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THE RELATION OF THE MEMBRANE POTENTIAL AND RESISTANCE OF NITELLA TO ION FLUXES

A Thesis
Presented to the Graduate School of
the State University of New York
College at Brockport

As partial fulfillment of the
requirements for the Degree of
Master of Science
in
Botany

By

Thomas E. Ryan

August 1973

THESIS DEFENSE

THE RELATION OF THE MEMBRANE

POTENTIAL AND RESISTANCE

OF NITELLA TO ION FLUXES

by Thomas E. Ryan

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INTRODUCTION

Ion fluxes through Characean membranes are, by classical analysis, divided into two distinct components: active energy-requiring transport and passive diffusion. Early studies (Hope and Walker, 1960, 1961; Hope, 1963) considered the electrical potential developed across the membrane a result of the passive component while active fluxes served to maintain the concentration gradients or driving force for the passive fluxes. If the active fluxes are electrically neutral, the diffusion potential developed across the selectively permeable membrane is given by the Goldman equation:

$$E_m = \frac{R\ T}{F} \ln \frac{(P_K\ K_o + P_{Na}\ Na_o + P_{Cl}\ Cl_i)}{(P_K\ K_i + P_{Na}\ Na_i + P_{Cl}\ Cl_o)} \qquad (1)$$

where R = universal gas constant, 1.99 cal/mole degree
T = absolute temperature, ^oK
F = Faraday constant, 23,060 cal/mole volt
P = permeability of the membrane to the ion, cm/sec
K_i, K_o, etc. = concentration of the ion inside and outside the cell, respectively, molar
E_m = resting potential, volts.

According to this equation the levelof the potential is limited by the concentrations of diffusible ions in contact with the cell membrane. K^+, Na^+ and Cl^- comprise 90% of the intracellular electrolytes in the Characeae (Barr, 1965).

MacRobbie (1970) has compiled a table of ion concentrations and potentials from work reported by many authors. In all cases the potential was within the range allowed by the passive diffusion model, when external K^+ concentration was

0.1 mM. Hope and Walker (1961) found that changes in external K^+ and Na^+ concentrations did affect the potential as predicted by the Goldman equation but only when Ca^{++} was excluded from the external solution. Williams and Hogg (1970) conducted similar experiments and concluded that the Goldman equation is applicable only if permeability coefficients are concentration dependent. Spanswick, Stolarek and Williams (1967) found that in the presence of Ca^{++} the potential did not follow a Goldman relationship when external K^+ concentration was increased. In Ca-free solution the potential was controlled by external K^+ but only over a limited range. Both Kitasato (1968) and Spanswick (1972) have tested the effect of 1.0 to 100 mM external Na on the resting potential and found little or no effect.

More recent work in 1.0 mM K^+ solution (Rent, Johnson and Barr, 1972) demonstrated that the potential is more negative than the limit imposed on it by the Goldman equation. Spanswick (1972) has reviewed cases similar to this and also established conditions in his own work (increased external $[K^+]$) where the potential is much more negative than predicted.

A further problem in the study of Characean membranes has been the discrepancy which exists between electrically measured conductance and the conductance calculated from flux measurements. According to Williams, Johnston and Dainty (1964) electrical conductance should be related to passive fluxes since ions carry the current flow when current is passed through the membrane by means of an external circuit.

The calculated partial conductance of a given ionic species is based on the following relationship:

$$g_i = \frac{F^2}{R\,T} J_i \tag{2}$$

where g_i = partial conductance of ion i, μmhos cm^{-2}
J_i^i = unidirectional flux of i when i is in electrochemical equilibrium, moles $cm^{-2}sec^{-1}$

The electrochemical equilibrium potential of an ion is that potential at which no net movement of the ion occurs (influx and efflux are balanced). If K^+ fluxes predominate over all other ionic fluxes as suggested in the literature (Walker and Hope, 1961) the Goldman equation becomes:

$$E_m = \frac{R\,T}{F} \ln \frac{K_o}{K_i} \tag{3}$$

where E_m is equal to the K^+ equilibrium potential (E_K). Because E_m for most Characean cells is close to E_K and K^+ efflux is thought to be entirely passive (see MacRobbie, 1970), equation (2) may be used to calculate the membrane conductance contributed by K^+. The value obtained for K^+ conductance should be very close to the total membrane conductance as the permeability of the membrane to Na^+ is only 10% of the K permeability (Hope and Walker, 1961), and the chloride permeability is even less (Kitasato, 1968; Walker and Hope, 1969).

Williams, Johnston and Dainty (1964) found the measured

conductance of Nitella translucens to be ten times the value calculated from K^+ and Na^+ fluxes. Hope (1963) obtained similar results working with Chara corallina. Spanswick (1970) has included the value for Cl efflux obtained by Hope, Simpson and Walker (1966) and found a discrepancy of less than two between calculated and electrically measured conductance. Spanswick (1970) explained this difference on variation between batches of cells. The conductance values of Spanswick (1970) were very low, approximately 7 μmho cm^{-2} compared to 21μmho cm^{-2} reported by Walker and Hope (1969) and 46μmho cm^{-2} reported by Williams et al. (1964). Conductances measured by Brown, Ryan and Barr (1973) and also Paszewski, Stolarek and Gebal (1968) are considerably higher than Spanswick's (1970) value. Subsequently Spanswick has obtained smaller conductances under the same conditions(Spanswick, 1972).

In 1968, Kitasato proposed a hypothesis that accounts for the previously described departures from the passive model in both potential and conductance. He postulated that H^+ fluxes account for 95% of the ionic traffic across the Nitella membrane. When Kitasato (1968) clamped the membrane potential at E_K and changed the pH of the external solution from 6 to 5 the current needed to hold the potential at E_K was consistant with a net positive influx. Since he also found partial conductances calculated from K^+ and Cl^- fluxes were negligible compared to the measured conductance and nothing but the pH of the external solution was changed, he concluded that H^+ must account for the major fraction of membrane conductance. Increased Na permeability was eliminated on the

basis of the small effect of increased $[Na]_o$ on the membrane potential.

Allowing that the diffusion potential for H^+ is positive (based on intracellular pH measurements of Hirakawa and Yoshimura, (1964)), and the observed potential negative Kitasato (1968) proposed an electrogenic H^+ efflux pump in combination with the passive H^+ influx which sets the level of the membrane potential.

Support was lent to Kitasato's hypothesis when alternating bands of acid and base formation were detected along the length of a _Nitella_ cell by Spear, Barr and Barr (1969). Estimates of H^+ extrusion from their work are very close to the value obtained by Kitasato (1968), these being 5-20 x 10^{-12} mole $cm^{-2}sec^{-1}$ and 40 x 10^{-12} mole $cm^{-2}sec^{-1}$, respectively. Lucas and Smith (1973) have recently measured the pH along the length of the alkaline and acid regions of _Chara corallina_ and found 10^4 fold changes in H^+ concentration between the two regions. The sharp peaks they found in the alkaline regions led them to conclude that a localized OH^- efflux is superimposed on an apparently uniform H^+ efflux system. They suggest OH^- is made available by uptake and subsequent splitting of HCO_3^- making CO_2 available for photosynthesis while OH^- is released to the external solution. This interpretation differs from that of Spear et al. (1969) who suggested that the alkaline zones were due to H^+ influx.

Lannoye, Tarr and Dainty (1970) have also studied the effect of pH on the membrane potential and ionic fluxes. They found a 100-fold increase in external $[K^+]$ or $[Na^+]$

(0.1 to 10.0 mM) resulted in only slight depolarizations of the membrane when the pH remained constant. Their flux studies led them conclude that E_m changes at low pH were brought about by a selective permeability increase to Na^+. The Na^+ fluxes observed by Lannoye et al. (1970) are much larger than those reported elsewhere in the literature for the Characeae (MacRobbie, 1962; Walker and Hope, 1961, 1969; Barr, 1965). Spanswick (1972) has studied the effect of increased external $[Na^+]$ on the membrane potential at low pH and concluded that the conclusions of Lannoye et al. (1970) are erroneous.

Walker and Hope (1969) have criticized Kitasato's (1968) conclusion that H^+ fluxes account for most of the membrane conductance although their flux studies show K, Na and Cl account for only one-third of the total membrane conductance. They suggested the discrepancy is possibly due to non-independent movement of ions. Equation (2) is therefore modified to include the factor n:

$$g_i = n \frac{F^2}{R\,T} J_i \qquad (4)$$

where n represents ionic interference (possibly the number of ions in a pore of the membrane). Walker and Hope (1969) also believe that fluxes have been underestimated due to backfluxes of tracer and discrepancies between the concentrations of ions immediately bounding the membrane and the external and internal concentrations on which flux calculations are based.

Walker and Hope (1969) also have suggested that potentials more negative than diffusion potentials may arise when the concentration of K^+ immediately bounding the outside of the membrane is lowered due to active influx of K^+ which must somehow be balanced by another charge movement. In view of the potentials observed by Spanswick (1972) and Brown et al. (1973) this would require an approximately 10-fold reduction in external $[K^+]$. The fact that changes in external $[K^+]$ rapidly changed the potential under certain conditions (Hope and Walker, 1961) indicates the region bounding the membrane is extremely permeable to K^+ and hence a 10-fold depletion in concentration seems unlikely.

MacRobbie (1971) has criticized the reasoning used by Walker and Hope (1969). She believes that if tracer buildup does occur near the inside and outside of the membrane causing backfluxes the underestimates in influx and efflux will cancel each other out. This will result in no error in calculating the net flux on which membrane chord conductance is based. In reviewing the work of Spear et al. (1969) and of Smith (1970) who found acid extruding regions in Chara corallina, MacRobbie (1970; 1971) concluded that H^+ fluxes seemingly account for most of the membrane conductance.

In light of Kitasato's (1968) evidence for a high H^+ permeability Rent et al. (1972) interpreted the rise in pH and the amount of acid addition necessary to maintain a low pH in the solution bathing cells as a net H^+ influx. The initial pH for these experiments was 4.5, chosen so the change in external pH due to CO_2 loss from solution would be negli-

gible. At higher pH the results become more difficult to interpret. As Smith (1967) has stated the pH change depends on the balance between HCO_3^- loss from solution, OH^- efflux into solution, CO_2 exchanges due to photosynthesis, respiration and partial equilibration with air and also the movement of organic ions into solution. The use of pH 4.5 eliminated CO_2 as an important factor in pH changes in the medium. Possible harmful effects of low pH on the cell have still not been determined. It is known that the mean lifespan of cells in pH 4.7 solution is about 3 days (Barr, unpublished observation).

A further problem observed by Rent et al. (1972) was the failure of the membrane to depolarize as rapidly as expected due to the initial high net H^+ influx. This indicates some other current is very nearly balancing the H^+ inward current or the H^+ missing from solution is being absorbed on the outer surface of the membrane. Perhaps it is trapped near the membrane by the large negative charge density of the cell wall (Dainty, Hope and Denby, 1960). The results of Kitasato (1968) and Lannoye et al. (1970) would appear to eliminate an increased Cl^- influx or a decreased Na^+ efflux as the balancing current. Since the potential at higher pH was above E_K K^+ efflux is not involved in slowing down the initial depolarization. Raven (1973) has suggested that low internal pH may cause the H^+ efflux pump to work faster; however the H^+ influx measured by Rent et al. (1972) was a net flux and therefore this cannot be the balancing current. The possibility

of an organic cation efflux remains. Zarlengo and Schultz (1966) have shown that organic anion efflux can balance H^+ extrusion in certain bacteria.

Brown, Ryan and Barr (1973) have calculated H^+ fluxes using an equation adapted from Kitasato (1968):

$$J_{H^+} = \frac{E_H - E_m}{R_a F} \qquad (5)$$

where J_{H^+} = net passive H^+ influx = H^+ extrusion when E_m is steady, moles $cm^{-2}sec^{-1}$
E_H = H^+ equilibrium potential, volts
R_a = areal membrane resistance, Ωcm^{-2}

This formula is a variation of Ohm's law with the difference between the H^+ equilibrium potential and the membrane potential as the driving force for H^+ current through the measured membrane resistance. A question arose regarding the validity of the very high fluxes they calculated, averaging 150×10^{-12} mole $cm^{-2}sec^{-1}$ in light. They suggested that these fluxes may be overestimated if the membrane conductance relates not only to passive H^+ influx as is assumed in equation (5) but also to the opposing H^+ pump. Membrane conductance is determined by the displacement of the resting potential when an applied current of known areal density is passed through the membrane, i.e.,

$$G = \frac{I_D}{\Delta E} \qquad (6)$$

where G = membrane conductance, mho cm^{-2}

I_D = applied current density amp cm^{-2}
ΔE = change in resting potential, volts

A serious error in conductance would occur if the electrogenic pump is extremely sensitive to the potential displacement, changing its electrogenicity to render the potential nearly the same as in the resting state. The effect of this would be a very small measured ΔE and a correspondingly large conductance. By this explanation conductance values relate mainly to the effect of an applied current on the electrogenic pump rather than on the passive ion movements as assumed in equation (5).

Slayman (1965) has proposed that the electrical properties of Neurospora are determined by the ionic emf and resistance in parallel with the metabolic emf or H^+ pump and the resistance of the pump. Spanswick (1972) has proposed a similar model for Nitella in which he suggests the resistance of the pump is much lower than the resistance calculated from passive fluxes of the major ions. Since he observed no resistance changes when the pH was increased to neutrality (putative decrease in current carrier) Spanswick (1972) reasoned that the passive permeability of the membrane to H^+ is low. Hence equation (4) cannot be used to calculate the electrogenic pump flux since it is based on the idea that membrane resistance relates only to the passive H^+ influx. As an alternative explanation Spanswick (1972) suggests that currents passed through the membrane during resistance measurements traverse the membrane as changes in the outward H^+ flux through the pump. The basis of this idea was first given by Finkelstein (1964) who

demonstrated that an electrogenic pump has a definite conductance and the flux through this pump is voltage dependent. The pump can thus be looked at as a self-limiting mechanism.

Spanswick (1972) has suggested that when a depolarizing current is applied to the resting cell the voltage dependent pump speeds up to hold the potential near the resting level. Alternatively a hyperpolarization would reduce the rate of H^+ extrusion. These changes in pumping rate account for the net current crossing the membrane while the external circuit prevents charge buildup. Spanswick (1972) has tentatively identified the current required to reduce the potential to E_K as the flux through the electrogenic H^+ pump. He estimated this at 20 x 10^{-12} moles $cm^{-2}sec^{-1}$ agreeing very well with the visual observations of Spear et al. (1969).

From Spanswick's (1972) idea it follows that a cell in the hyperpolarized state ($E_m > E_K$) has a healthy H^+ pump and a correspondingly low resistance or high conductance. Conditions which lower the potential should therefore increase the resistance.

The effects of inhibitors, temperature and light on the resting potential and resistance have been used to further the idea of a metabolically controlled electrogenic mechanism which determines the level of the membrane potential. Kitasato (1968), working with 2,4-dinitrophenol, and Spanswick (1973), working with azide and CN^-, found that these depolarized the

membrane to about E_K and increased the resistance. It was presumed that these inhibitors stopped or retarded the electrogenic pump. The observations fit well with the idea that most of an applied net current travels through the pump. With the pump retarded by darkness or chemical inhibition the relative increase in resistance should be roughly equal to the relative decrease in pumping rate. If the pump is completely stopped both the resistance and potential should be able to be predicted from the passive model.

The potential will be close to E_K if the membrane is not passively permeable to H^+ as Spanswick (1972) suggests. If the membrane is passively permeable to H^+ as suggested by Kitasato (1968) and Brown et al. (1973) the potential should fall between E_K and E_H as given by the following equation:

$$E_m = \frac{RT}{F} \ln \frac{K_o P_K + H_o P_H}{K_i P_K + H_i P_H} \qquad (7)$$

Since the H^+ equilibrium potential is positive this potential is below E_K, and a much larger than normal K efflux should be evident if indeed the membrane is passively permeable to H^+. This point will be tested experimentally, by using 2,4-dinitrophenol in the external solution. The results should either confirm Spanswick's (1972) idea that the membrane is not passively permeable to H^+ or give a new measure of H^+ influx equal to K efflux minus K influx under the same conditions. The amount of H^+ coming out passively in DNP solution is negligible since the potential is still much greater than E_H.

The effect of decreased temperature on the membrane potential and resistance have been studied by Hope and Aschberger (1970) and Spanswick (1972). Both found that in light temperature has a much greater effect on the potential than expected from equation (1). Equally interesting was the finding that in the dark a 10°C decrease in temperature had no effect on membrane resistance while the resistance doubled for the same decrease in temperature in the light. These tests support the idea of a metabolically controlled potential and further show that the mechanism involved in this plays a key role in determining membrane resistance.

Brown et al. (1973) have found that the membrane potential and resistance are changed by light/dark transitions and pH changes. Essential to their interpretation that this change is caused by changes in H^+ fluxes are the assumptions that 95% of the membrane conductance is due to H^+ and measured conductance relates only to passive fluxes. The results of Walker and Hope (1969) indicate this is not the case. When they altered the potential via an external circuit the K^+ influx increased at a rate much greater than predicted by the Goldman equation.

PROPOSED STUDIES

The present work was designed to test the hypotheses that H^+ is the main current carrier and that membrane resistance relates only to passive ionic movements. Potassium and chloride fluxes were monitored while a hyperpolarizing current was passed

through the membrane. Flux measurements on resting cells were made so these results could be compared to what is expected from the passive model. The fraction of current carried by K^+ and Cl^- are also reported.

The effect of low pH, which is assumed to eliminate the electrogenic component of the membrane potential (Brown et al. 1973), on K fluxes was also studied. Its effect on passive fluxes are discussed in terms of the passive model. The effect on active fluxes is related to a H^+/H^+ or H^+/K^+ exchange pump proposed by Poole (1973).

Both Spanswick (1972) and Brown et al. (1973) have reported approximately 50 mv depolarization of the potential in the dark at pH 5.7 - 6.0, and increases in resistance from 16 $K\Omega\ cm^2$ in light to 153 $K\Omega\ cm^2$ and 55 $K\Omega\ cm^2$, respectively, in darkness. K fluxes were measured in darkness to determine if passive fluxes are changed under conditions which change membrane resistance. This work also measured resistances in the dark for the length of time over which these fluxes were measured.

Potentials and resistances were also measured in a solution containing DNP, a condition which supposedly renders the H^+ pump inoperable (Kitasato, 1968). If this is the case both the resistance and potential should be close to the values predicted by the passive model. K^+ and Cl^- fluxes were monitored in DNP to determine if this is true. These fluxes were compared with the resting fluxes to try to establish the H^+ permeability of the membrane.

