ may be important in transient nonequilibrium situations. An Appendix deals with the interpretation of simple nonsteady-state tracer kinetic data.

## INTRODUCTION

*Acetabularia* is a genus of giant unicellular green algae whose peculiar life cycle and uninucleate character have made it one of the most favored organisms for the study of nucleocytoplasmic interactions. Little is known about other aspects of its physiology and the work described in this paper is part of the outcome of an investigation of ionic regulation in *A. mediterranea*. *A. mediterranea* commonly reaches a length of 4 cm and a diameter of 0.3 mm. Equilibrium potentials, contents, and fluxes have been presented in another paper (Saddler, 1970) and are summarized in Table I. Permeabilities to $K^+$, $Na^+$, and $Cl^-$ have been calculated from the constant field equation, assuming that $K^+$ influx, $Na^+$ influx, and $Cl^-$ efflux are entirely passive. Measurement of the time courses of influx and efflux of $Na^+$ and $Cl^-$ showed that both ions exchange as a single intracellular compartment. It has been concluded from these results that *Acetabularia* resembles many other plants in

## TABLE I

### SUMMARY OF THE IONIC RELATIONS OF

*Acetabularia mediterranea* TAKEN

### FROM SADDLER (1970)

The range of experimental values of fluxes and permeabilities is given. $K^+$ efflux was not calculated for reasons explained in the text.

| Ion | ASW concentration | Vacuolar concentration | Vacuolar Nernst potential | Plasmalemma influx | Plasmalemma efflux | Permeability |
|---|---|---|---|---|---|---|
| | $mM$ | $mM$ | $mv$ | $pmoles\ cm^{-2} \cdot sec^{-1}$ | $pmoles\ cm^{-2} \cdot sec^{-1}$ | $cm \cdot sec^{-1}$ |
| $Na^+$ | 470 | 60 | −48 | 11–49 | 15–37 | $0.5–1.2 \times 10^{-8}$ |
| $K^+$ | 10 | 360 | −93 | 11–40 | ? | $2–5 \times 10^{-7}$ |
| $Cl^-$ | 550 | 490 | −6 | 200–790 | 290–850 | $1–3 \times 10^{-7}$ |
| $SO_4^{--}$ | 28 | 5 | −25 | 0.17 | ? | ? |
| Oxalate | 0 | 65 | — | — | ? | ? |

$Eco = -170$ mv, $Evc = 0$.  Eco is plasmalemma potential, Evc, tonoplast potential.

having a $Cl^-$ influx pump and a $Na^+$ efflux pump, both of which are at the plasmalemma. Efflux results for $K^+$ are complex and cannot be interpreted in terms of simple kinetic models; they are discussed elsewhere.[1] However, consideration of the long life (at least 6 months) and slow rate of growth of the *Acetabularia* cell makes it clear that a $K^+$ influx of 40 pmoles·$cm^{-2}$·$sec^{-1}$ must, over periods of more than a few hours, be balanced by a similar efflux.

The present paper is concerned with the factors controlling the membrane potential of *Acetabularia mediterranea*. Gradman and Bentup (1970) have described some experiments with *A. crenulata*. They state that $K^+$ is the only cation which affects the membrane potential $(E_m)$ and that $E_m$ is normally much more negative than $E_K$. They show that under conditions which inhibit metabolic activity the potential comes rapidly to a second steady level (termed $E_m'$) close to $E_K$, and attribute the difference between $E_m$ and $E_m'$ to an electrogenic mechanism. Results of a somewhat similar kind have been reported by Marmor and Gorman (1970) in a giant ganglial cell of the mollusc *Anisodonis nubilis*. It appears that in both preparations the membrane potential can be described by the summation of an ionic and an electrogenic component. The results presented in this paper confirm and amplify those of Gradman and Bentup (1970). It is shown that the very large active $Cl^-$ influx is strongly electrogenic.

The distinction between neutral and electrogenic pumps affords one way of classifying such pumps. In molecular terms, an electrogenic pump is a mechanism whereby an ion is transported across a membrane neither in association with nor in strict (stoichiometric) exchange for another charged

[1] Saddler, H. D. W. 1970. Fluxes of sodium and potassium in *Acetabularia mediterranea*. *J. Exp. Bot.* 21: In press.

species. Thus the pump generates an electric current across the membrane, which interacts with passive ion fluxes in such a way that a potential may be generated as a direct consequence of pump activity. The size of the potential in relation to the pump flux (current) will depend on the passive permeability of the membrane; it will also be affected by the nature of the chemical linkage, if any, between the fluxes. In the simplest situation, linkage between the electrogenic flux and all other fluxes is solely electrical, but the possibility of changes in passive permeability makes the system a complicated one nevertheless.

A system like the $Na^+$ pump can be said by definition to be electrogenic if the pump is uncoupled or has a coupling ratio greater than 1:1, and this can be investigated experimentally. Present knowledge of ion transport mechanisms in plant cells is not sufficient for this approach to be used. The best direct evidence for the existence of an electrogenic pump therefore depends on the demonstration of a quantitative relationship between the active flux of the pumped ion and short-circuit current. However, this ideal situation can almost never be attained. A great many preparations are not amenable to the short-circuit technique, and even when they are, interpretation of the results may often be ambiguous (Ginzburg and Hogg, 1967). Potential measurements must therefore be used for proof of electrogenicity. In general, a sufficient though not a necessary condition for the active flux of an ion being electrogenic is that the membrane potential be a function of the active flux. It is not a necessary condition for electrogenicity because certain combinations of permeability may prevent the operation of the pump being manifest as a contribution to the potential; for example, an electrogenic $K^+$ influx pump could be completely short-circuited by passive $K^+$ efflux if the permeability of the membrane to $K^+$ were sufficiently high.

Examples of well-substantiated electrogenic pumps are few. The most extensively investigated is electrogenic $Na^+$ efflux when the coupling ratio of $Na^+$ efflux to $K^+$ influx in the $Na^+$ pump is greater than one. This has been shown in frog muscle (Kernan, 1962; Mullins and Awad, 1965; Adrian and Slayman, 1966), snail nerve cells (Kerkut and Thomas, 1965), crayfish neuron (Nakajima and Takahashi, 1966), and nonmyelinated mammalian nerve (Rang and Ritchie, 1968). In all these preparations the $Na^+$ pump makes only a small contribution to the membrane potential and its electrogenic character cannot easily be demonstrated except by artificially increasing the internal $Na^+$ concentration, thereby greatly stimulating the $Na^+$ pump. In gastric mucosa, the active flux of $Cl^-$ from the nutrient (blood) side to the secretory side consists of at least two components, one of which is electrogenic (Heinz and Durbin, 1957; Durbin and Heinz, 1958). The $H^+$ secretion process also seems to be electrogenic (Rehm, 1966). An electrogenic $K^+$ efflux pump has been found in several insect preparations, for example silk-

worm midgut (Harvey, Haskell, and Nedergaard, 1968); see the review by Keynes (1969) for more examples. The only convincing example of an electrogenic pump in a plant cell is the active transport of $K^+$ and $Na^+$ from the cytoplasm to the vacuole of *Valonia* (Gutknecht, 1967). Both potential and short-circuit current in perfused cells fall rapidly in the dark.

## MATERIALS AND METHODS

A culture of *Acetabularia mediterranea* was maintained in the laboratory; culture techniques are described in detail elsewhere (Saddler, 1970). About 24 hr before starting an experiment cells were transferred from the enriched natural seawater medium to an artificial seawater of the following composition (mN): $Na^+$ 467, $Mg^{++}$ 110, $Ca^{++}$ 20, $K^+$ 9.8, $Cl^-$ 549, $SO_4^{--}$ 55, $HCO_3^-$ 2.3, $Br^-$ 0.8. All experiments were performed in this solution which will henceforward be abbreviated as ASW. Cells without caps were used for all measurements.

Most of the experimental techniques have been described at length in another paper (Saddler, 1970) and only the most important aspects will be dealt with here. Intracellular potential was measured by standard microelectrode techniques. Reservoirs of bathing solution were connected to the experimental chamber via a six-way tap, which made rapid changes of solution possible. It was not always certain whether the electrode tip was in the cytoplasm or the vacuole of the *Acetabularia* cell, but since the potentials in the two phases are not significantly different this was not a problem.

Membrane resistance was measured by the method of Hogg, Williams, and Johnston (1968) using two intracellular electrodes. This method was checked in several cases by using three electrodes and determining the cell space constant. It is necessary to assume that the cell is a uniform circular cylinder, but many cells actually vary quite considerably in diameter along their length. It was estimated that absolute resistance values might be in error by a factor of two either way, but relative changes measured on the same cell are much more accurate.

Chloride influx was measured by the uptake of $^{36}Cl$ into whole cells. Detailed time course measurements of $Cl^-$ influx and efflux show that exchange of $Cl^-$ occurs as a single intracellular compartment with a half-time of about 80 min and that tracer uptake is linear over the first 30 min. A true measure of plasmalemma $Cl^-$ influx can therefore be obtained from the $^{36}Cl$ content of a cell after a time of less than 30 min in active solution. Four or five cells were used for each flux measurement. Before being placed on planchettes for end window counting, cells were allowed 90 sec washing out in unlabeled ASW; tracer washout experiments show that more than 90% of the free space for $Cl^-$ is exchanged in this time. Cell surface area was found by measuring the cells under a microscope. The use of ASW made it possible to prepare $^{36}Cl$ solutions of relatively high specific activity without changing the ionic composition of the solution, so that influx could be quite accurately measured over times as short as 5 min.

Chloride efflux values were obtained from the rate constant of the single exponential curve which was obtained when whole cells were loaded with $^{36}Cl$ and then washed out in unlabeled seawater. The cells were loaded for long enough to ensure that they were virtually fully labeled with $^{36}Cl$; $Cl^-$ efflux could therefore be ob-

tained directly from the washout curves. Chloride fluxes are so large relative to content that, under conditions which significantly change either influx or efflux, intracellular Cl⁻ content will not be constant during a washout. The interpretation of simple tracer kinetic data with varying content is considered in an Appendix.

A number of experiments were performed with a solution containing 21 mN Cl⁻ and 580 mN SO₄⁻, all the other items being the same as in ASW; this solution will be called low Cl⁻ ASW. Some solutions were also prepared with different ratios of K⁺ to Na⁺ but with the same total concentration of K⁺ plus Na⁺ as in ASW (477 mN). Solutions containing CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) at 5 × 10⁻⁶ M were prepared by evaporating the ethanol solvent before adding ASW, so that control solutions did not need to have ethanol added to them. CCCP was obtained from Calbiochem, Los Angeles, Calif., U.S.A. Except where specified, experiments were performed at 25°C in the light.

Some flux measurements were made with what I have called nonvacuolated cell fragments. These are prepared by centrifuging an *Acetabularia* cell at about 300 *g* for 6 min so that the cytoplasm flows to the apical end. The cell is then tied off with a fine hair at the cytoplasm vacuole interface, and the basal portion is cut away. One is left with a piece of cell up to 1.0 cm or more in length consisting only of cytoplasm (including a large volume of chloroplasts) surrounded by the plasmalemma and cell wall, i.e. a mature giant algal cell with no vacuole. These fragments survive for at least 2 wk and can be used for flux experiments.