The results of this study are discussed in terms of Kitasato's (1968) hypothesis that H^+ fluxes are largely responsible for the maintainence of the resting potential. It has attempted to determine if the measured conductance of the membrane reflects the active or passive properties of the membrane. An answer to this question depends largely on whether or not the membrane is passively permeable to H^+ since conductance calculated from fluxes of K, Na and Cl account for only a amall portion of the membrane conductance. The possibility remains that an applied current drastically changes the properties of the membrane, but there apparently is no way to investigate this idea.

MATERIALS

Nitella clavata was cultured in 3.0 liter glass containers with aeration under illumination of about 125 foot-candles measured at the solution surface. Sixteen hours of light provided by Sylvania Gro-lux and Cool-white fluorescent lamps alternated with 8 hours of darkness. The composition of the nutrient solution is given in Table 1A. The nutrient solution for cells used in Experiments 46, 58 and the E_m and R_a measurements was changed to that listed in Table 1B.

Internodal cells, the second or third from the growing tip, were separated from the plant by cutting away neighboring internodal cells and branch cells. They ranged between 2 and 5 cm in length and were 600 to 800 μm in diameter.

After being harvested, the cells were placed in K solution; the composition in millimoles per liter was 1.0 KCl, 0.1 NaCl, 0.1 $MgCl_2$, and 0.1 $CaCl_2$. The pH of this solution was 5.7. The cells were kept in an incubator at 22°C and approximately 45 foot-candles cool white fluorescent light. The length of preconditioning (postharvest age) varied from 6 to 10 days.

In certain experiments the concentration of KCl in K solution was changed. This was due to errors in the specific activity of ^{42}K made by Union Carbide Corp. (see Appendix). These are indicated as 0.44 mM K, 0.86 mM K etc. The concentrations of NaCl, $MgCl_2$ and $CaCl_2$ remained constant. In the Na solution the concentrations of K and Na were reversed; K^+ was 0.1 mM, and Na^+ was 1.0 mM.

TABLE 1

Composition of Culture Solutions

A

4.0	mM	tris-(hydroxymethyl)-aminoethane neutralized with HCl to pH 7.0
1.0	mM	$MgSO_4$
1.9	mM	NaCl
0.2	mM	KCl
3.0	mM	$CaCl_2$
0.1	mM	$NaHCO_3$
0.2	mM	KNO_3
8.6	μM	streptomycin
10.0	μM	KH_2PO_4
3.7	μM	H_3BO_3
1.0	μM	Na_2MoO_4
1.0	ml/l	micronutrient stock solution

B

4.0	mM	tris-(hydroxymethyl)-aminoethane neutralized with HCl to pH 7.0
1.0	mM	$MgSO_4$
1.0	mM	NaCl
0.3	mM	KCl
1.5	mM	$CaCl_2$
1.0	mM	$NaHCO_3$
0.1	mM	KNO_3
0.2	mM	$NaNO_3$
8.6	μM	streptomycin
10.0	μM	KH_2PO_4
3.7	μM	H_3BO_3
48.7	μM	nitriloacetic acid
3.4	μM	$FeCl_3$
0.27	μM	$MnCl_2$
0.20	μM	$ZnSO_4$
0.05	μM	$CoCl_2$
0.04	μM	$CuSO_4$
1.0	μM	Na_2MoO_4
1.0	ml/l	micronutrient stock solution

Micronutrient stock solution: filtrate from 50 g Brockport brown soil boiled 30 minutes in 1.0 l of 6.0 mM ethylenediamine tetraacete at pH 8.0.

METHODS

Membrane Potential and Areal Resistance

Membrane potentials and resistances were measured with the cell resting on filter paper (Whatman No. 2) in a narrow plexiglass trough (60 x 8 x 3 mm.). Experimental solution filled the trough and was delivered via polyethylene tubing and removed by a filter paper siphon. The entire volume of solution was replaced approximately every 2 minutes.

Two microelectrodes were inserted into the vacuole of the cell with the aid of a Titan 1218 dissecting microscope. One served for potential measurements and the other for current delivery. These were made from microcapillary glass pulled to an outside tipe diameter of 5 to 10 μm by a Narashige PN-3 glass microelectrode puller.

The potential-measuring Ag/AgCl electrode was filled with "artificial cell sap" (80.0 mM KCl, 30.0 mM NaCl and 5.0 mM $CaCl_2$) which simulates the vacuolar contents of the cell and reduces the possibility of a diffusion potential arising at the tip. A similar glass microelectrode was used for reference. These were connected to a Keithley 604 electrometer. The electrometer output was delivered to a Leeds and Northrup Speedomax XL680 strip chart recorder. It was found that electrical artifacts were minimized by using the differential inputs of the electrometer and grounding the trough to earth through a Ag/AgCl electrode inserted into a Coleman reference electrode reservoir.

The potential difference between the measuring and reference microelectrodes was measured before and after each experiment. This was done by mimicking the experimental conditions; i.e., placing the measuring electrode in "artificial cell sap" and the reference electrode in K solution, with a filter paper salt bridge joining the solutions. Values obtained in this manner were usually between -10 and +10 mv. Microelectrodes having larger potentials were discarded.

The current delivery microelectrode was a Ag/AgCl electrode inserted into a microcapillary tube, filled with 3.0M KCl to minimize tip resistance. This was connected to an 81 volt DC source in series with a suitable number of 100 MΩ resistors to produce the desired current, as monitored by a Simpson taut band microammeter. Current was returned by a silver wire placed along the length of the cell. Harco repeat cycle multi-gang timer switches obtained from Herbach and Rademan, Inc. were used to program the delivery of negative and positive currents at chosen intervals.

In the resistance measurements small current pulses of 10^{-6} to 10^{-7} amperes and 10 second duration were passed through the cell membrane causing a displacement of the membrane potential of 5 to 10 mV. Negative and positive currents were separated in time by a few seconds of zero current. If rectification occurred the two voltage displacements were averaged. Using Ohm's law the resistance of the entire membrane was calculated knowing the change in voltage across the membrane and the current delivered to the cell. The resistance

of a unit area (areal resistance) was then calculated. It should be noted that the membrane potential (E_m) and areal resistance (R_a) are actually the potential and resistance across the cell wall, cell membrane and vacuolar membrane in series (the microelectrodes are inserted into the large central vacuole), but for convenience are refered to as the membrane potential and membrane resistance, respectively.

Corrections for the leaky cable properties of characean cells were avoided by employing the procedure outlined by Hogg, Williams and Johnston (1969). With this method displacement of membrane potential is measured at the place where the average current is passing through the membrane. If the current is delivered to the center of the cell this point is equal to 0.21 x (cell length) away from the center and it is here that the potential-measuring electrode is inserted. The reference electrode is placed as close as possible to this point outside the cell to avoid a potential drop across the solution bathing the cell.

Potassium Fluxes

Potassium fluxes were measured with ^{42}K at an initial specific activity of 1.3×10^4 mCi mole^{-1}. Experiments were continued until about 95 per cent of the radioactivity was lost by decay. About 1.7×10^{-4} moles of tracer was available for each experiment.

Cells were initially exposed to tracer solution for the period indicated in Tables 2-5 after which ^{42}K was removed

from the cell surface by a 3-phase 2-hour rinse in non-tracer solution. Radioactivity was then assayed by placing the cell in a specially designed holder directly beneath a Geiger-Muller tube. Counting was done with a Nuclear-Chicago 8703 decade scaler. Appropriate corrections for geometry were made (see Appendix). In some experiments cells were placed back into tracer solution and influx determined a second time.

Potassium efflux was measured by collecting ^{42}K lost from cells loaded with tracer during influx experiments. Individual cells were placed in 4.0 ml of non-tracer solution. At the end of a specified time period (see Tables 2-5) solution was collected and replaced for a second consecutive efflux period. At the end of the second efflux period solution was again collected and the samples from the first and second efflux periods were prepared for radioassay. From the amount collected 1.0 ml was placed in a planchette and a drop of 1.0M $NaHCO_3$ added to it. Samples were then dried on a hot plate and counted on a Nuclear-Chicago low background, gas flow beta counter. The length of time the cells were rinsed before each efflux period varied. Normally 6-8 hours are necessary to wash out the free spaceof the cell wall and cytoplasm (Hope, 1963). Longer rinses were made to be sure the effluxes were steady. Preconditions are given in Tables 2-5.

Specific activity for unidirectional efflux measurements depends on the internal potassium concentration of the cell. This was determined for individual cells by placing them in 4.0 ml of distilled water and breaking them open. Potassium

activity of this solution was then measured with a Corning monovalent cation electrode (No. 476220) with an apparent K^+/Na^+ selectivity of 8. Sodium interference was also minimized by using standards which were suitable dilutions of artificial cell sap. An average internal potassium concentration determination was also made using a Perkin-Elmer model 303 Atomic Absorption Spectrophotometer. This was done for experiment 32 (Table 2).

Potassium Influx with an Applied Current

Potassium influx was measured while a hyperpolarizing current was being applied to a *Nitella* cell. Current was delivered in the same manner described under Membrane Potential and Areal Resistance. Radioassay was by a Geiger-Muller tube mounted with its axis perpendicular to the axis of the cell and separated from contact with the radioactive solution in the trough by a plexiglass partition.

The normal potassium influx (control) was first established during two 30 or 60 minute influx periods. To do this the trough was sealed off (no solution flow) and filled with tracer solution. At the end of an influx period tracer solution was removed and the cell and trough rinsed repeatedly with non-tracer solution. Rinsing continued until radioassay of the cell showed no fluctuation. This was usually after a period of 20 to 30 minutes during which time the total volume of the trough was replaced with non-tracer solution approximately 40 times.

Following the controls the potassium influx was measured

while a negative current of about 4.0×10^{-7} ampere was being delivered to the cell. The duration of this period was also 30 or 60 minutes. The procedure was the same as in the controls. A final control was then obtained following the current delivery period.

At the end of the final influx period the electrodes were removed and the cell was transferred to the specially designed holder (see Potassium Fluxes) for radioassay under the standardized geometry procedure. This enabled the same geometry corrections employed in the previously described influx measurements to be used (see Appendix). The fraction of measured radioactivity entering the cell during each 30 or 60 minute influx period in the trough was then multiplied by the total cpm in the cell as determined under the standard geometry procedure, in order to obtain influx values for each influx period.

Chloride Fluxes and Chloride Efflux with an Applied Current

The same methods employed under Potassium Fluxes were used to measure unidirectional $^{36}Cl^-$ fluxes. The specific activity of $^{36}Cl^-$ in K solution was 114 mCi $mole^{-1}$.

Chloride efflux was also measured while a negative current was being delivered to the cell. Cells of *Nitella* were loaded with $^{36}Cl^-$ during the preconditioning period. Preconditioning was the same as described in Materials. Current was delivered in the same manner described under Membrane Potential and Areal Resistance in this section. Before insertion of the electrodes cells were given a 3-phase 2-hour rinse to remove external $^{36}Cl^-$.

To measure chloride efflux the trough was sealed off and filled with non-tracer K solution. After a designated period 1.0 ml of this solution was drawn off and prepared for radioassay in the manner previously described for potassium efflux. Efflux was measured both with and without applied current. Current varied from 4 to 10 x 10^{-7} ampere.

Specific activity was obtained by counting the whole cell in the manner described under Potassium Fluxes in this section. The internal chloride concentration was measured using a Orion solid state chloride electrode. Samples were prepared for assay by breaking the cells open and drawing up 3μl of cell sap with a microcapillary. This was then diluted to 300μl with distilled water. The average value obtained from 17 cells was used in the calculations. Appropriate corrections for geometry were made and are included in Appendix.

As in all experiments K solution was the preconditioning and experimental solution. Experimental lighting throughout this work was 45 foot-candles cool white fluorescent light.

Statistics

Variation is expressed as the standard error of the mean in all cases except where otherwise indicated.

RESULTS

Membrane Potentials and Areal Resistance

The records of the membrane potentials (E_m) and areal resistances (R_a) of 7 cells in both light and darkness are shown in Figures 1 - 7. Dark periods of 16 to 65 hours in length were used to obtain values which could be related to ^{42}K fluxes under similar conditions. Previous work from this laboratory (Brown et al., 1973) presented the electrical properties of the membrane in darkness over much shorter time periods.

The average E_m of 30 cells used in various experiments throughout this work was -152 ± 3 mv in light at pH 5.7. The average postharvest age of these cells was 7 days. Cell age is important since Figure 8 shows that internal $[K]$ increases with the time cells were conditioned in K solution. E_K calculated for a 7-day cell ($[K^+]_i$ = 85 mM), from Equation (3) is -114mv. This value is 38 mv more positive than the average E_m, a strong indication that an electrogenic pump is responsible for the resting potential in _Nitella_.

The average membrane resistance of 11 cells in light at pH 5.7 was 17 ± 2 $K\Omega cm^2$ (from Figures 1 - 7, 9 - 12). This value agrees well with resistances measured by Brown et al. (1973) and Spanswick (1972) which were, respectively, 15.5 $K\Omega cm^2$ for _Nitella clavata_ and 17.0 $K\Omega cm^2$ for _Nitella translucens_. The measured resistances are much lower than the value of about 300 $K\Omega cm^2$ which Hope (1963) and Williams, Johnston and Dainty (1964) calculated from fluxes of the major ions. Under our

present conditions $[K^+]_o$ is relatively high, 1.0 mM, and one might expect a K^+ flux that would be consistant with the low measured resistance. However, according to Equation (8):

$$R_a = \frac{E_m - E_K}{J\ F} \quad (8)$$

where J = net flux of K, moles $cm^{-2}sec^{-1}$
the net cationic influx predicted is 23 pequiv $cm^{-2}sec^{-1}$, while the measured net K^+ influx averaged 1.6 pmoles $cm^{-2}sec^{-1}$ (see RESULTS, Unidirectional K^+ Fluxes). There are two conclusions possible here: (a) some other cations or anions are carrying the major portion of current or (b) the use of resistance measurements for the calculation of passive fluxes is not valid. Equation (8) was introduced to relate resistance to an ionic flux because Equation (2) is valid only when the potential is at the equilibrium potential for the ion. The potentials obtained in our work are more negative than E_K; therefore the equation for chord conductance must be used (see Kitasato, 1968; Hope, 1971).

The typical initial response of the cells to darkness (Figures 1 - 7) was a depolarization of E_m and an increase in R_a. However, after 10 to 25 hours of darkness E_m and R_a typically returned to levels fairly close to the steady values observed in light. The average initial depolarization for 6 of the cells took place immediately after the lights were switched off. The average peak value of R_a in darkness was 65 $K\Omega cm^2$, representing about a 3-fold increase.

Two exceptions to the general trend of an increased R_a in the dark were noted. In one case, where R_a in light was very high, darkness initially caused a large decrease in R_a, but a level similar to the light level was eventually established (Figure 4). In the other no immediate change was observed, but after 15 hours of darkness a high resistance peak appeared (Figure 7). This was associated with the usual depolarization of E_m. In one cell a large amount of rectification was observed and R_a was calculated and plotted for both inward and outward applied currents.

It is important to note that cells in which an atypical E_m change occurred in response to darkness (hyperpolarizing rather than depolarizing), there was a typical initial increase in R_a (Figure 2). The reverse was true for the cell shown in Figure 4 where a typical initial depolarization was accompanied by an atypical decrease in R_a.

The changes im E_m and R_a induced by darkness are not strictly coordinated, but there is some basis for assuming that they are both associated with H^+ extrusion (see INTRODUCTION). It is possible that, as Spanswick (1972) has suggested, an applied current does pass mainly through the H^+ extrusion pump, but in a manner that does not relate in any simple way to the normal electrogenic current. This point is illustrated by the fact that the initial high measured R_a in darkness still does not come anywhere near the value predicted from the passive model. This indicates that even when there is no evidence for electrogenic H^+ pumping (as shown by the low E_m and lack of acid regions in darkness (Spear et al., 1969))

most of the applied current still travels through some channel other than the passive channels of the membrane.

E_m and R_a were also measured in K solution plus 0.05 mM 2,4-dinitrophenol (DNP) in the light at pH 5.7. Records of these cells are shown in Figures 9 - 12. In cases where large amounts of rectification occurred R_a calculated from both hyperpolarizing and depolarizing currents was plotted (Figures 9, 10). Normally the applied current (3×10^{-7} amp cm^{-2}) was not reduced when DNP solution was used. This resulted in very large (30 - 40 mv) changes in potential as compared to the normal 5 - 10 mv change. In one case (Figure 12) the current was reduced to obtain changes in potential within the normal range. R_a measured in this way compares favorably with R_a calculated from larger displacements of E_m.

Figures 9 - 12 show that R_a is several times greater than normal in DNP solution. This seems to be consistant with the idea that an applied current passes through the H^+ pump easily only when it is operating normally. Since DNP interferes both with the normal conductance and with the maintainence of a normal resting potential it would appear these are closely related as Spanswick (1972) has suggested. The fact that DNP blocks the conductance by 80% or more suggests the possibility the H^+ pump may be inhibited to the same degree; however all of the fluxes of major ions must be considered before conclusions are drawn.

The average E_m in DNP solution was -104 mv, a value very close to E_K. If passive K^+ fluxes serve to maintain this potential a 10-fold decrease in $[K]_o$ should result in E_m becoming 59 mv more negative. This was not the case as shown in

Figure 13, where Na solution plus DNP was substituted for K solution plus DNP. This result was repeatable and demonstrates that the potential in DNP solution is not a K^+ diffusion potential. This means that when DNP is present the level of E_m is somewhere in the neighborhood of E_K but appears not to be determined by passive K^+ movement.

The simplest way to find out how the stabilization of E_m at about -100 mv is achieved in the presence of DNP is to measure the fluxes of Cl^- and K^+ and determine whether: (a) a large, balanced net flow of K and Cl out of the cell occurs (making the $[K]_o$ change above a negligible factor in the determination of E_m), or (b) a substantial net efflux of Cl^- occurs which is balanced by the electrogenic mechanism, operating at a reduced rate under these conditions. An alternative explanation would be that passive inward movement of H^+ coupled with a partial loss of electrogenicity is responsible for the observed depolarizations. Implicit in this statement and alternative (b) is the assumption that there is an electrogenic H^+ extrusion pump. If this assumption is true and either of these possibilities is correct, it would be impossible to predict the level of the resting potential since this requires knowledge of the decrease in the electrogenic pumping rate and the change in P_{Cl} or P_H. Only P_{Cl} can be determined unambiguously from experimental data.

Unidirectional K^+ Fluxes

The rate of ^{42}K uptake was measured under varying conditions of light, darkness, pH, added DNP and $[K]_o$. The results

of these experiments are shown in Tables 2, 3, 4 and 5. The cells loaded with ^{42}K in light were later used to measure ^{42}K effluxes under the same conditions as influx measurements.

The average ^{42}K influx for 100 cells in 0.86 to 1.0 mM K solution, in light at pH 5.7 was 1.6 pmoles $cm^{-2}sec^{-1}$ (pmole = 10^{-12} mole). The average influx for different cell groups ranged from 1.0 to 2.6 pmoles $cm^{-2}sec^{-1}$. The differences observed between consecutive influx periods in light are scattered and difficult to interpret. At times the initial rate of uptake was faster than the subsequent uptake period (Table 4, Exp. 40A). In one case the reverse was true (Table 3, Exp. 46A), and in another experiment (Table 2, Exp. 32A) no difference was found. Long term uninterrupted influxes fell between or were lower than the consecutive periods (Tables 3, 4, 5). This suggests handling the cells between influx periods had no adverse effect on ^{42}K uptake.

There was no significant effect of darkness on K influx. The average ^{42}K influx for 64 cells in darkness at pH 5.7 was 1,5 pmoles $cm^{-2}sec^{-1}$ with the same range of variation among different cell groups as in light, 1.0 to 2.6 pmoles $cm^{-2}sec^{-1}$. The duration of dark conditioning before the start of an influx period ranged from 0 to 22 hours and this had no significant effect on the rate of ^{42}K uptake. This contrasts sharply with the work of MacRobbie (1965) who found a 10-fold decrease in K^+ influx in the dark. From this she concluded that K^+ uptake was supported by light energy through utilization of ATP produced in photophosphorylation. The results of this work indicate that under our conditions (where $[K]_o$ is 1.0 mM as

compared to the usual 0.1 mM) this is not the case. The level of the potential in the dark and the very high influx/efflux ratio allow the possibility that K^+ influx may be active, perhaps linked to H^+ extrusion.

The results shown in Tables 2 - 5 indicate that the mean values for K influxes, among similar experiments done at different times, seem to fall into two discrete categories: Those having high influxes (2.15 to 2.65 pmoles $cm^{-2}sec^{-1}$) and those having low influxes (1.0 to 1.5). Presumably the nutritional status of the cells is responsible for this difference. Usually a fast initial period was followed by a slower period but this was not always the case. The same degree of variation which was seen in the potential and resistance measurements in dark was also found for K^+ influx in both light and dark. This variation makes correlations between resistance measurements and K^+ influxes difficult. The only evidence to suggest such a correlation may exist is from work in which increasing the external $[K]$ results in an increased K influx, a decreased R_a and presumably a somewhat depolarized E_m (Hope, 1965).

^{42}K influx in 1.0 mM K solution plus 0.05 mM DNP at pH 5.7 was about one-fifth of that for the control solution. (Table 5, Exps. 58C, 70B, 70C). It is generally thought that active transport processes are blocked in the presence of DNP, perhaps by interference with ATP synthesis. The large decrease in K^+ influx is greater than that calculated for passive movement from the observed change in potential in DNP solution; this supports the idea that most of the K^+ influx is active and that DNP is effective in blocking this component.

The K influx in solution at pH 4.7 averaged about one-fourth that of cells at pH 5.7 (Tables 3, 4; Exps. 40D, 46E). The observed influxes were very close to the values observed in DNP solution suggesting H^+ likewise interferes with active K^+ transport. The most likely mechanism for this inhibition is H^+ competition for sites on the K^+ influx transport mechanism. The effect of $[H^+]_o$ on E_m (Kitasato, 1968; Brown et al., 1973) leads one to suspect that the H^+ efflux pump and K^+ influx mechanism are related. That is, the reduction in K^+ influx caused by low pH should in itself be hyperpolarizing since it reduces the positive inward current; the actual effect of low pH is a depolarization indicating perhaps an interference with H^+ extrusion. However, other possible explanations will also be considered in the DISCUSSION.

It should also be mentioned that the differences in influx at differing concentrations of external $[K^+]$ are within the range of experimental error and normal variation among cells for $[K]_o$ = 0.86 to 1.0 mM. Excluding any fast influx periods the ^{42}K influx in 0.44 mM K solution is close to the values obtained at higher concentrations (Table 2, Exp. 32A). The E_m's at this concentration also are similar to E_m's at higher concentrations (see RESULTS, Ion Fluxes with an Applied Current). Thus no extreme changes in cells are noted at an external $[K]$ similar to that used by Spanswick (1972).

^{42}K effluxes in light and darkness and at pH 5.7 and 4.7 are also shown in Tables 2 through 5. After 8 hours of rinsing in tracer-free solution the efflux became constant, averaging 0.16 pmole $cm^{-2}sec^{-1}$ for 28 cells. Longer rinsing (25

hours) resulted in no significant change in efflux (Table 4, Exp. 40A). This is consistant with the work of Hope (1963) who found 8.3 hours were needed to wash out the free-space of the cell wall and cytoplasm.

This short washout period disagrees with Barr's (1965) work in which 2 days of rinsing were necessary before constant effluxes were attained; the effluxes were much lower in that work and reflect the differences in solution composition. It may be that the small nodal cells at the end of each Nitella internode (described by Barr, 1965) are washed out faster than previously reported.

^{42}K efflux in darkness was measured on two groups of cells (Tables 3, 4; Exps. 46C, 40C). In one group (Table 4, Exp. 40C) the efflux in dark was one-half the value observed in light (difference significant at 0.05), after 6 hours of rinsing and 2 hours of prior dark conditioning. After 20 hours of darkness and 25 hours of rinsing the efflux in dark was one-third of the value observed in light (difference significant at 0.01). In another experiment (Table 3; Exp. 46C) no significant difference was observed between light and dark cells with 0 hours of prior dark conditioning. It appears that changes in K permeability are not responsible for the high resistance peaks observed during the first 10 hours of darkness. The long term effects show an inverse relationship since resistance in darkness typically decreases to the normal (light) value while K efflux decreases.

^{42}K efflux in the light at pH 4.7 was twice the value observed in light at pH 5.7 (Table 3; Exp. 46D). This dif-

ference is significant at the 0.05 level and will be related to the effect of H^+ on K permeability in DISCUSSION.

Figure 14 shows the ^{42}K efflux in light at pH 5.7 in both K solution and K solution plus 0.05 mM DNP. Ten cells were used for each solution; five cells were used to obtain the effluxes for a full 3-hour period (shown by the dotted line), while for the other five cells the temporal course of the efflux was followed in detail. This figure shows the effluxes were high during the shorter periods at the beginning of the 3 hours. This is possibly due to mechanical disturbances caused by handling of the cells. Very steady effluxes were obtained for both solutions after the first 45 minutes. These agree very well with the long term effluxes. The efflux in the DNP solution was about 0.8 pmoles $cm^{-2}sec^{-1}$. This value is five times greater than the overall average value for cells under control conditions (Tables 2, 3, 4) and four times greater than the control cells in this experiment. The significance of this large efflux can be better analyzed together with the net chloride efflux observed under this condition (see RESULTS, Unidirectional Chloride Fluxes).

Unidirectional Chloride Fluxes

Chloride influx and efflux were measured with ^{36}Cl in K solution at pH 5.7 and 4.7 and in K solution plus 0.05 mM DNP. Efflux was also measured in K solution at pH 5.7 in the dark and in K sulfate solution; i.e., where $SO_4^=$ was substituted for Cl^-. The results of these experiments are shown in Tables 6 and 7.

The normal resting influx in 3-hour experiments at pH 5.7 was 0.09 pmoles $cm^{-2}sec^{-1}$ for a total of 17 cells in experiments 73 and 74 (Tables 6, 7). The average resting efflux of 15 cells under the same conditions, prior to any experimental treatment was 0.24 pmoles $cm^{-2}sec^{-1}$ (Tables 6, 7). This efflux is the same as the value obtained by DiGregorio, Barr, Endress, Sherwin and Mannhardt (1973) under the same conditions; however in their work they found no net efflux of Cl^-. The low influx and hence net efflux obtained in this work perhaps reflects the very short (3 hour) influx periods used. If most of the $^{36}Cl^-$ is still in the cytoplasm much of it may be washed out during the 1.5-hour rinse in non-tracer solution. On this basis the results are lower than the true influx values; a comprehensive study involving varied influx and rinse periods are needed to confirm this.

Both in low pH (4.5 - 5.1) and in the DNP solution at pH 5.7 the Cl^- effluxes are increased to about 1.8 pmoles $cm^{-2}sec^{-1}$ while influx increases to 0.8 pmoles $cm^{-2}sec^{-1}$ and 0.7 pmoles $cm^{-2}sec^{-1}$, respectively. Since at low pH the K^+ fluxes are nearly balanced (see RESULTS, K Fluxes) there is a net Cl^- efflux of 0.8 pmoles $cm^{-2}sec^{-1}$. This may account for the E_m depolarizations observed by Kitasato (1968), Lannoye et al. (1970) and Rent et al. (1973). A similar situation exists for the DNP solution where there is net negative outward current of 0.6 pmoles $cm^{-2}sec^{-1}$, as calculated from the algebraic sum of the K^+ and Cl^- fluxes. In fact the large K efflux in this solution may be a passive effect as E_m becomes less than E_K due to an increase in P_{Cl}. Kitasato (1968) was aware of a

large Cl^- efflux at low pH but considered this insignificant in comparison to the H^+ fluxes he calculated from resistance and voltage clamp data. Evidence is presented below which casts doubt on the existance of very large H^+ fluxes and correspondingly makes more important consideration of the net Cl^- efflux as the cause of the depolarization.

Table 7 also shows Cl^- efflux is relatively unchanged in K sulfate solution. This suggests Cl^- fluxes are not due to an exchange mechanism and tends to confirm the passive independent movement of Cl^- outward; on this basis Cl^- efflux may be used to calculate Cl^- conductance.

The effects of low pH and DNP on the Cl^- permeability of the membrane appear to be similar; however, Brown et al. (1973) report little or no change in R_a at pH 4.7 while DNP causes an average 10-fold increase in R_a. The fact that little change in resistance occurs at low pH while P_{Cl} increases 7-fold indicates measured resistance bears little or no relationship to passive Cl^- fluxes. The increase in resistance caused by DNP can then be viewed as an effect on the channel through which current easily passes in the normal state. This doesn't necessarily mean that a complete stoppage of H^+ pumping occurs since Cl^- efflux can account for the observed depolarization in DNP solution.

Net Positive Influx

It has been shown from tracer studies that a net K^+ influx occurs in the resting state; i.e., K solution at pH 5.7. This net influx was about 1.4 pmoles $cm^{-2}sec^{-1}$. Analysis of

vacuolar sap shows that $[K]_i$ increases with the time cells were conditioned in K solution (Figure 8). The net influx calculated from the average slope of the line in Figure 8 was 1.1 pmole $cm^{-2}sec^{-1}$. Something must balance the inward net positive charge carried by K^+ since E_m is steady in the resting state. The Cl^- flux work shows that Cl^- fluxes are essentially balanced; therefore net Cl^- influx cannot be the balancing current. It has also been demonstrated that E_m is much more negative than E_K. The easiest way of accounting for charge balancing and the high E_m is the electrogenic H^+ efflux pump first proposed by Kitasato (1968).

Ion Fluxes with an Applied Current

^{42}K influx, $^{36}Cl^-$ efflux and net H^+ influx were measured while a hyperpolarizing current of 0.4 to 0.6 microamp was passed through the membrane of single cells. Control fluxes (no current) were also measured on the same cells to provide a basis for relating the increase in ionic current carried by the ion in question to the magnitude of the applied current. The transference number (TN) was calculated by dividing the increment in flux during an applied current by the total current (expressed in pequiv $cm^{-2}sec^{-1}$).

^{42}K influxes with and without applied current are presented in Table 8. Also shown are the TN, E_m and the ΔE_m caused by the applied current. The TN averaged 0.53 in experiments where $[K]_o$ ranged from 0.44 to 1.0 mM. The highest values were in experiments completed in Spring, 1973 in 1.0 mM K solution. The average TN in these experiments (0.67) was

found to be higher than in experiments completed in Fall and Winter, 1972 (avg TN $<$ 0.5). This difference is understandable in two cases where $[K]_o$ was 0.44 mM but not in 0.86 to 0.98 mM $[K]_o$.

Figures 15 - 20 show the E_m, resting K^+ influx, K^+ influx during an applied current, and the applied current for 5 of the cells represented in Table 6. It can be seen from these diagrams that applying a current for 30 to 60 min. has no detrimental effect on either E_m or resting K^+ influx as judged by the behavior following the period of the applied current. An instantaneous deflection of E_m was observed upon switching the current on. This usually remained steady, but, at times the potential hyperpolarized slowly during the first 5 minutes of applied current. This second slow hyperpolarization at times raised the total ΔE_m caused by the applied current to double the instantaneous change. This same effect was also observed in reverse when the current was switched off. The values listed in Table 6 are the difference in E_m between the stabilized hyperpolarized level and the level attained shortly after the current is turned off.

Three experiments were undertaken in attempts to measure the K transference number in the presence of DNP (Table 9). These experiments were started immediately after influx experiments in non-DNP solution. If ^{42}K did carry much of the current significant counting differences would have been obtained. In only one experiment did K^+ carry a significant amount of current (TN = 0.3). In the other two experiments no significant differences were observed. The final control

in experiments 61 and 63 (Table 9) showed a negative influx. The most probable interpretation for this result is that a high specific activity in the cytoplasm (due to the short rinse period in non-tracer solution) allowed the ^{42}K efflux to mask the influx. Since ^{42}K efflux in DNP solution is quite high (Figure 14), this explanation appears adequate to account for erroneously small or negative transport numbers under these conditions. It is also possible $^{36}Cl^-$ carries a substantial portion of the current in DNP solution as P_{Cl} increases about 7-fold under these conditions.

Table 10 shows the effect of an applied current on Cl^- efflux in 4 cells. These results are presented graphically in Figures 21 - 26. The average TN in these experiments was 0.07. ΔE_m and the resting E_m were very close to those in the $^{42}K^+$ influx experiments. One cell with a potential considerably more positive than typical had about the same TN as other cells. The same was true in the ^{42}K influx experiments where the TN for a cell near E_K was little different from a cell with E_m values in the normal range (Table 8; Exps. 42, 44).

Net H^+ influxes with and without applied current were measured by following the pH change in the external solution. This was done in the same apparatus used for ^{42}K influx with an applied current. These measurements were made on 3 cells with a control pH change preceding the pH change with applied current. In both the control (no current) and experimental (applied current) the pH changed from 5.7 to 6.2 in about 25 minutes. If H^+ was carrying the current as Kitasato (1968) suggests the pH change observed (5.7 to 6.2) should have taken

place in 4 minutes for the experimental current of 0.8 μamp. Since the Cl^- and K^+ applied current experiments show Cl^- and K^+ carry 60 to 70% of the current the observed pH change for H^+ carrying the remaining fraction should have taken place in about 10 minutes. The fact that no difference between the control and experimental was observed in 3 cases is good evidence that the membrane is not passively permeable to H^+. The significance of the applied current experiments will be analyzed under DISCUSSION.

Table 2. K Fluxes of Nitella. Experiment 32

Influxes: K_o = 0.44 mM
Effluxes: K_o = 1.00 mM

Cell Group	Special Pre-conditioning	Experimental Conditions lighting	pH	Flux Period hrs	K Influx pmoles $cm^{-2}s^{-1}$	K Efflux pmoles $cm^{-2}s^{-1}$
A	none	light	5.7	0- 3	1.03±0.13 (10)	
	none	light	5.7	3-19	0.79±0.08 (10)	
	3 hr rinse	light	5.7	19-23		0.42±0.07 (6)
	8 hr rinse	light	5.7	23-40		0.15±0.03 (8)

Variation expressed as the standard error of the mean. Values in parentheses are the number of cells used. Light refers to the standard light condition (see METHODS). Post-harvest age 8 days.

Table 3. K Fluxes of Nitella. Experiment 46

Influxes: K_o = 0.86 mM
Effluxes: K_o = 1.00 mM

Cell Group	Special Pre-conditioning	Experimental Conditions lighting	pH	Flux Period hrs	K Influx pmoles $cm^{-2}s^{-1}$	K Efflux pmoles $cm^{-2}s^{-1}$
A	none	light	5.7	0- 4	1.28±0.07 (12)	
	none	light	5.7	4-17	2.15±0.17 (12)	
	5 hr rinse	light	5.7	17-26		0.22±0.05 (10)
	14 hr rinse	light	5.7	26-37		0.15±0.03 (10)
B	none	dark	5.7	0- 4	1.57±0.11 (10)	
	8 hr dark	dark	5.7	4-17	1.38±0.06 (10)	
C	none	light	5.7	0-21	1.02±0.06 (10)	
	5 hr rinse	dark	5.7	21-30		0.16±0.02 (10)
	14 hr rinse, 10 hr dark	dark	5.7	30-41		0.09±0.01 (10)
D	none	light	5.7	0-21	1.01±0.07 (10)	
	5 hr rinse	light	4.7	21-24		0.47±0.06 (10)
E	none	light	4.7	0- 3	0.37±0.04 (10)	

Variation expressed as the standard error of the mean. Values in parentheses are the number of cells used. Light refers to the standard light condition (see METHODS). Post-harvest age 7 days.

Table 4. K Fluxes of *Nitella*. Experiment 40

Influxes: K_o = 0.98 mM
Effluxes: K_o = 1.00 mM

Cell Group	Special Pre-conditioning	Experimental Conditions lighting	pH	Flux Period hrs	K Influx pmoles $cm^{-2}s^{-1}$	K Efflux pmoles $cm^{-2}s^{-1}$
A	none	light	5.7	0- 5	2.63±0.25 (13)	
	none	light	5.7	5-17	1.40±0.08 (13)	
	6 hr rinse	light	5.7	17-35		0.22±0.05 (10)
	25 hr rinse	light	5.7	35-47		0.18±0.02 (10)
B	14 hr dark	dark	5.7	0- 5	1.06±0.08 (12)	
	22 hr dark	dark	5.7	5-17	1.20±0.11 (12)	
C	none	light	5.7	0-21	1.87±0.07 (10)	
	6 hr rinse, 2 hr dark	dark	5.7	21-40		0.10±0.01 (10)
	25 hr rinse, 20 hr dark	dark	5.7	40-51		0.05±0.01 (8)
D	none	light	4.7	0- 4	0.43±0.01 (7)	

Variation expressed as the standard error of the mean. Values in parentheses are the number of cells used. Light refers to standard light condition (see METHODS). Post-harvest age 6 days.