### RESULTS

#### Ion Fluxes and the Membrane Potential

30 sec after the entry into the measuring chamber of an ASW solution containing CCCP the potential begins to depolarize from −170 mv, and within a further 1.5 min it reaches a steady value of −90 mv. Placing the cell in the dark has a similarly large and even more rapid effect on potential. The same response is observed in low Cl⁻ solution and in normal ASW at low temperature (6°C). Except in the case of CCCP, the depolarization is immediately reversed on returning the cell to normal ASW at 25°C in the light. Fig. 1 shows the effects of low temperature on the membrane potential. The other treatments give very similar potential traces, but have not been plotted because of the difficulty of monitoring concentrations adjacent to the cell wall. Gradman and Bentup (1970) have reported that darkness, low temperature, and DNP produce similar depolarization effects in *A. crenulata*.

As explained above, Cl⁻ influx can be measured over periods as short as 5 min. This enables a fairly good time resolution to be made of the effects of various treatments on Cl⁻ influx. Table II lists the results of several experiments to determine the effects of CCCP, low Cl⁻, darkness, and low temperature on Cl⁻ influx. Controls were at 25°C and all treatments (except darkness) were in normal light conditions. It can be seen that all four treatments produce large and rapid inhibitions of Cl⁻ influx.
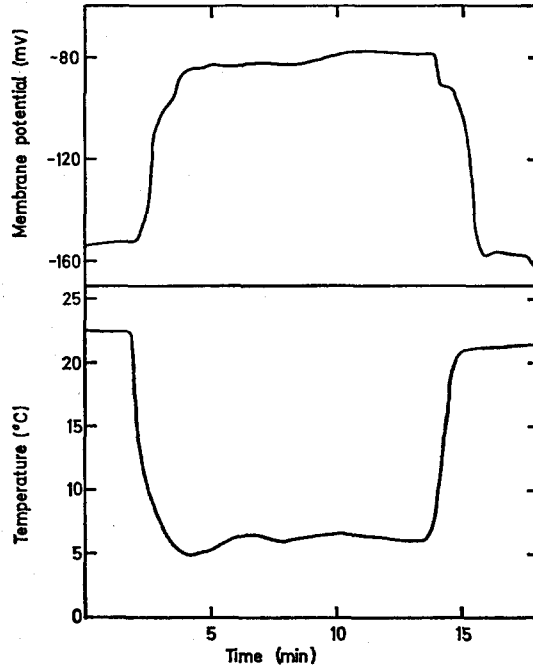
FIGURE 1. The relationship between temperature of bathing solution and the membrane potential of *Acetabularia*.

TABLE II

CHLORIDE INFLUX TO WHOLE CELLS

Time in minutes from beginning of treatment. All measurements at 25°C in light unless stated otherwise. Fluxes in pmoles. $cm^{-2}$. $sec^{-1}$; all values $\pm$ standard error of the mean (number of replicates).

| Time | Control | CCCP | Dark | Low Cl⁻ | 5°C |
|------|---------|------|------|---------|-----|
| 0–5 | 680±53 (4) | | | 17±5.5 (4) | |
| 9–14 | | | | 29±4 (4) | |
| 0–9 | 420±80 (4) | 90±11 (4) | | | |
| 12–21 | | 105±11 (3) | | | |
| 0–8 | 200±28 (4) | 115±48 (4) | | 10±2 (4) | 197±10 (4) |
| 12–20 | | 77±14 (4) | | 76±14 (4) | 50±10 (3) |
| 0–230 | 470* | | | | |
| 0–12 | | | 52±11 (5) | | |

* Value obtained from slope of semilog plot with four samples of four cells (Fig. 8 in Saddler [1970]).

The membrane resistance under normal conditions is about 100 $\Omega$ $cm^2$. In the depolarized cell it falls at first to a much lower level—too low, in fact, to be measured with accuracy. It increases again after a short time. These resistance changes under various conditions are shown in Table III.

CCCP is an uncoupler of oxidative phosphorylation in isolated mitochondria and photophosphorylation in isolated chloroplasts (Heytler, 1963).

The effects of CCCP, low temperature, and darkness on the potential are far too rapid to be caused by running down of a diffusion potential as a result of changes in ion content when active fluxes are inhibited. This rapid depolarization must be attributed either to a sudden increase in membrane permeability, as in an action potential, or to the inhibition of an electrogenic pump. The effects of the inhibitory treatments on Cl⁻ influx indicate that this is an active process, as was concluded from the electrochemical measurements. Hence a variety of treatments which inhibit Cl⁻ influx are also associated with changes in potential and resistance. Before concluding that the electrical effects are a direct consequence of Cl⁻ fluxes, it is necessary to consider cation efflux which might also be important in controlling the membrane potential. Table IV gives a summary of the effects of the various treatments on Na⁺ and K⁺ effluxes; these results are presented in greater detail elsewhere.[1] It was explained above that the complexity of the ⁴²K washout curves prevents the quantitative estimation of K⁺ efflux.

Of the three fluxes considered, Cl⁻ influx, Na⁺ efflux, K⁺ efflux, only Cl⁻ influx is inhibited by each of the treatments which cause depolarization. CCCP inhibits neither Na⁺ nor K⁺ efflux, and low temperature actually stimulates the latter. Only Cl⁻ influx, therefore, can be invoked in any consideration of a possible electrogenic component of the membrane potential.

TABLE III

MEMBRANE RESISTANCE FOLLOWING DEPOLARIZATION

These measurements were made at low temperature and in low Cl⁻ ASW. The times and associated potentials are approximate only; the precise time course varied with different cells.

| Time | Potential | Resistance |
|---|---|---|
| | *mv* | |
| Before depolarization | −170 | 100 Ω cm² |
| Up to about 10 min after depolarization | −70 | Very low |
| From 5 or 10 min after depolarization | −80 | 1,000 Ω cm² |
| After repolarization | −160 | 150 Ω cm² (darkness, CCCP) |
| | | 1,000 Ω cm² (low Cl⁻, low temperature) |

TABLE IV

EFFECTS OF INHIBITORY TREATMENTS
ON K⁺ AND Na⁺ EFFLUX

Summary of results presented in Saddler.[1] They are given as percentage inhibition (−) or no change (0).

| Flux | Darkness | Cold | CCCP | Low Cl⁻ |
|---|---|---|---|---|
| Na⁺ efflux | −75 | −85 | 0 | ? |
| K⁺ efflux | 0 | Stimulated | 0 | ? |

To this must be added $H^+$ efflux. No $H^+$ content or flux measurements have been made, but it appears that the pH of the vacuolar sap is very low; published estimates include 4–5 (Crawley, 1963), 2–3 (Tandler, 1962), and 1–2 (Bouck, 1964). The possible importance of $H^+$ efflux is discussed below.