Table 5. K Fluxes of *Nitella*

Influxes: K_o = 1.00 mM

Cell Group	Special Pre-conditioning	Experimental Conditions lighting pH (other)		Flux Period hrs	K Influx pmoles $cm^{-2}s^{-1}$
58A	none	light	5.7	0-13	1.75±0.16 (10)
58B	none	light	5.7	0-13	1.51±0.14 (10)
58C	none	light (+0.05 mM DNP)	5.7	0- 3	0.35±0.01 (11)
59A	none	dark	5.7	0- 3	2.65±0.23 (10)
	see below*	dark	5.7	3-22	1.01±0.09 (10)
59B	none	light	4.7	0- 3	0.71±0.07 (10)
70A	none	light	5.7	0-12	1.74±0.1 (20)
70B	none	light (+0.05 mM DNP)	5.7	0-.5	.48±0.07 (5)
70C	none	light (+0.05 mM DNP)	5.7	0- 3	.19±0.01 (5)

*This group was exposed to 3 hrs of dark followed by 3 hrs of light prior to the influx measurement.

Variation expressed as the standard error of the mean. Values in parentheses are the number of cells used. Light refers to the standard light condition (see METHODS). Postharvest age 8 days. Each cell group number refers to a separate experiment (harvest of cells). Letters refer to separate groups within the same harvest.

Table 6. Experiment 73

$^{36}Cl^-$ INFLUXES: 9 cells each in 73A and 73B, mean surface area 0.7 cm^2/cell

Exp. No.	Treatment	Cell Age, Days	pH	Flux Period hrs	Influx pmole $cm^{-2}sec^{-1}$
A	K solution	6	5.7	0- 3	0.09±0.01
B	K solution+ 0.05 mM DNP	6	5.7	0- 3	0.95±0.04

$^{36}Cl^-$ EFFLUXES: 8 cells each in 73C and 73D, mean diameter 0.072 cm

Exp. No.	Treatment	Cell Age, Days	pH	Flux Period hrs	Efflux pmole $cm^{-2}sec^{-1}$
C	K solution	6	5.7-?	0- 3	0.22±0.04
	K solution	6	4.7-5.1	3- 6	1.79±0.17
	K solution	7	5.7-5.4	6-23	0.37±0.05
	K solution	7	4.5-4.7	23-26	1.88±0.07
	K solution	8	5.7-5.3	26-46	0.35±0.05
	K solution	9	5.6-5.4	46-70	0.27±0.04
	K solution	10	5.7-5.4	70-95	0.22±0.02
D	K solution+ 0.05 mM DNP	6	5.7-?	0- 3	1.81±0.34
	K solution	6	5.7-7.2	3- 6	0.31±0.04
	K solution	7	5.7-5.5	6-23	0.13±0.01
	K solution+ 0.05 mM DNP	7	5.7-5.6	23-26	1.82±0.09
	K solution	8	5.7-5.3	26-46	0.19±0.02
	K solution Dark	10	5.7-5.6	70-95	0.45±0.04

Table 7. Experiment 74

$^{36}Cl^-$ INFLUXES: 8 cells, mean surface area 0.54 cm^2/cell

Exp. No.	Treatment	Cell Age, Days	pH	Flux Period hrs	Influx pmole $cm^{-2}sec^{-1}$
A	K solution	8	5.8	0- 4	0.17 ± 0.04
	K solution	8	4.8	4- 8	0.93 ± 0.13
	K solution	8	5.6-5.4	8-16	0.57 ± 0.08
	K solution	9	5.8-5.5	16-48	0.45 ± 0.06

$^{36}Cl^-$ EFFLUXES: 7 cells, mean diameter 0.071 cm

Exp. No.	Treatment	Cell Age, Days	pH	Flux Period hrs	Efflux pmoles $cm^{-2}sec^{-1}$
B	K solution	9	5.7-7.2	0-25	0.28 ± 0.03
	K solution	10	4.8	25-29	1.41 ± 0.26
	K solution	10	5.7-5.6	29-50	0.27 ± 0.03
	K solution	11	5.9	50-62	0.35 ± 0.05
	K $SO_4^=$ soln	12	5.8	62-73	0.32 ± 0.05
	K $SO_4^=$ soln	12	4.9	73-76	0.96 ± 0.13
	K solution	13	5.8-5.6	76-84	0.42 ± 0.05
	K solution	13	5.9-5.8	84-90	0.26 ± 0.02

Table 8. K Influxes: Applied Current Experiments

Exp. No.	K_o mM	Avg. Resting Influx pmoles cm^{-2} sec^{-1}	Influx with applied current pmoles $cm^{-2}sec^{-1}$	Applied current pequiv $cm^{-2}sec^{-1}$	Em -mv	Hyperpole Em -mv	Transference No.
33	0.44	0.90	4.55	7.64	145	12	0.48
34	0.44	1.04	3.60	6.94	145	9	0.38
42	0.98	1.07	3.56	6.70	113	50	0.37
44	0.98	0.90	3.05	5.00	144	17	0.43
48	0.86	2.86	4.85	4.40	163	17	0.45
61	1.00	2.28	6.85	7.10	174	22	0.65
62	1.00	2.88	8.53	9.33	149	30	0.60
63	1.00	1.66	8.50	9.00	152	25	0.76
64	1.00	1.66	8.15	9.42	159	23	0.69

Each Experiment no. refers to one cell. Data from Exps. 33, 34, 62, 63 and 64 were taken from Figs. 15, 16, 19, 18, 17, respectively. Avg. resting influxes were the average of 2 or 3 60 min. influx periods except Exps. 63 and 64 where 30 min. periods were used. Influx with applied current is the influx of one period. The average cell age was 8 days.

Table 9. Experiments 61, 62, 63

^{42}K INFLUXES: in K solution +0.05 mM DNP with and without applied current

Exp. No.	Treatment	Applied current pequiv $cm^{-2}sec^{-1}$	Em -mv	Influx pmoles $cm^{-2}sec^{-1}$
61	60 min No current	0	84	1.8 ± 0.27
	60 min Applied current	2.73	141	0.27 ± 0.27
	60 min No current	0	81	-0.67 ± 0.27
62	30 min No current	0	97	0.44 ± 0.19
	30 min Applied current	9.33	162	3.20 ± 0.19
63	30 min Applied current	3.50	219	0.36 ± 0.19
	30 min No current	0	123	-0.36 ± 0.19

Table 10. Cl^- Effluxes: Applied Current Experiments

Exp. No.	Cell Age Days	Avg. Resting Efflux pmoles cm^{-2} sec^{-1}	Efflux with applied current pmoles $cm^{-2}sec^{-1}$	Applied current pequiv $cm^{-2}sec^{-1}$	Em -mv	Hyperpole Em -mv	Transference No.
14	6	0.07	1.50	9.4	155	32	0.09
	6	0.07	3.20	9.4	155	32	0.26
	7	0.25	0.40	11.1	150	27	0.01
	7	0.25	0.90	11.1	150	27	0.06
	8	0.50	1.10	14.5	155	20	0.04
	8	0.50	1.20	14.5	155	20	0.05
	8	0.50	1.20	14.5	155	20	0.05
	8	0.50	1.20	14.5	155	20	0.05
17	8	0.15	0.30	4.5	145	15	0.03
	8	0.15	0.30	4.5	145	15	0.03
	8	0.15	0.50	4.5	145	15	0.08
	8	0.15	0.40	4.5	145	15	0.06
20	6	0.35	0.75	7.3	118	29	0.05
	6	0.35	1.10	7.3	118	33	0.10
21	5	0.50	1.10	5.5	150	8	0.11
	5	0.50	0.85	5.5	150	12	0.06
Avg.		0.41	0.90		146 ± 3	21 ± 2	$.071 \pm 0.01$

Each Experiment no. refers to one cell. Average resting effluxes are the average of 5 or more 30 min. efflux periods. Efflux with applied current is one 30 min. efflux period.

Table 11. Expected Passive and Actual Increases in K^+ Influx During Applied Current

Exp. No.	Calculated Passive K^+ Influx at Resting Potential, pmoles $cm^{-2}sec^{-1}$	Calculated Expected increase in Passive K^+ Influx during Applied current pmoles $cm^{-2}sec^{-1}$	Actual Increase in K^+ Influx during Applied Current pmoles $cm^{-2}sec^{-1}$
33	0.28	0.02	2.56
34	0.28	0.03	3.65
42	0.50	0.21	2.49
44	0.63	0.07	2.15
48	0.62	0.07	1.99
61	0.77	0.10	4.57
62	0.66	0.14	5.65
63	0.68	0.11	6.84
64	0.71	0.10	6.49

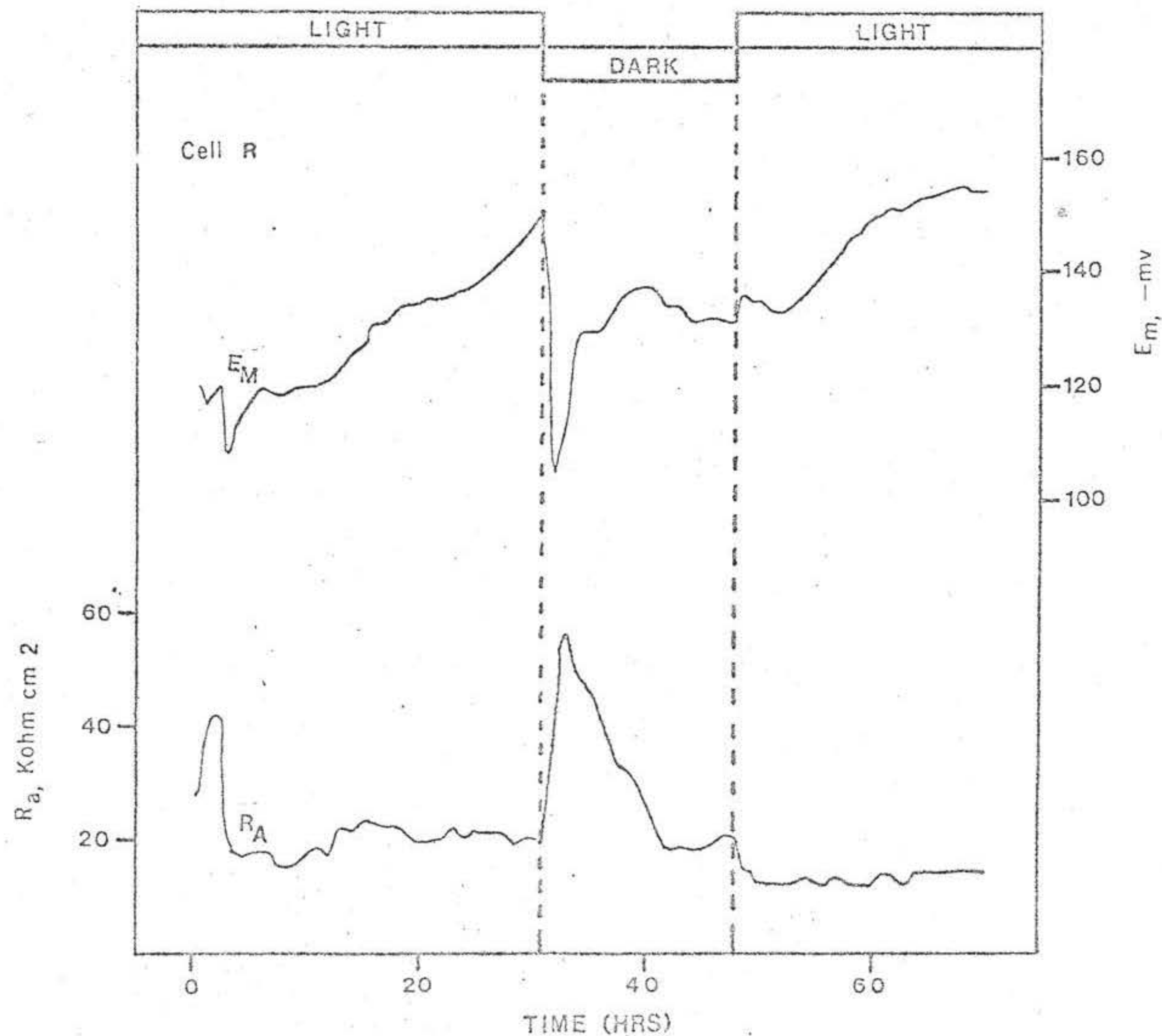


Figure 1. Areal resistance and resting potential measurements of a three day old *Nitella* cell in light and darkness at pH 5.7. This cell shows the typical initial dark depolarization and increase in R_a. After a number of hours of darkness R_a returns to the light level and E_m partially recovers. Actual R_a measurements were made every o.5 hour. The high initial R_a in light is presumably related to the insertion of the microelectrodes and the time required for recovery.

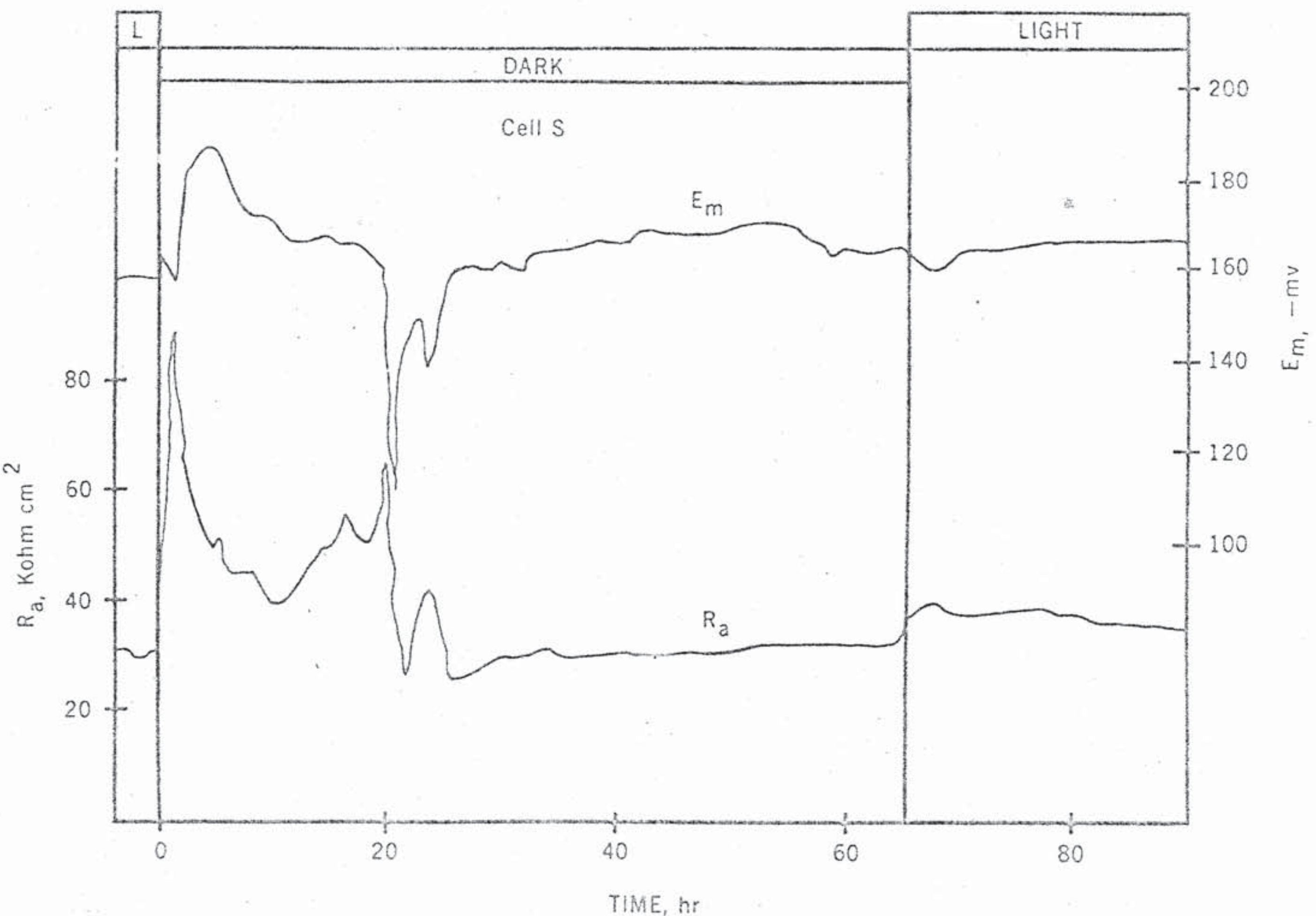


Figure 2. Areal resistance and resting potential measurements of an 8 day old <u>Nitella</u> cell in light and darkness at pH 5.7. This cell shows the typical initial increase in R_a with an atypical hyperpolarization. After about a day in darkness both potential and resistance returned to light levels. Actual R_a measurements were made every 0.5 hour. The record begins after the cell has completely recovered from the insertion of the microelectrodes (see Fig. 1).

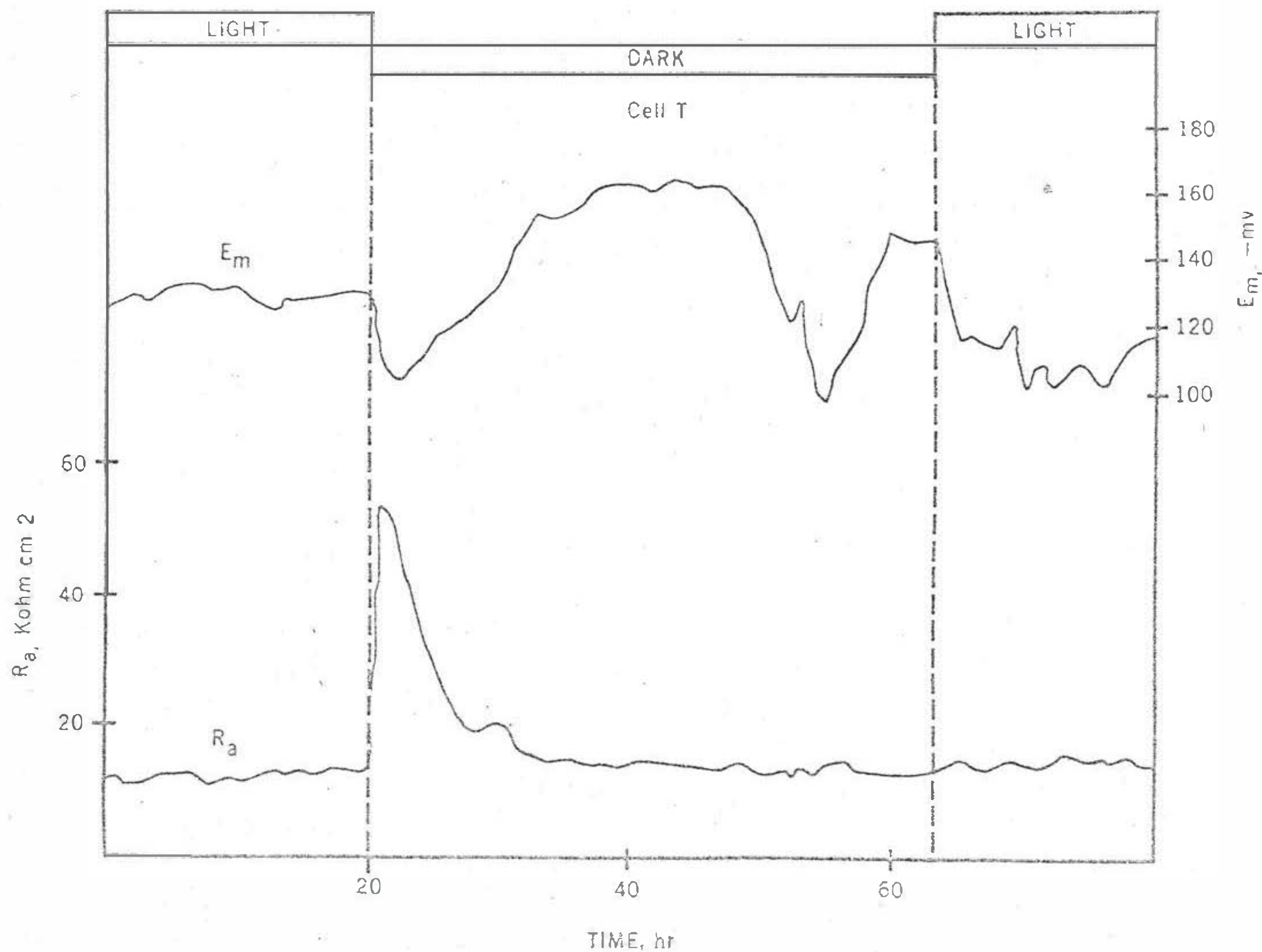


Figure 3. Areal resistance and resting potential measurements of a 4 day old <u>Nitella</u> cell in light and darkness at pH 5.7. The initial changes in darkness are both typical; after this the potential varies appreciably while the resistance remains constant. See Figs. 1 and 2 for further details.

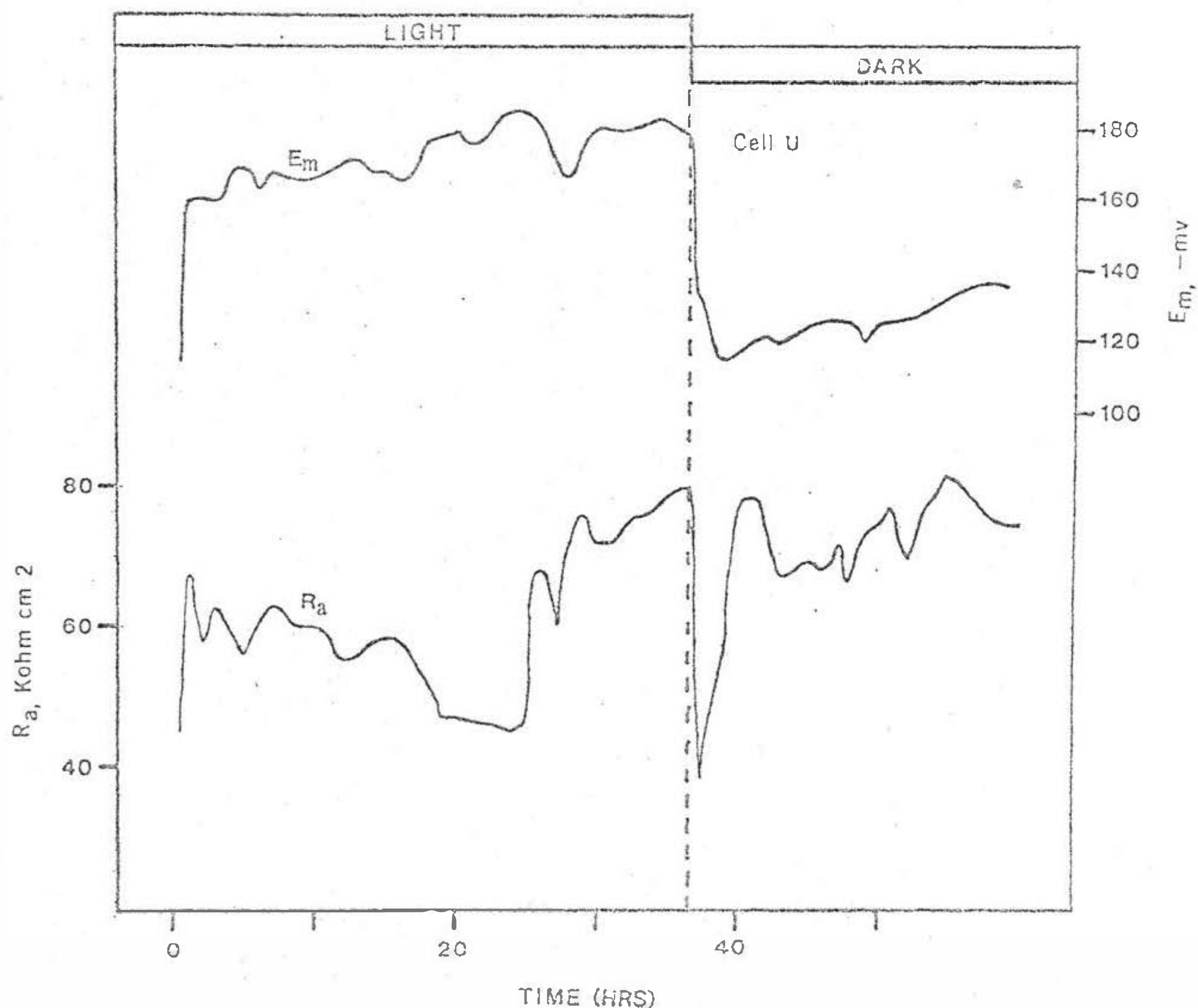


Figure 4. Areal resistance and resting potential measurements of a 7 day old <u>Nitella</u> cell in light and darkness at pH 5.7. This cell shows the typical initial dark depolarization, but the cell was atypical in that a large resistance decrease, rather than an increase was the initial response to darkness; this may be related to the unusually high resistance in light. See Figs. 1 and 2 for further details.

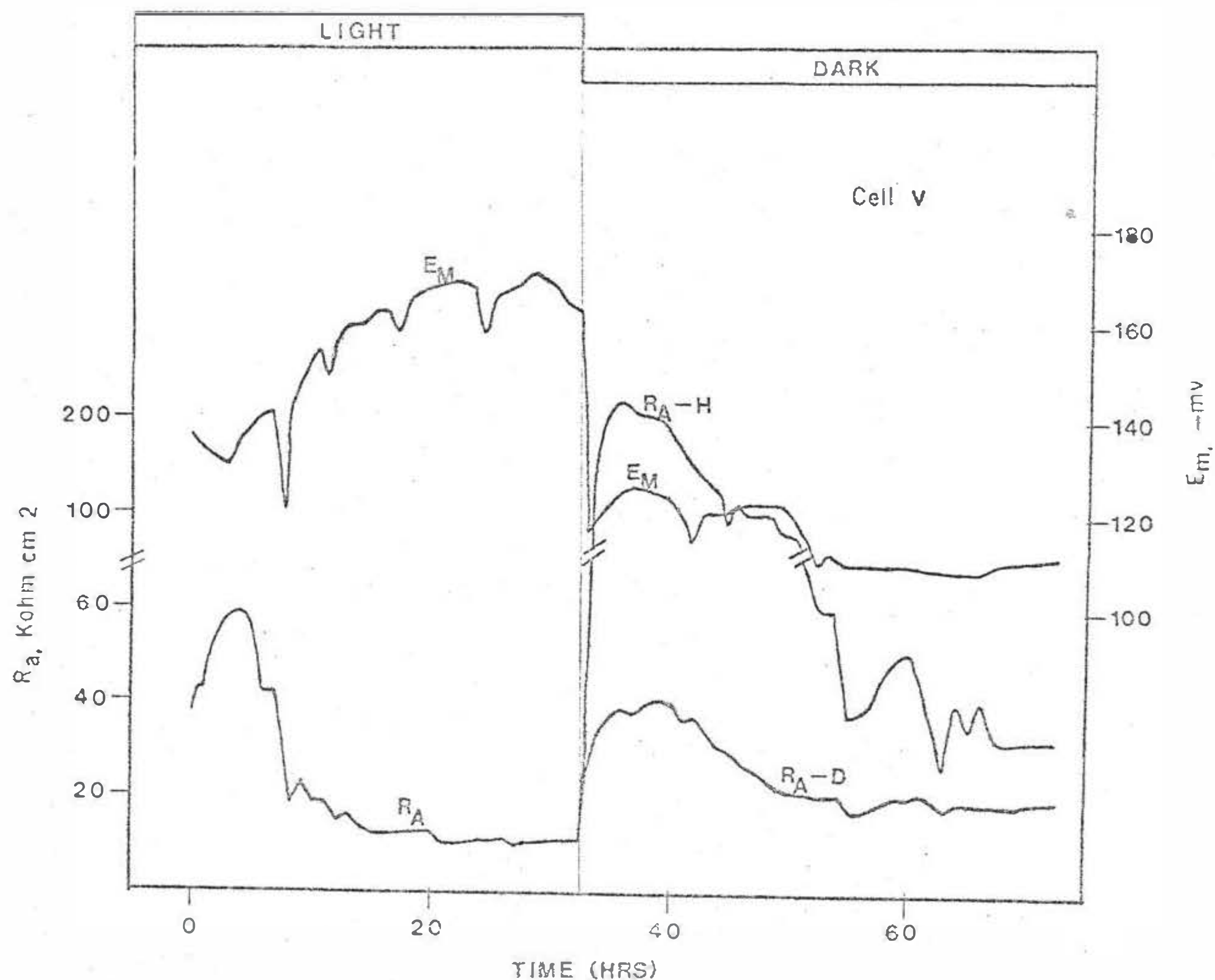


Figure 5. Areal resistance and resting potential measurements of a 6 day old Nitella cell in light and darkness at pH 5.7. A large amount of rectification was observed in darkness therefore R_a was plotted for both inward (R_a-H) and outward (R_a-D) currents. This cell shows typical initial dark responses after which the resistance partially recovers to the light level while the potential remains depolarized. See Figs. 1 and 2 for further details.

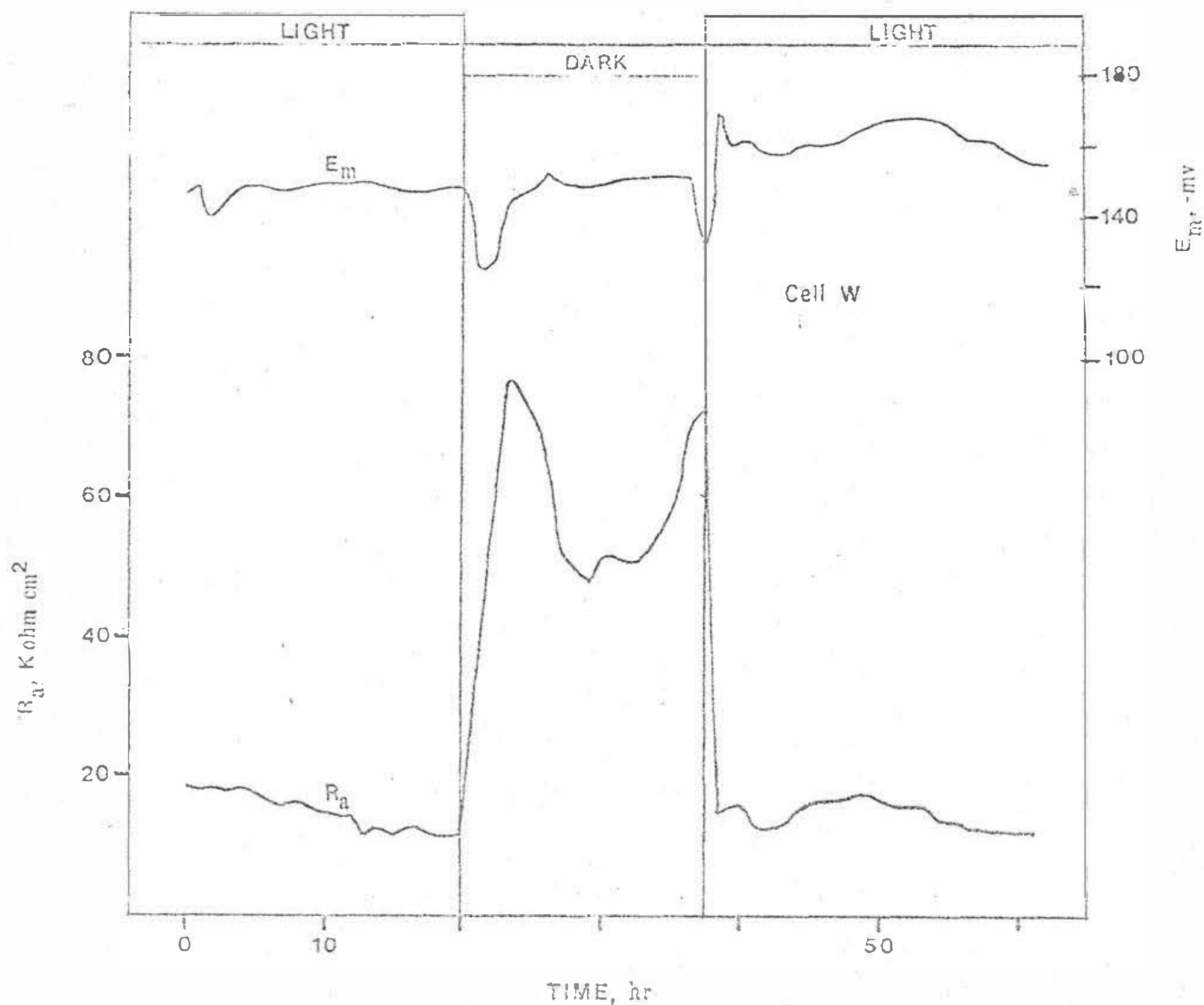


Figure 6. Areal resistance and resting potential measurements of a 4 day old <u>Nitella</u> cell in light and darkness at pH 5.7. The typical initial dark responses were followed by recovery of the potential while the resistance remained high. See Figs. 1 and 2 for further details.

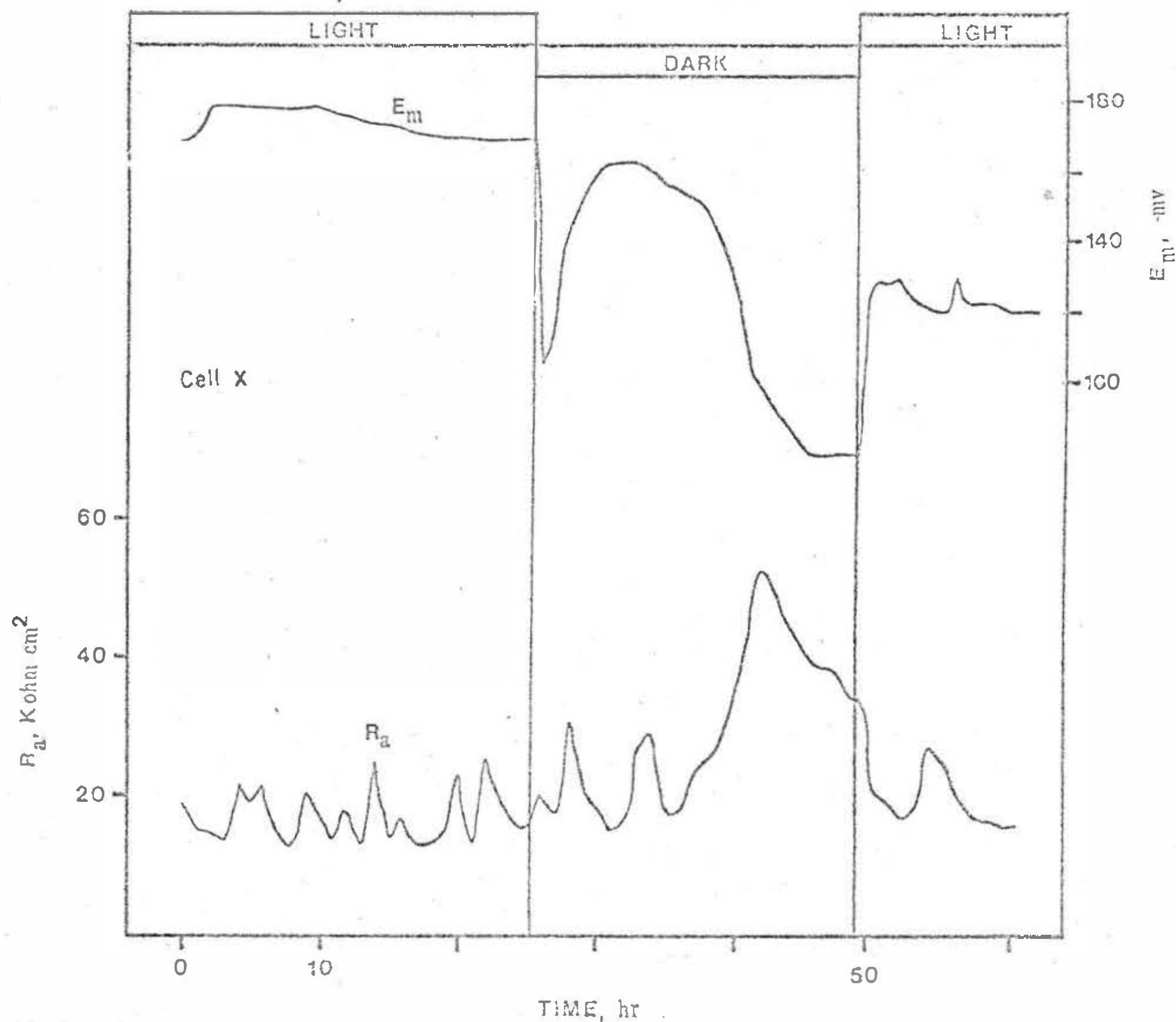


Figure 7. Areal resistance and resting potential measurements of a 6 day old Nitella cell in light and darkness at pH 5.7. This cell shows the typical initial depolarization while little or no initial change in resistance takes place. See Figs. 1 and 2 for further details.