*Repolarization*

Chloride influx remains at a low level for at least 3 hr in all four treatments. The membrane potential, however, stays at the depolarized level of about $-90$ mv for a relatively short period only; the membrane then repolarizes to between $-140$ and $-170$ mv. The membrane resistance increases before repolarization to a high value and in some conditions stays high after repolarization. In other conditions (darkness, CCCP) resistance falls after repolarization to near the normal level. Details of these changes are given in Table III. The precise extent of this repolarization and the time after depolarization at which it occurs also depend on the treatment. In darkness it happens within a few minutes, whereas at low temperature it may take several hours. Cells in CCCP die after about 36 hr, but the membrane potential, after repolarization, remains at about $-150$ mv until very shortly before death. Other aspects of this phenomenon have been investigated in some detail and a mathematical model of the system has been developed; this shows that it may be explained by a general decrease in membrane permeability, provided that there is a small residual activity of an electrogenic pump. These results are too extended to be given here, but are presently being prepared for publication.

*Chloride Fluxes*

Influx of $Cl^-$ has been followed up to 3 hr with the various inhibitory treatments. In CCCP, in darkness, and at 5°C the inhibition remains at about the same level as in the typical experiments listed in Table II. The maximum inhibitions observed are: $5 \times 10^{-6}$ M CCCP, 95%; in darkness, 90%; at 5°C, 95%. In low $Cl^-$ the influx gradually rises after the initial severe inhibition and after about 1 hr reaches a level representing a 70% inhibition (30% of control flux). This is a new equilibrium condition, since cells will survive several months in this solution, though turgor is greatly reduced. Some cells were kept in low $Cl^-$ for 24 days. At the end of this time $Cl^-$ influx was $207 \pm 15$ pmoles·cm²·sec⁻¹. On transfer to normal ASW $Cl^-$ influx rose within 2 hr to $640 \pm 20$ pmoles·cm⁻²·sec⁻¹. All flux measurements were made in the middle of the day so as to eliminate the possibility of interference from diurnal rhythms.

Firm evidence that the $Cl^-$ influx pump is located at the plasmalemma has been obtained from experiments with nonvacuolated cell fragments. 20 hr after preparation some fragments were given 8 min pretreatment in unlabeled CCCP solution and in the dark. They were then transferred to active

solutions with CCCP and in the dark, and the influxes were measured over an 8 min period. The results are shown in Table V. The control flux is similar to control fluxes in normal cells. The 75% inhibition of Cl⁻ influx in CCCP is comparable to the 60–90% inhibition that CCCP produces in whole cells; the inhibition in the dark is about 15% less than is found with whole cells. Both inhibitions are significant at the 5% level.

As was explained in the Methods section, fluxes of Cl⁻ are so large that cells can be fully labeled with $^{36}$Cl before starting a washout experiment. The efflux under various conditions can therefore be measured by loading cells in the light and applying the experimental treatment at the beginning of the washout period. Results of efflux experiments are shown in Table VI. Comparison with the Cl⁻ influx results shows that a large net efflux occurs at first. This must cause Cl⁻ content to fall, and since the rate constant does not change, a situation of flux balance is eventually attained. With some

TABLE V

CHLORIDE INFLUX TO NONVACUOLATED CELL FRAGMENTS

Time in minutes from beginning of treatment. Fluxes in pmoles·cm⁻²·sec⁻¹.

| Time | Control | CCCP | Dark |
|------|---------|------|------|
| 0–8 | 545±145 (5) | 140±15 (3) | 165±30 (5) |

TABLE VI

CHLORIDE EFFLUX UNDER VARIOUS CONDITIONS

Typical results for Cl⁻ efflux in CCCP, 5°C, and darkness calculated from the rate constant of the washout curve by the method explained in the Appendix. Influx values on which the calculation depends were estimated from the results of measurements with other cells. Permeability was calculated in the usual way, assuming that Cl⁻ efflux is entirely passive and using the values of potential shown which were estimated from measurements on other cells.

| Treatment | Control flux | Permeability, assuming PD of −170 mv | Time from beginning of washout | Efflux | Assumed PD | Permeability |
|-----------|-------------|------------------------------|-------------------|--------|-----------|--------------|
| | pmoles·cm⁻²·sec⁻¹ | cm·sec⁻¹ | min | pmoles·cm⁻²·sec⁻¹ | mv | cm·sec⁻¹ |
| CCCP | 560 | 1.7×10⁻⁷ | 0 | 560 | −80 | 8×10⁻⁸ |
| | | | 120 | 190 | −150 | 1.5×10⁻⁷ |
| | | | 240 | 120 | −150 | 1.5×10⁻⁷ |
| 5°C | 730 | 2.1×10⁻⁷ | 0 | 770 | −80 | 3×10⁻⁷ |
| | | | 120 | 200 | −80 | 3×10⁻⁷ |
| | | | 240 | 6 | −80 | 1.2×10⁻⁸ |
| Darkness | 300 | 9×10⁻⁸ | 0 | 270 | −80 | 4×10⁻⁸ |
| | | | 120 | 186 | −170 | 7×10⁻⁸ |
| | | | 240 | 115 | −170 | 7×10⁻⁸ |
| | | | 360 | 85 | −170 | 7×10⁻⁸ |

treatments the rate constant also falls, so that Cl⁻ flux equilibrium will be reached more quickly. Fig. 2 shows examples of these two situations: CCCP (no change in rate constant) and low temperature (change in rate constant). The analysis of simple nonsteady-state tracer kinetic data, on which the calculation of effluxes from these curves is based, is set out in the Appendix. It should be noted that when the potential falls from −170 to −80 mv the outward driving force on Cl⁻ ions is decreased by a factor of 2.2. Therefore, if efflux does not change, permeability to Cl⁻ must increase by this factor; calculated permeability values in Table VI show these changes. They do not seem to be reflected in the resistance values of Table III but this might
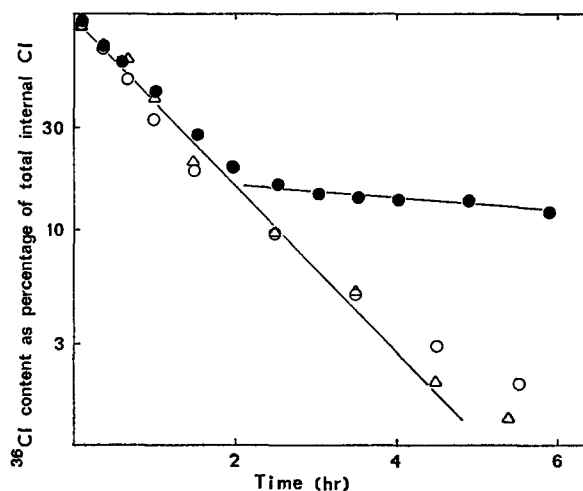


FIGURE 2. ³⁶Cl content of *Acetabularia* cells being washed out into unlabeled solutions of normal ASW at 25°C (○), ASW with CCCP at 25°C (△), and ASW at 5°C (●). Note the change of rate content during the 5°C washout.

be because of the simultaneous decreases of the permeability to other major ions such as H⁺ or K⁺.

It has been noted that when cells are first placed in solutions such as low Cl⁻ or CCCP, Cl⁻ efflux is very much greater than influx, and efflux only falls gradually as content is depleted. Influx and efflux of Cl⁻ must therefore be completely independent of each other; in other words, only a very small proportion of the very large Cl⁻ fluxes can be attributed to exchange diffusion. The large, transient net efflux of Cl⁻ under these various conditions represents a large outward negative electric current. Measured fluxes of Na⁺ and K⁺ are an order of magnitude smaller than Cl⁻ fluxes, and there is no evidence of the huge stimulation of cation effluxes that would be needed to balance the Cl⁻ current. It seems probable that H⁺ efflux is important in this context; this is considered in the Discussion section below.

*Effects of External Cation Concentration*

Normal ASW contains 467 mM Na+, 10 mM K+, and 550 mM Cl−. A series of solutions was prepared in which the concentrations of Na+ and K+ were changed without changing Cl− or any of the other five ions and the total concentration of Na+ plus K+ was kept at 477 mM. The K+ concentrations used were 1.0, 10, 50, 100, 200, 400, and 457 mM, and the corresponding Na+ concentrations were 476, 467, 427, 377, 277, 77, and 20 mM. The effect of these solutions on the membrane potential was measured at 23 and 5°C. At 23°C the potential is at its normal level of −160 to −180 mv and the cell is presumably in flux equilibrium, K+, Na+, and Cl− all being actively transported. At 5°C the potential is usually between −50 and −80 mv; there is a net efflux of K+ and Cl−, and a net influx of Na+. The solution in the measuring chamber could be changed in less than 30 sec and the corresponding changes in membrane potential usually took about 3 min to complete. Solutions were always left longer than this so as to be certain that the potential had reached a true quasi-steady level. All changes in potential were completely reversible, and for a given cell the potential in a particular solution was the same whether arrived at by increasing K+ concentration from a lower or decreasing it from a higher level. Fig. 3 shows a typical potential trace.

The relative values of K+ and Na+ flux and content are such that a new flux equilibrium distribution of these ions could not possibly be reached in 3 min. The apparently stable potential level reached is therefore a quasi-steady state. In some experiments the cell was left in the same solution for periods of about 30 min, during which time a steady drift of potential could be observed. It is most probable that the quasi-equilibration time of 3 min is the time needed for ion exchange in the cell wall. The difference in total
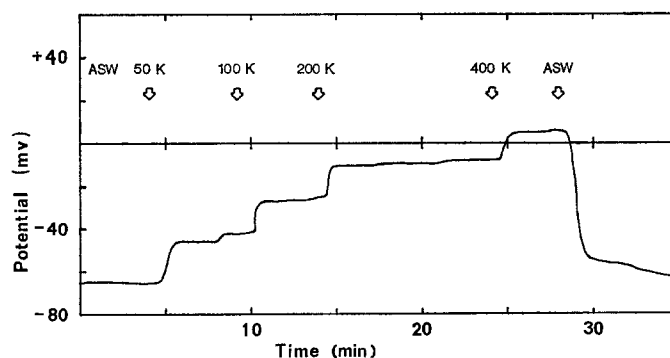


Figure 3. Recorder trace of the effect of changing [K+]o on the membrane potential of an *Acetabularia* cell at 5°C, showing the time needed for quasi-equilibration. See text for details of composition of the solutions.

cation concentration between cell wall and external solution will be quite small for a system bathed in seawater, so that cell wall exchange is not nearly so great a problem as it is with a cell bathed in freshwater. A time of 3 min for partial exchange of $Na^+$ and $K^+$ is consistent with the slightly longer time needed for total exchange of radioactive $Na^+$ and $K^+$ (15 min for $Na^+$, 3 min for $K^+$ in ASW) (Saddler, 1970).