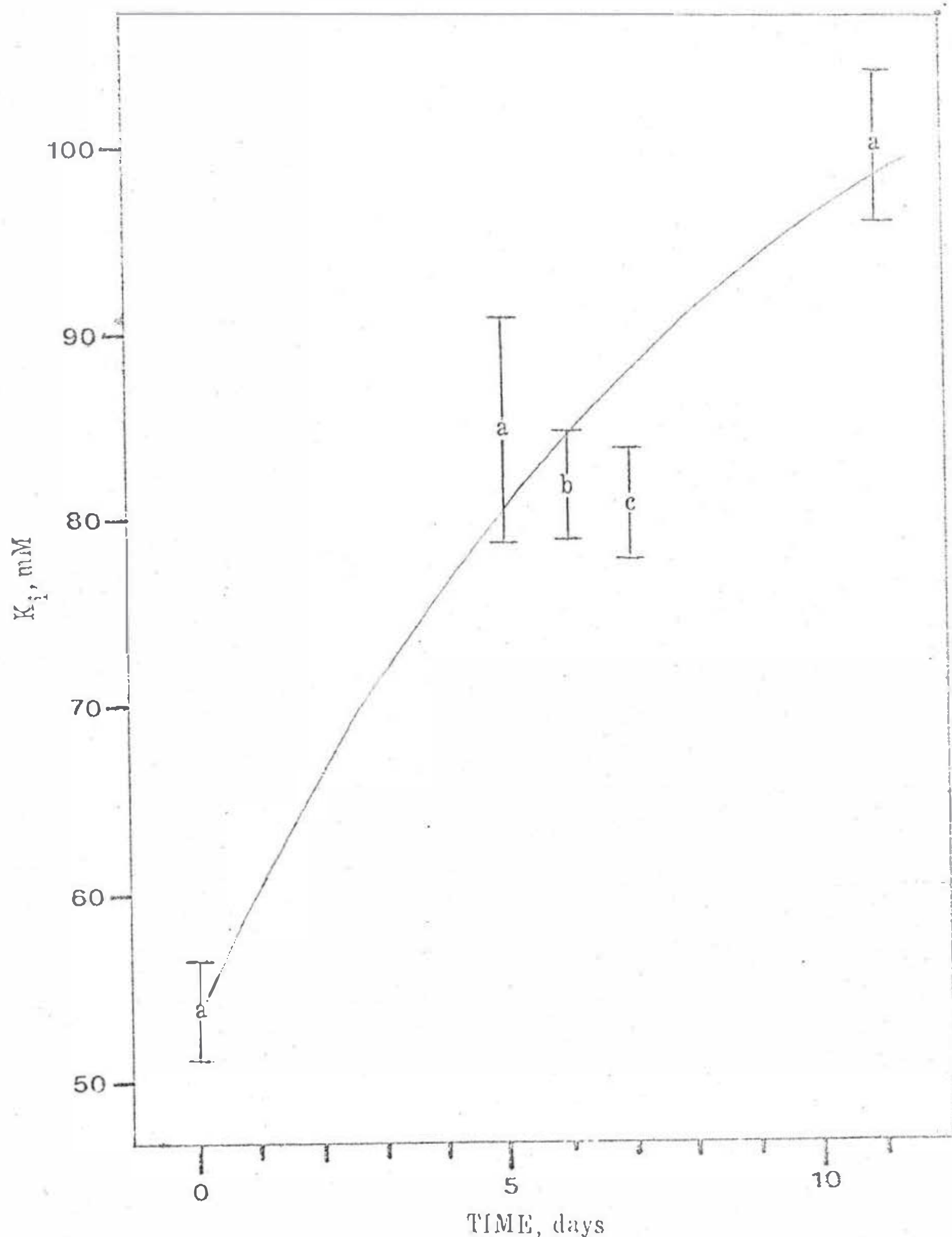


Figure 8. Internal K of Nitella cells maintained in K solution for the indicated period of time. Point (a) at 0 days was taken immediately after harvesting from culture solution B (see MATERIALS). Points labeled (a) represent 10 cells harvested and conditioned together. The values indicated by (b) and (c) represent 30 and 20 cells, respectively, and were obtained from cells used in experiments 40 and 46 (Tables 3,4). Variation is expressed as the standard error of the mean.

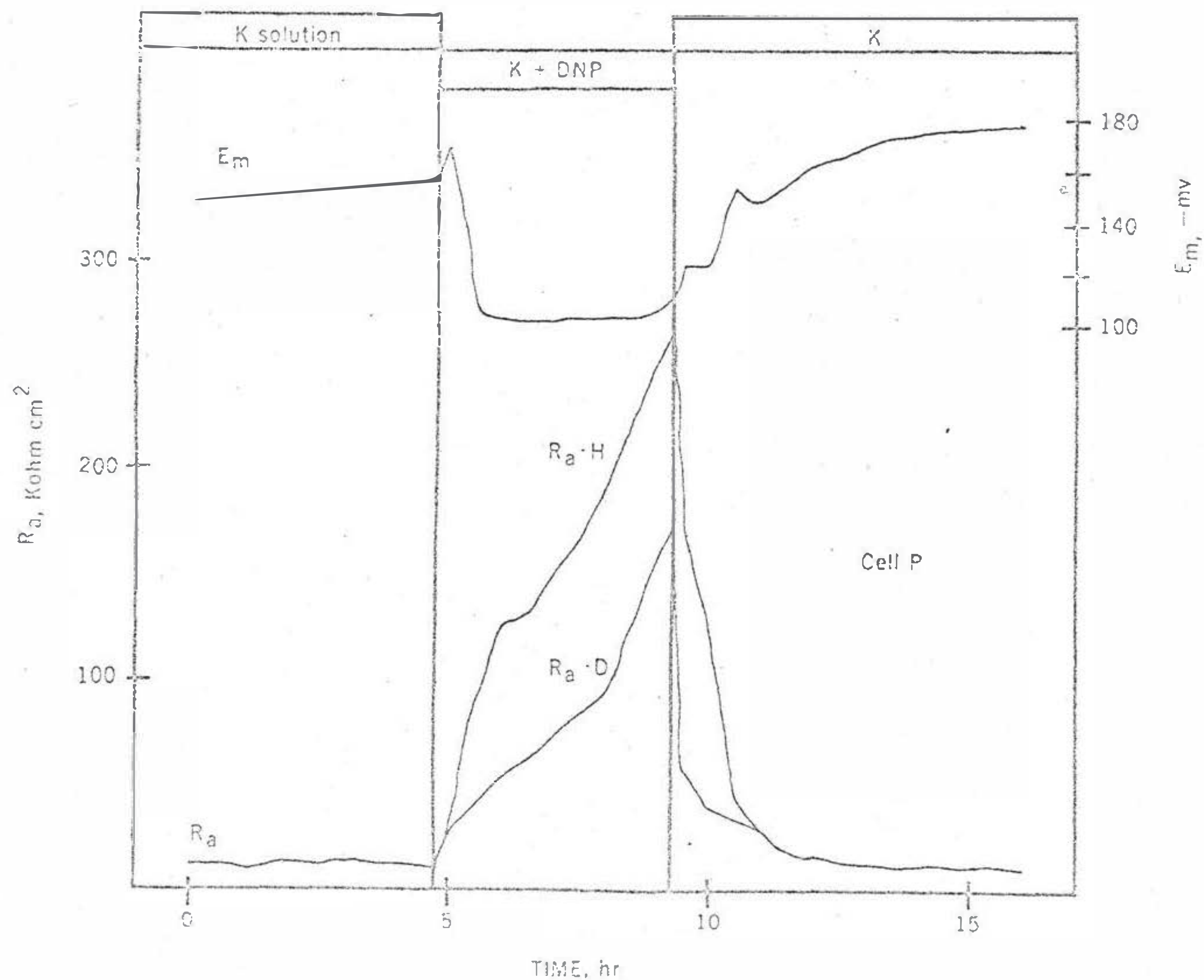


Figure 9. The effect of 0.05 mM DNP on the resting membrane potential and areal resistance of a 4 day old Nitella cell. The resistance values for both the inward (R_a-H) and outward (R_a-D) applied currents are presented since rectification was appreciable. See Figs. 1 and 2 for further details.

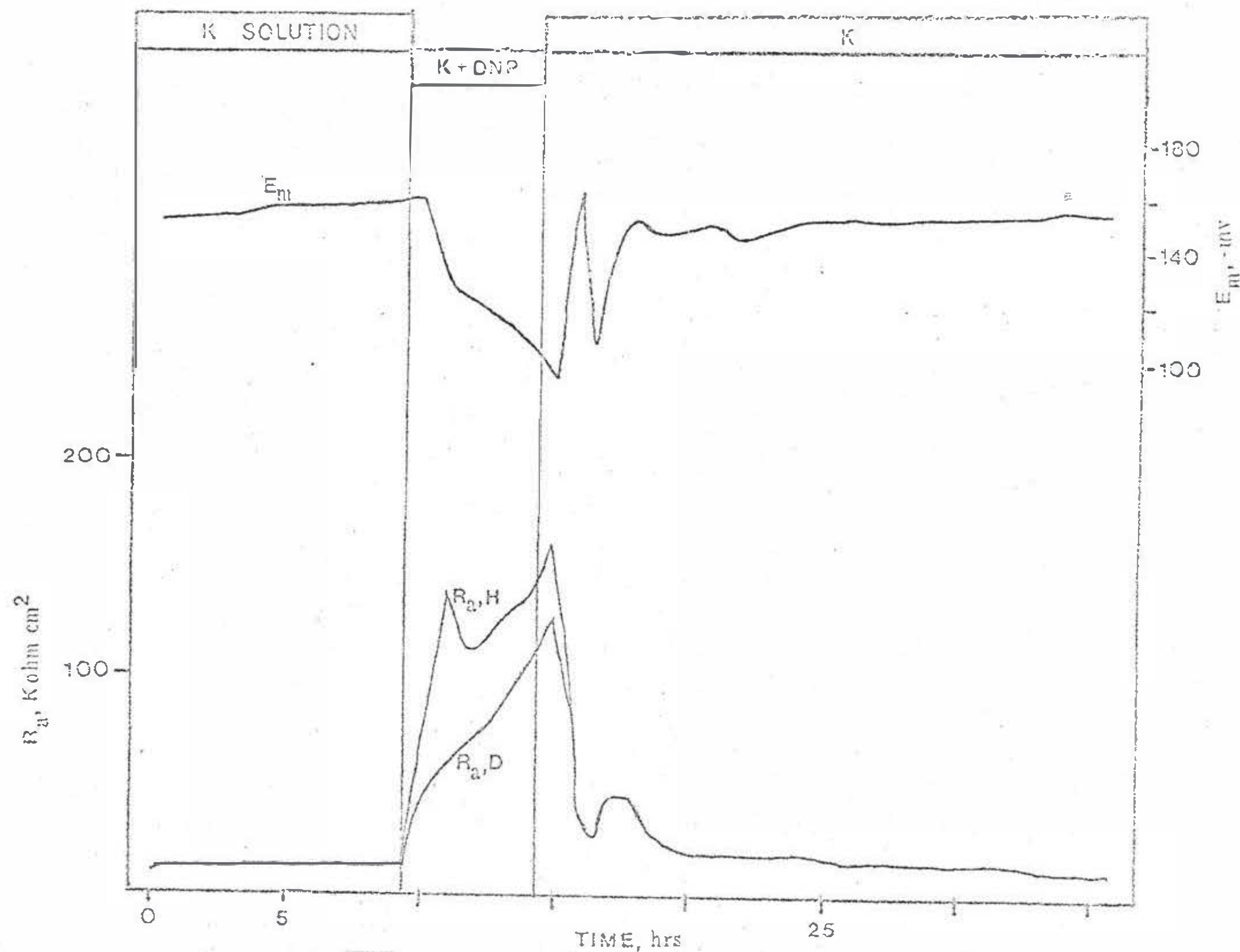


Figure 10. The effect of 0.05 mM DNP on the resting membrane potential and areal resistance of a 3 day old *Nitella* cell. The resistance values for both the inward (R_a-H) and outward (R_a-D) applied currents are presented since appreciable rectification occurred. See Figs. 1 and 2 for further details.

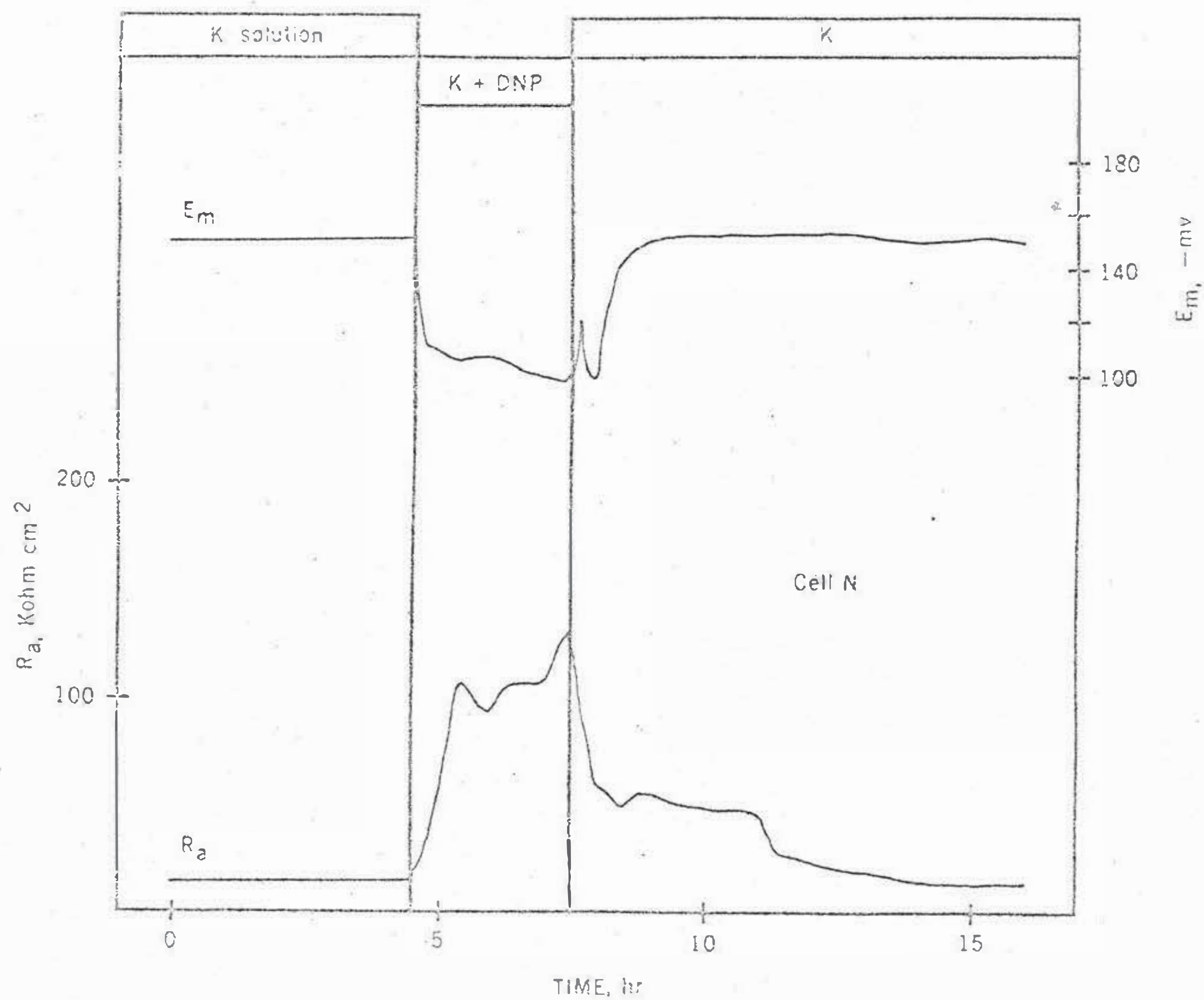


Figure 11. The effect of 0.05 mM DNP on the resting membrane potential and areal resistance of a 6 day old Nitella cell. See Figs. 1 and 2 for further details.

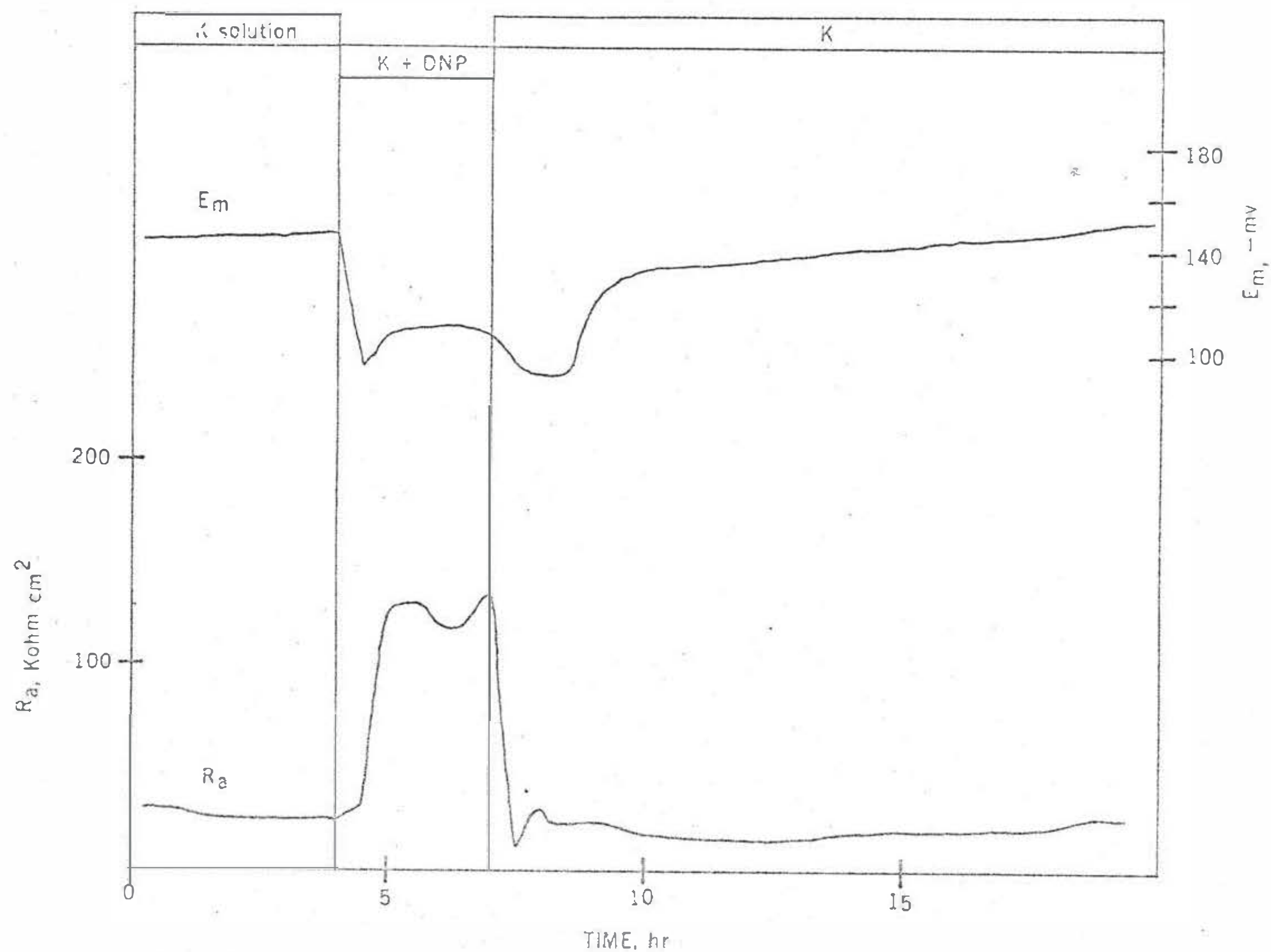


Figure 12. The effect of 0.05 mM DNP on the resting membrane potential and areal resistance of a 9 day old Nitella cell. See Figs. 1 and 2 for further details.