It was always found that increasing external $K^+$ concentration depolarized the cell membrane both at 23 and 5°C. Decreasing external $K^+$ usually caused a small hyperpolarization, but the potential tended to become rather unstable so no useful numerical values could be obtained. Some typical results are shown in Fig. 4. The positive potentials found in cells at 5°C
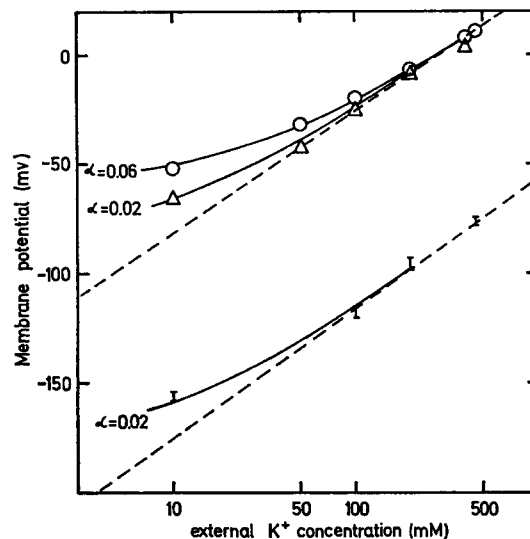


FIGURE 4. The effect of $[K^+]_o$ on the membrane potential at 5°C (upper curves) and 23°C (lower curves). The lines of limiting slope have gradients of 56 mv per 10-fold concentration change at 5°C and 59 mv per 10-fold concentration change at 23°C. See text for further details.

bathed in high $K^+$ solutions are particularly noteworthy. Potential is rather less stable at 23 than at 5°C so the values are represented by small bars. The results at 5°C can be described to a first approximation by an equation of the form

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

where $E$ is membrane potential, $R$ the gas constant, $T$ absolute temperature, $F$ the Faraday, $[K]_o$ external $K^+$ concentration, $[K]_i$ internal $K^+$ concentration, $[Na]_o$ external $Na^+$ concentration, $[Na]_i$ internal $Na^+$ concentration. $\alpha$ is the ratio $P_{Na}/P_K$ where $P$ is permeability. This equation was originally derived by Hodgkin and Katz (1949) from the constant field assumptions of Goldman (1943), but, as many authors have since pointed out, it does not

depend on the assumption of constant field and simply requires that $K^+$ and $Na^+$ be the only permeant ions. Mullins and Noda (1963) have called it the "equation for membrane potential with net ion flow." For fitting the curves to the 5°C results the value of $[K]_i + \alpha[Na]_i$ has been obtained from the point where $E = 0$. The values are 290 mM for the $\alpha = 0.06$ curve and 350 mM for the $\alpha = 0.02$ curve; these are well within the range of values of intracellular $K^+$ and $Na^+$ concentrations found by analysis (Introduction to this paper and Saddler, 1970). The range of values of $\alpha$ represents the extremes of all the values that have been obtained. The line of limiting slope is for $\alpha = 0$ and the gradient is 56 mv per 10-fold concentration change.

The results at 25°C can be fitted to a simple modification of equation (1), obtained by adding a constant term $-X$ to the right side. The curve shown in Fig. 4, with $\alpha = 0.02$, was obtained on the same cell that gave $\alpha = 0.02$ at 5°C. In this case there is more scope for uncertainty, since values have to be chosen both for $[K_i + \alpha Na_i]$ and for $X$. The curve shown is for $X = 85$ mv and $[K_i + \alpha Na_i] = 350$ mM. The gradient of the line of limiting slope is 59 mv per 10-fold concentration change. The unusual and, as will be shown below, inexplicable aspect of these results is the constancy of the term $X$.

The experimental curves can also be fitted by a modification of equation (1) which includes a term for the permeation of $Cl^-$. This requires that $\beta$ $(P_{Cl}/P_K)$ be less than 0.06. It must be emphasized, however, that the fitting of experimental data to a particular theoretically derived formula does not imply that the theoretical assumptions are validated for the system. Such a fit, particularly to so simple an expression as equation (1), may well be merely fortuitous. Any theory which can yield a similar curve is equally plausible, and the choice between alternatives must be made on other grounds. Several possibilities are considered below.

DISCUSSION

An *Acetabularia* cell which has been in the light for some hours is in a state of flux equilibrium (zero net flux) with respect to all the major ions. Any diffusion theory predicts that the membrane potential of such a cell must lie within the range of the equilibrium (Nernst) potentials of the permeating ions. Yet the *Acetabularia* potential is more negative than the Nernst potentials of $K^+$, $Na^+$, and $Cl^-$. Is it also more negative than $E_H$? The pH of ASW solution is 7.8 (culture medium has pH 8.2), and the potential across the plasmalemma membrane is $-170$ mv. If this is to equal $E_H$, then the cytoplasmic pH must be 4.8. It is highly improbable that all the normal metabolic activities of a cell can be maintained in an environment where the pH is so low, in which case $E_H$ must be less negative than $-170$ mv. In other words, the steady-state membrane potential of *Acetabularia* lies outside the range of the Nernst potentials of all the permeating ions. This is the central problem

posed by the results of this investigation of the membrane potential of *Acetabularia mediterranea*, and it is one which I have been unable to explain. It is possible, nevertheless, to identify the factors which have some influence on the potential, and these will now be considered for various equilibrium and dynamic situations.

### Flux Equilibrium, Large Cl⁻ Influx

It must be emphasized once more that a cell can be kept in continuous light for several days with no ill effects. If the light intensity is less than optimal the rate of growth will be much less than 1% per day. Yet the ion fluxes and permeabilities listed in Table I are very large. Net fluxes must therefore be at least an order of magnitude smaller than one-way fluxes. That is, the cell is in a true equilibrium situation. What are the factors affecting the potential?