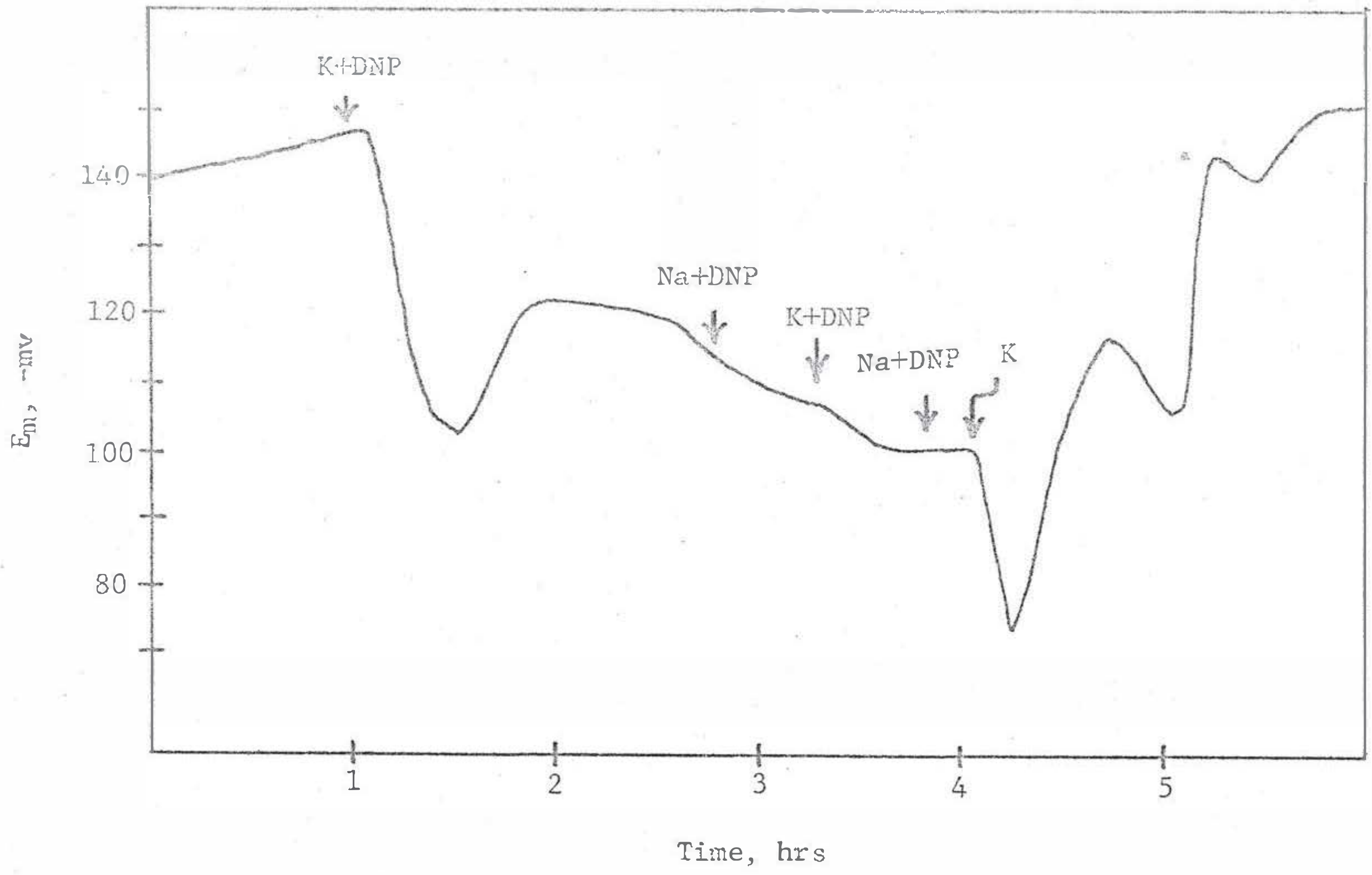


Figure 13. The effect of reducing the external K^+ concentration from 1.0 mM (K solution) to 0.1 mM (Na solution) in the presence of 0.05 mM DNP at pH 5.7 on the resting potential. The Nitella cell used was 12 days old.

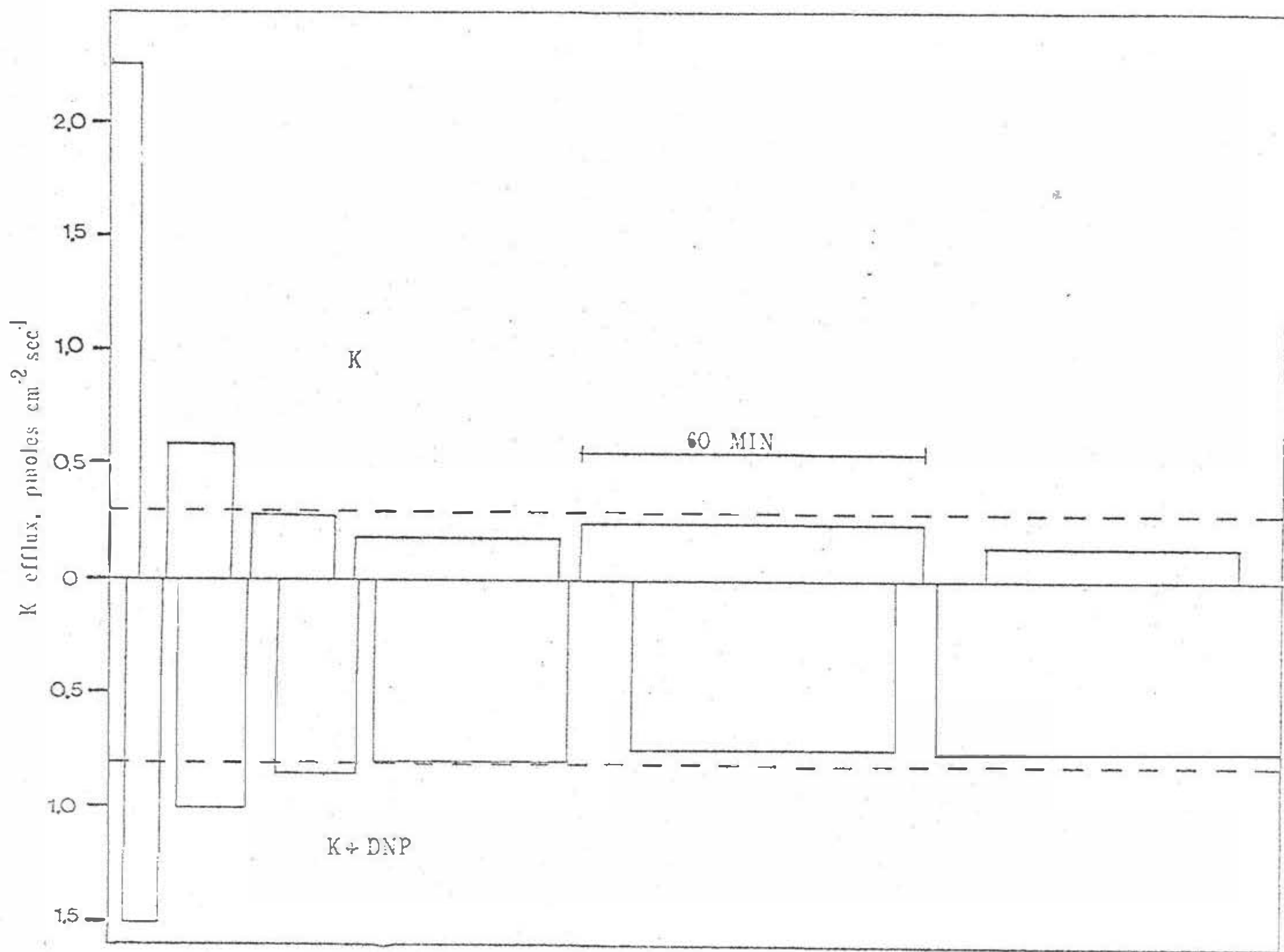


Figure 14. K^+ efflux in K solution (above the zero line) and K solution plus 0.05 mM DNP (below the zero line). Bars represent successive efflux periods in both solutions and are the average effluxes of 5 cells. Dotted lines represent the average efflux of 5 cells for a 3 hour uninterrupted period. Standard error was 0.04 pmoles cm^{-2} sec^{-1}. Postharvest age 7 days.

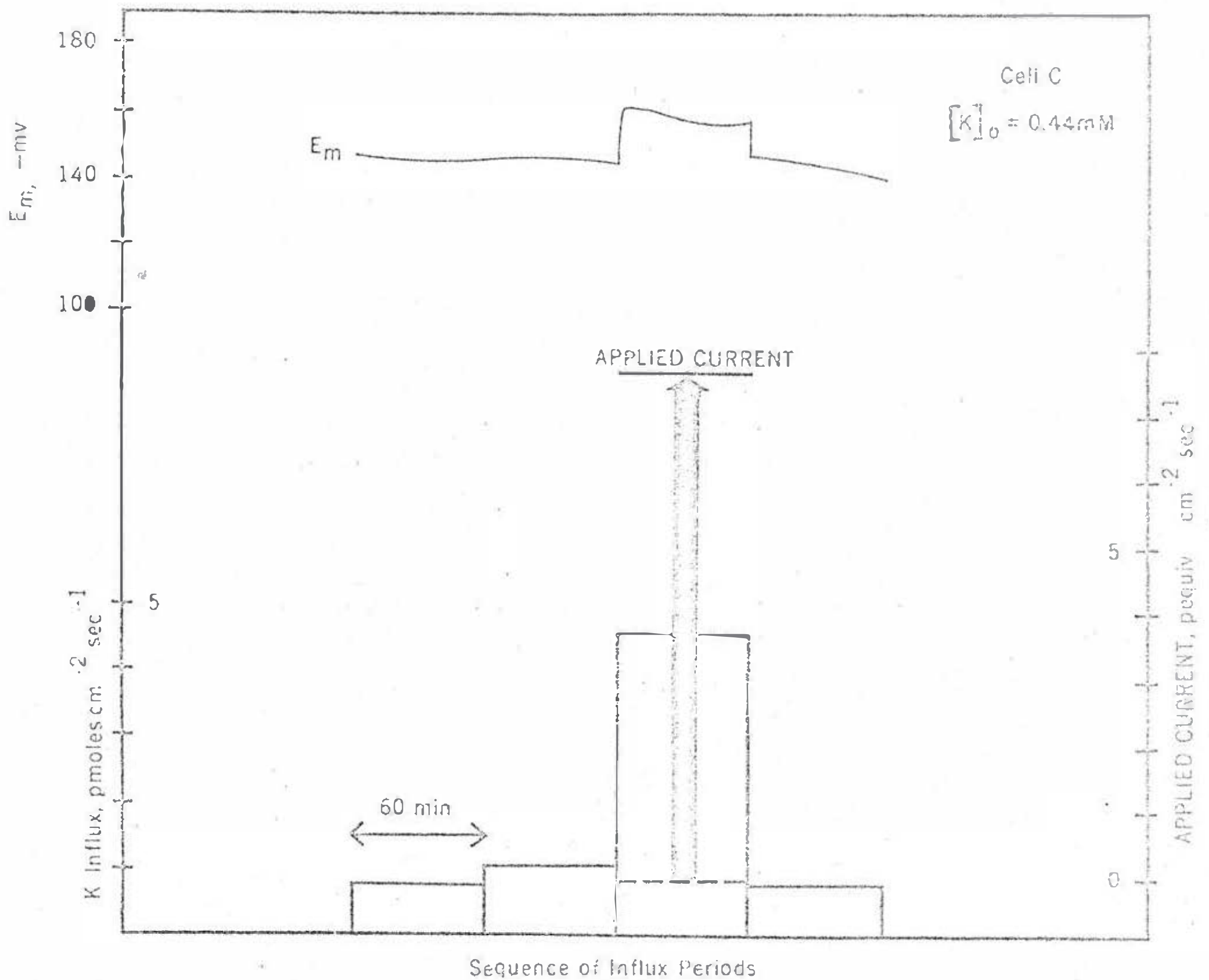


Figure 15. K^+ influx of a 6 day old Nitella cell in the resting state and during application of a hyperpolarizing (positive inward) current through the membrane. Plain bars represent resting influxes; the bar with the arrow represents influx during the applied current period. The height of the arrow indicates the density of the current applied. Shown in the upper part of the figure is the membrane potential at rest and during the application of the current. Standard errors are insignificant.

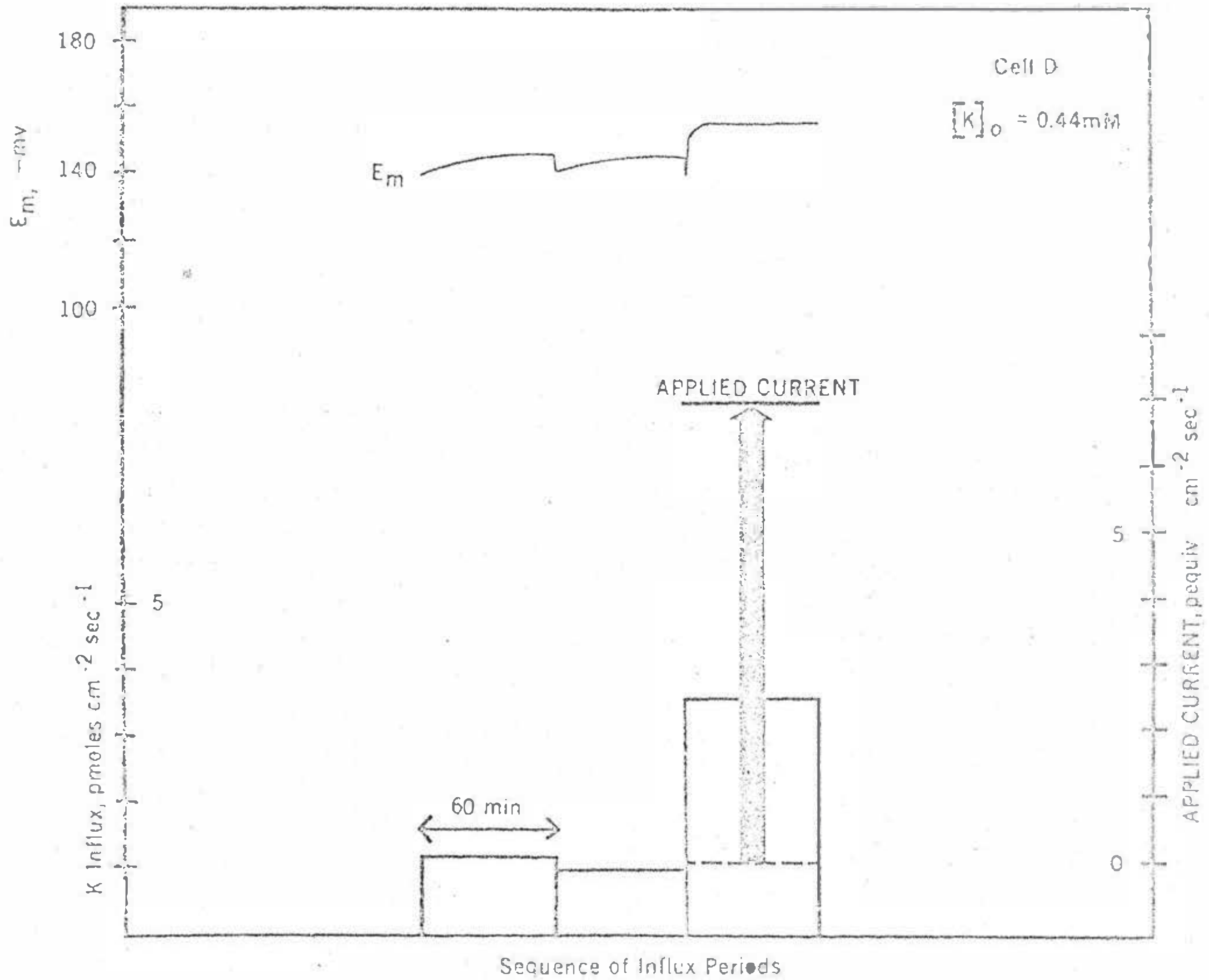


Figure 16. K^+ influx of a 7 day old Nitella cell in the resting state and during application of a hyperpolarizing (positive inward) current through the membrane. See Fig. 15 for further details.