There is evidently one component which is linked directly to metabolism. All inhibitory treatments which cause fast depolarization also inhibit active $Cl^-$ influx, and both the potential difference and the $Cl^-$ pump are located at the plasmalemma. The same correspondence has not been found for the $K^+$ and $Na^+$ effluxes; some treatments which depolarize the membrane do not also inhibit these fluxes. It seems probable that electrogenic $Cl^-$ influx is contributing to the membrane potential. An alternative explanation which must be considered, however, is that the rapid depolarization is caused by a sudden increase in membrane permeability to a specific ion such as $Cl^-$ or $Na^+$. It is not possible to dismiss this theory completely without a knowledge of the individual ion conductivities, but the fact that membrane resistance in a depolarized cell can be much higher than under normal conditions (Table III) makes such an explanation most improbable.

The fast changes in membrane potential might also indicate the presence of electrogenic $H^+$ efflux as well as, or instead of electrogenic $Cl^-$ influx. Kitasato (1968) has proposed that electrogenic $H^+$ efflux is an important factor controlling the membrane potential of the freshwater alga *Nitella*. But it is impossible to devise a satisfactory system in which $H^+$ efflux is the primary electrogenic process in *Acetabularia*. Consider what happens when a cell is placed in low $Cl^-$. We must suppose that $H^+$ efflux is inhibited and that by some means the experimentally observed $Cl^-$ influx inhibition follows. Positive outward current is thus drastically reduced. $H^+$ influx may also be reduced, but the observed large $Cl^-$ efflux represents a net positive inward current for which no balancing current can be found. It is much simpler to postulate $Cl^-$ influx as the primary electrogenic process, and to resort to $H^+$ fluxes in order to balance net $Cl^-$ fluxes in various dynamic situations. This is considered in more detail below.

It is observed that the membrane potential is also affected by the ratio of

external $K^+$ and $Na^+$ concentrations. $K^+$ has a proportionately greater effect than $Na^+$ and the potential responds as if it were a $K^+$-dominated diffusion potential. Curve fitting leads to a value of $\alpha$ $(P_{Na}/P_K)$ between 0.02 and 0.06, which is in good agreement with flux measurements which give $\alpha = 0.01$–0.05. But the simple diffusion equation also demands a value of 0.02–0.06 for $\beta$ $(P_{Cl}/P_K)$, whereas $\beta$ calculated from flux data is between 0.2 and 1.0.

An alternative explanation for the effect of $K^+$ is that it changes membrane resistance and hence changes the electrogenic potential. It is known that high $K^+$ decreases membranes resistance in the freshwater algae *Chara* (Hope and Walker, 1961) and *Nitella* (Spanswick, personal communication). In the limiting case such a change would reduce an electrogenic potential to zero. This explanation would be satisfactory for the 23°C results, but at 5°C the potential reaches positive values. $K^+$ diffusion would produce this effect, but a simple resistance change would not. The cell at 5°C is in a dynamic nonequilibrium situation which is discussed below, but the results do show that neither explanation for the effect of $K^+$ on membrane potential can account for all the observations.

### Flux Equilibrium, Small $Cl^-$ Influx

It is an experimental fact that *Acetabularia* cells can survive for some weeks in darkness, at low temperature, or in low $Cl^-$ ASW. They have a reduced $Cl^-$ influx and efflux and a lower content of $Cl^-$; cell turgor is almost zero. The membrane potential is $-140$ to $-170$ mv and the resistance may be as high as 1.5 k$\Omega$ cm². Few measurements of cation concentrations have been made under these conditions, but $Na^+$ is at least 150 mM and $K^+$ 100 mM or lower at low temperature. The time that a cell can survive under these conditions is presumably limited by the depletion of energy reserves. This is also a true flux equilibrium situation in which membrane resistance is higher and electrogenic $Cl^-$ influx lower than in normal conditions; the potential is consequently about the same.

### Dynamic Situations

These occur for the first few hours after conditions are changed. The simplest one to consider is the application of low $Cl^-$ solution, since the primary effect of this is on $Cl^-$ influx alone. The experimental observation is that $Cl^-$ influx is inhibited, the membrane is depolarized, but $Cl^-$ efflux is unaltered. As has been mentioned above, there is a consequent net $Cl^-$ efflux which is not balanced by the net fluxes of $K^+$ and $Na^+$.

It seems likely that a stimulated $H^+$ efflux could balance the $Cl^-$ efflux. Unless the cytoplasmic pH is less than 4.8 the electrochemical potential gradients for $H^+$ in the normal cell require an efflux pump at the plasmalemma and a cytoplasm to vacuole pump at the tonoplast for $H^+$. The de-

polarization of the plasmalemma will abolish the gradient against efflux at the plasmalemma and will not change the gradient at the tonoplast in favor of movement into the cytoplasm. One might therefore expect a large stimulation of $H^+$ efflux. It would be an electrogenic flux because, while in this dynamic situation it is balancing $Cl^-$ efflux, in flux equilibrium conditions $Cl^-$ efflux must be balanced by $Cl^-$ influx as explained above, and $H^+$ efflux should therefore be low. In other words, fluxes of $H^+$ and $Cl^-$ are electrically linked. Stimulated electrogenic $H^+$ efflux could well contribute to repolarization.

The high concentration of $H^+$ in the vacuole would serve as a reservoir to be used for balancing net $Cl^-$ efflux. Even if the pH is as low as 1.0, however, the reservoir will soon be exhausted in balancing a net $Cl^-$ efflux of 500 pmoles $\cdot cm^{-2} \cdot sec^{-1}$. The high oxalate concentration in the vacuole suggests another mechanism for balancing charge movement. Induction of oxalate synthesis in the cytoplasm, followed by active transport of oxalate ions into the vacuole and $H^+$ ions to the outside, would produce a positive outward current which could be maintained until the $Cl^-$ fluxes came into equilibrium again. According to this proposed mechanism, then, the over-all effect of placing a cell in low $Cl^-$ ASW would be a stimulated $H^+$ efflux and a rise in vacuolar pH, followed by accumulation of oxalate in the vacuole. When $Cl^-$ flux equilibrium was restored the vacuolar pH would return to a low value by pump activity, whereas most of the accumulated oxalate would stay in the vacuole.

The effect of external $K^+$ in the dynamic situation has already been mentioned. Measurements of $Cl^-$ fluxes in this paper and of $Na^+$ and $K^+$ fluxes[1] show that none of the six fluxes is chemically linked; their only interaction is via the membrane potential. The same considerations can therefore be applied to $Na^+$ and $K^+$ fluxes regardless of whether $Cl^-$ fluxes are in a dynamic or an equilibrium state. The positive potential attained in high $K^+$ solutions at $5°C$ cannot be ascribed to a change in electrogenic potential, nor, since $E_{Cl}$ has a small negative value, can it be caused by an increase of $P_{Cl}$ which would make the potential move towards $E_{Cl}$. It can only be a $K^+$ diffusion potential.

The experiments reported in this paper show that the activity of an electrogenic $Cl^-$ pump contributes to the normal membrane potential of *Acetabularia*. The pump is inhibited by CCCP and darkness, which presumably reduce the energy supply. The equal effects of these two treatments are in contrast to the findings of MacRobbie (1965, 1966) and Raven (1967) on *Nitella translucens* and *Hydrodictyon africanum*, respectively. They have shown that $Cl^-$ influx is insensitive to CCCP and seems to be directly coupled to light-stimulated electron transport.

I have also shown that in certain circumstances $K^+$ diffusion affects the

potential and it may do so under all conditions. The existence of an electro-
genic H+ efflux pump has been proposed, but there is no direct evidence for
its existence.

Thus the membrane potential of *Acetabularia mediterranea* behaves as the
combination of electrogenic and ionic components, as has been reported for
*A. crenulata* (Gradman and Bentup, 1970) and mollusc ganglial cell (Marmor
and Gorman, 1970). The relationship of these components to each other is
particularly complex and its exact nature has not been established.

### APPENDIX

*The Interpretation of Simple Nonsteady-State Tracer Kinetic Data*

The equations for the movement of tracer between a system of compartments can
only be solved analytically if compartmental content and flux are constant. Numeri-
cal or analogue methods must be used if they vary in time (Sheppard, 1962). How-
ever, the very simple two kinetic compartment system is an exception to this general-
ization. The exchange of tracer $Na^+$ and $Cl^-$ in *Acetabularia* can be described by a
system of two compartments, one intra- and one extracellular. The equations are

$$\frac{d}{dt}(QS) = -M_o S \tag{1}$$

$$\frac{dQ}{dt} = -(M_o - M_i) \tag{2}$$

where $M_o$ = efflux, $M_i$ = influx, $Q$ = content of intracellular compartment, and
$S$ = specific activity of compartment as a fraction of the maximum possible specific
activity (the specific activity of the labeling solution). Thus $0 \leqslant S \leqslant 1$ and $S$ is
dimensionless.

If the conditions are the same throughout the course of the experiment it must be
assumed that $M_i$ is constant. $M_o$, however, may well be a function of $Q$. It is most
commonly assumed (in the constant field theory, for example) that for a passive flux
$M$ is a linear function of the activity (which in the absence of any information about
activity coefficients must be assumed to be proportional to $Q$). At the other extreme,
$M$ may be independent of $Q$. This will be true when $M$ is a predominately active
flux showing saturation kinetics and $Q$ varies within the saturating range. There seems
little point in considering other relations between $M$ and $Q$ without knowing that
they do, in fact, occur.

(a) For $M_o$ a linear function of $Q$, say $M_o = aQ$. Then the equations become

$$Q\frac{dS}{dt} + S\frac{dQ}{dt} = -aQS \tag{3}$$

$$\frac{dQ}{dt} = -aQ + M_i \tag{4}$$

From (4)

$$Q = \frac{M_i}{a}\left[1 + \left\{\frac{aQ(O)}{M_i} - 1\right\}e^{-at}\right]$$

$\therefore$ From (3) $S = \dfrac{Q(O)S(O)ae^{-at}}{M_i\,[1 + \{aQ(O)/M_i - 1\}e^{-at}]}$     (5)

$\therefore QS = Q(O)\,S\,(O)e^{-at}$     (6)

and

$$\frac{d}{dt}(QS) = -aQ(O)S(O)e^{-at} \qquad\qquad (7)$$

In a washout experiment the results are in the form of graphs of $QS$ and $\dfrac{d}{dt}(QS)$ against $t$. These nonsteady-state solutions give simple exponential curves, so this situation will often be indistinguishable from flux equilibrium. However, if influx under the relevant conditions is known, it can be established whether the experimental results represent an equilibrium or a net flux situation, and they can then be appropriately analyzed. Most of the *Acetabularia* results give simple exponential curves in net flux situations. Substitution of (5) and (7) into equation (1) gives

$$M_o = M_i + \{aQ(O) - M_i\}e^{-at}$$

Hence efflux at any time can be calculated from the experimental values of $M_i$, $Q(O)$, and $a$.

 (b) For $M_o$ independent of $Q$ equations (1) and (2) stand as they are

From (2)       $Q = Q(O) - \Delta Mt$

where        $\Delta M = M_o - M_i$

$\therefore$ (1) becomes $[-\Delta Mt + Q(o)]\dfrac{dS}{dt} - \Delta MS = M_o S$

$$\therefore S = K\left[1 - \frac{\Delta M}{Q(O)}t\right]^{M_i/\Delta M}$$

When $t = 0$, $S = S(O)$

$$\therefore S = S(O)\left[1 - \frac{\Delta M}{Q(O)}t\right]^{M_i/\Delta M}$$

$$\therefore QS = Q(O)S(O)\left[1 - \frac{\Delta M}{Q(O)}t\right]^{M_o/\Delta M}$$

and

$$\frac{d}{dt}(QS) = -M_o S(O) \left[ 1 - \frac{\Delta M}{Q(O)} t \right]^{M_i/\Delta M}$$

$$= M_o S$$

These results give curved semilogarithmic plots. They were never encountered with *Acetabularia*.

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