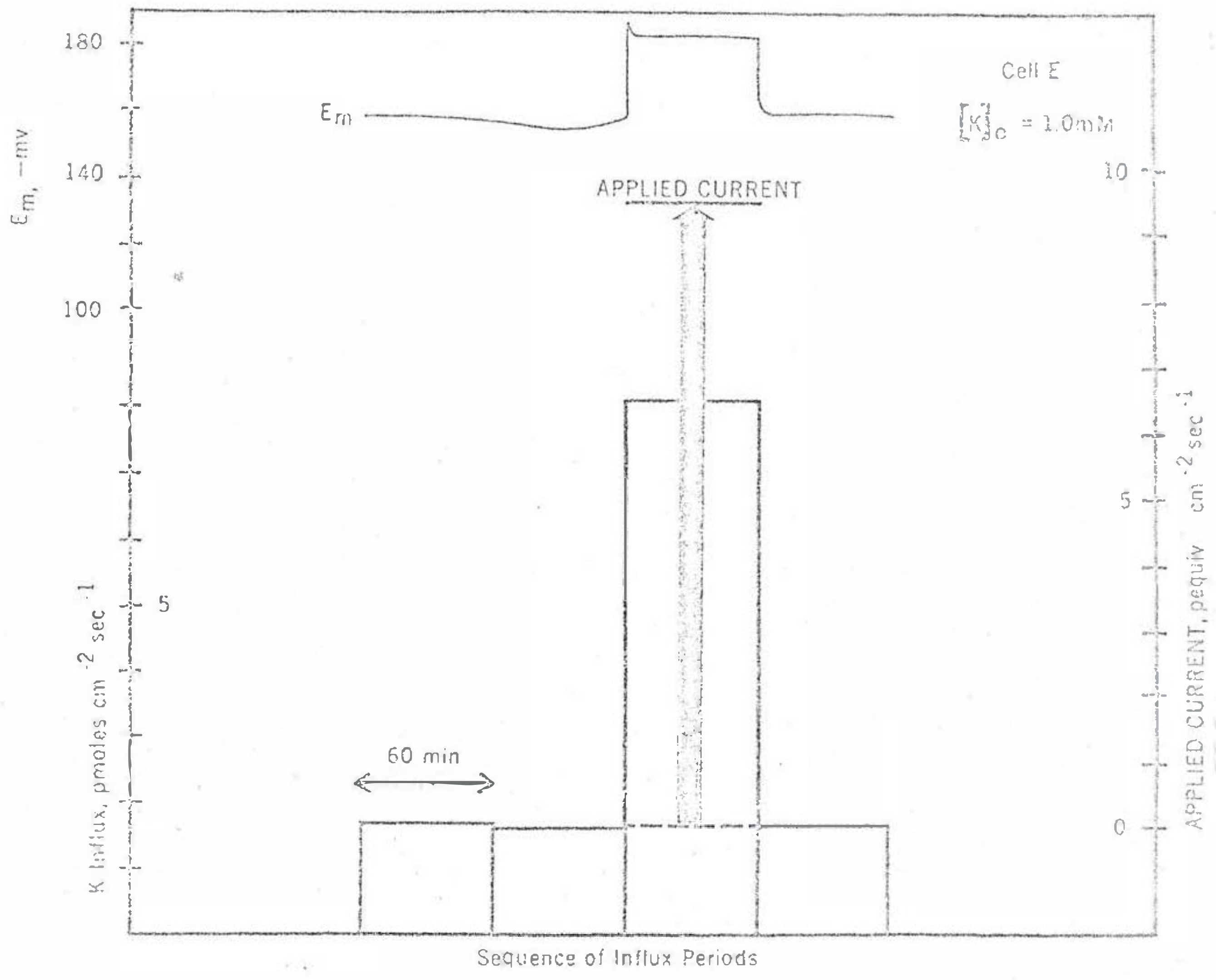


Figure 17. K^+ influx of a 10 day old Nitella cell in the resting state and during application of a hyperpolarizing (positive inward) current through the membrane. See Fig. 15 for further details.

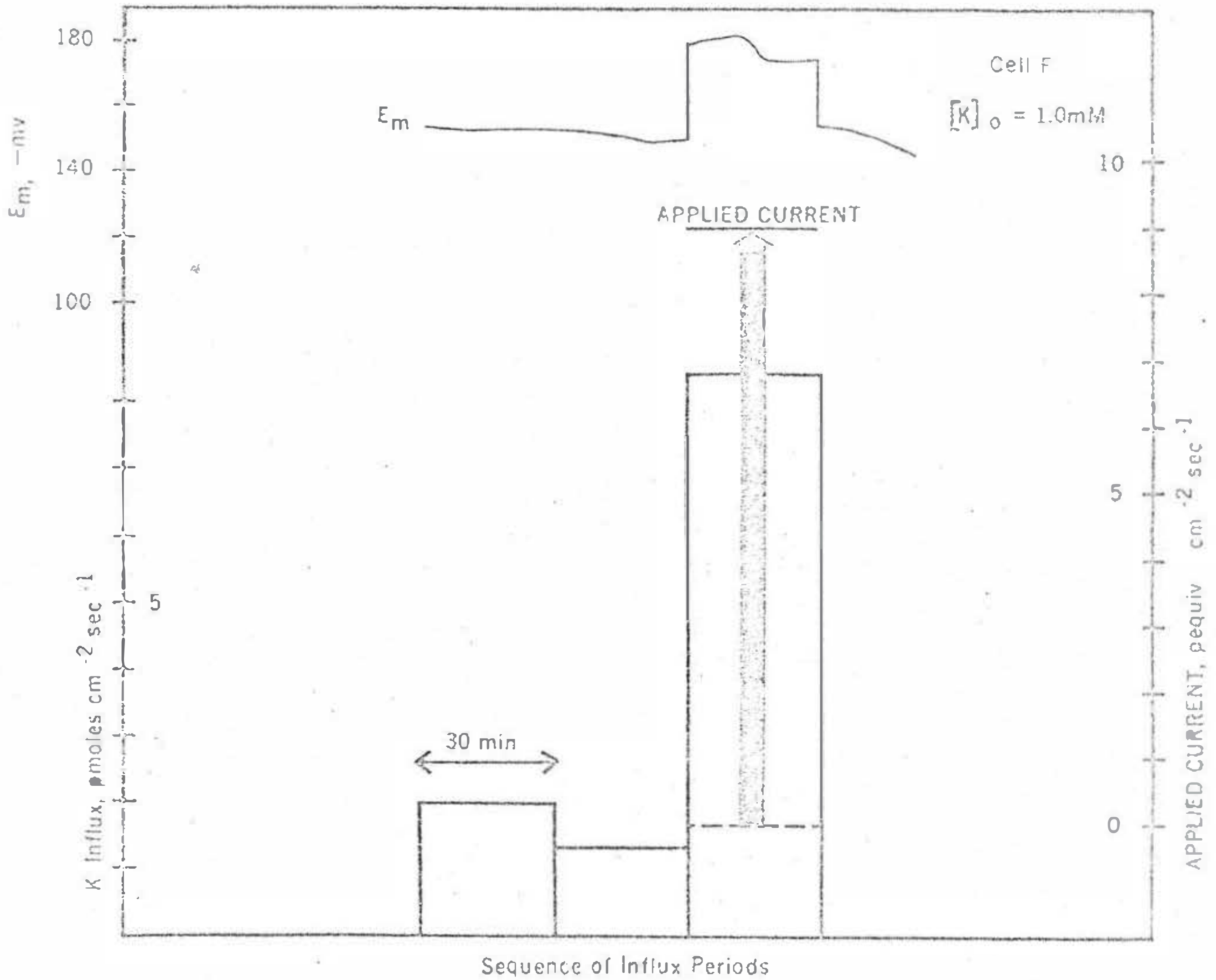


Figure 18. K^+ influx of a 9 day old Nitella cell in the resting state and during application of a hyperpolarizing (positive inward) current through the membrane. See Fig. 15 for further details.

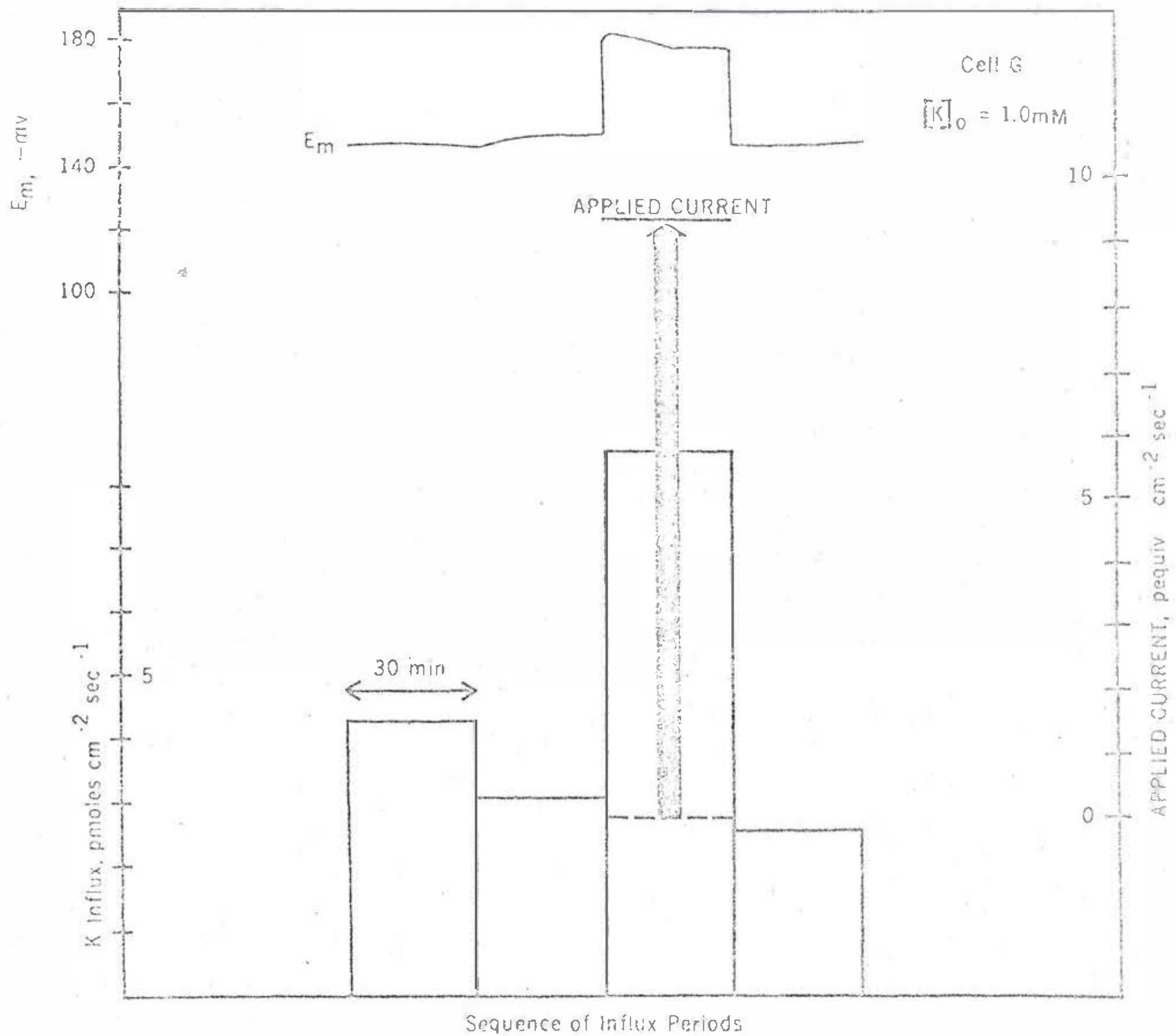


Figure 19. K^+ influx of a 8 day old Nitella cell in the resting state and during application of a hyperpolarizing (positive inward) current through the membrane. See Fig. 15 for further details.

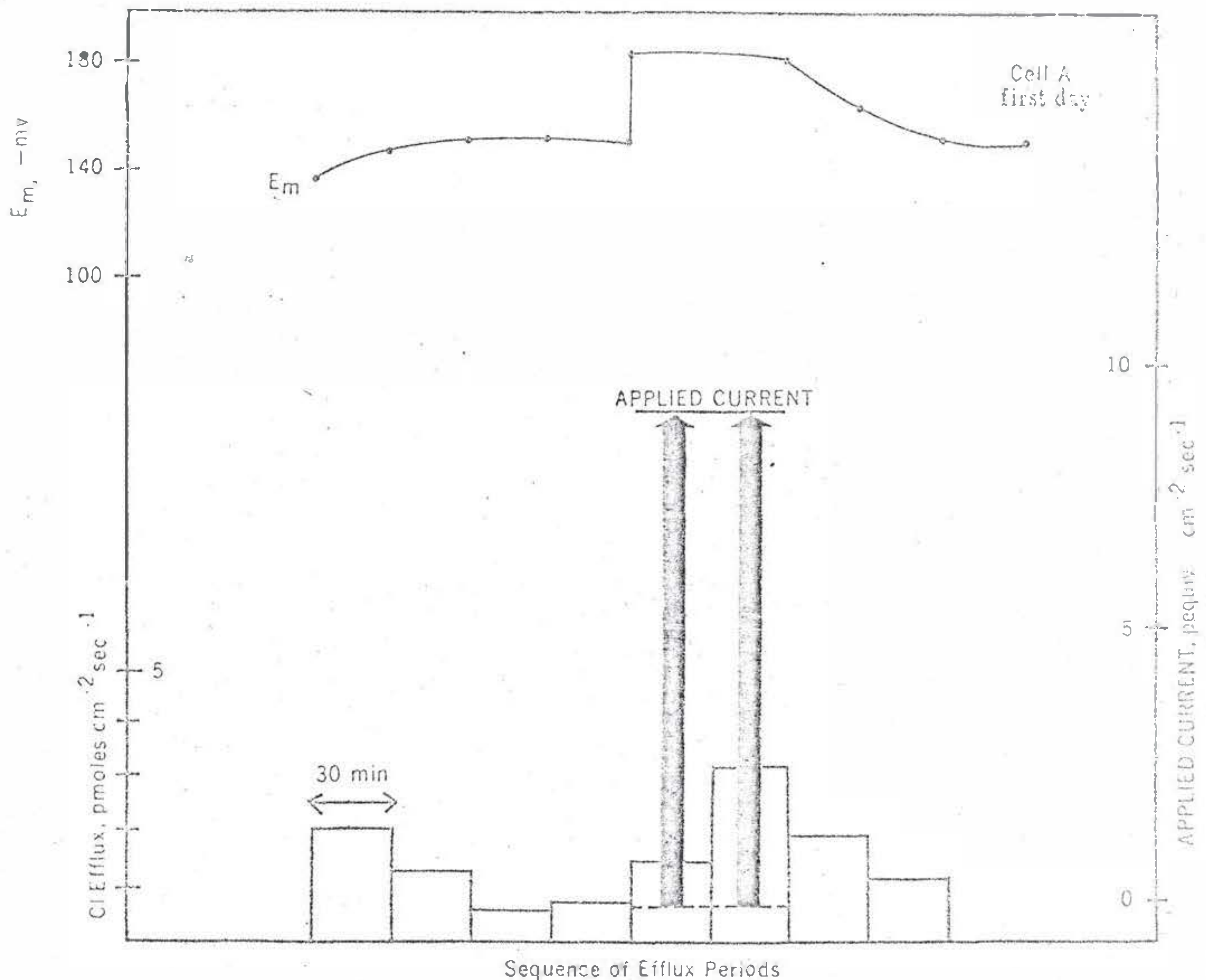


Figure 20. Chloride efflux of a 9 day old Nitella cell in the resting state and during application of a hyperpolarizing (negative outward) current through the membrane. Plain bars represent the resting efflux; the bars with arrows represent the efflux during the applied current periods. The height of the arrows indicates the density of the applied current. Also shown (above) is the membrane potential in the resting state and during the applied current periods. This cell was designated cell A, first day, indicating the effluxes were measured the day the microelectrodes were inserted. Standard errors are not significant.

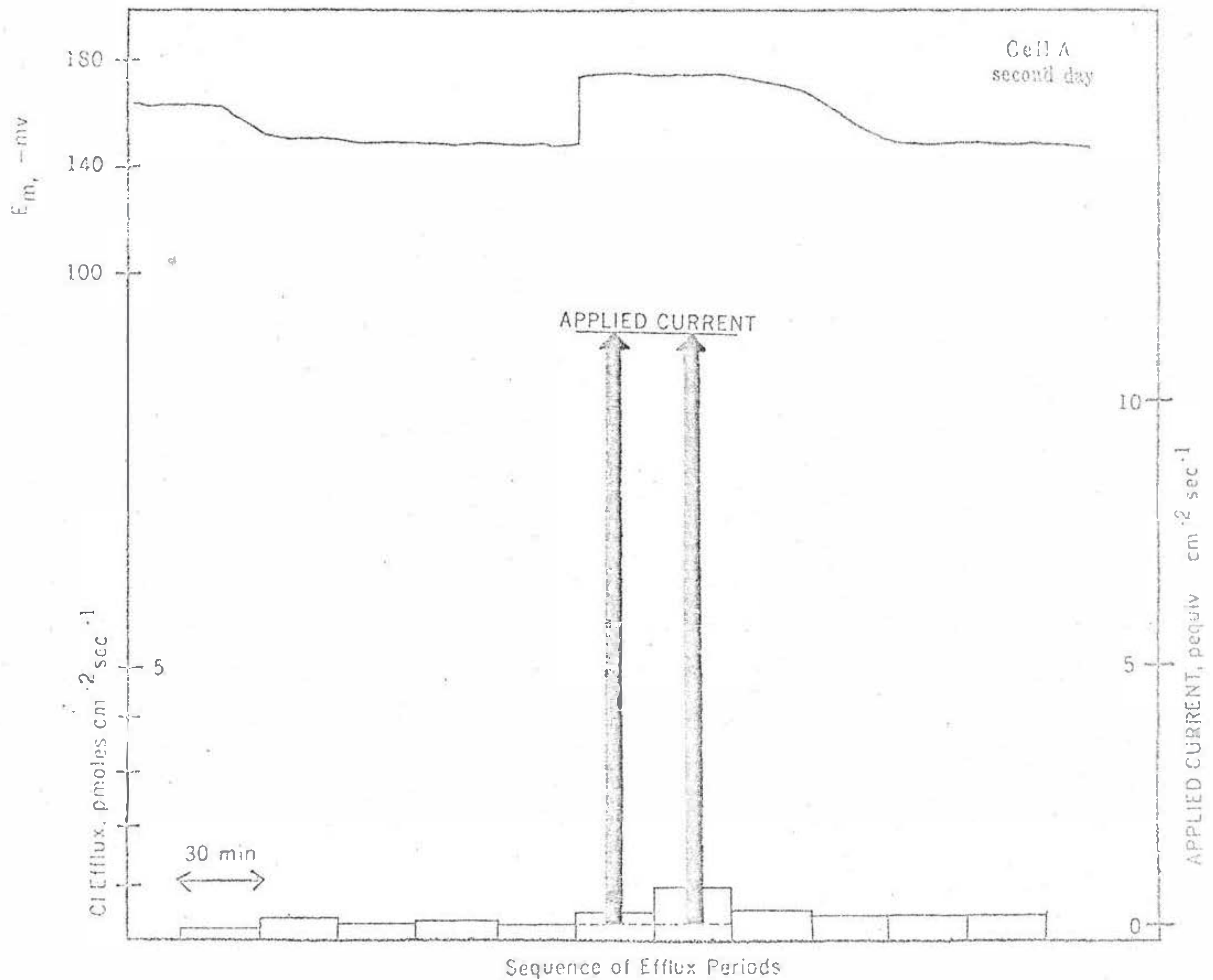


Figure 21. Chloride efflux of the same cell as used in the Fig. 20 results under similar experimental conditions. These measurements were made one day after those of Fig. 20, with the cell now 9 days old.

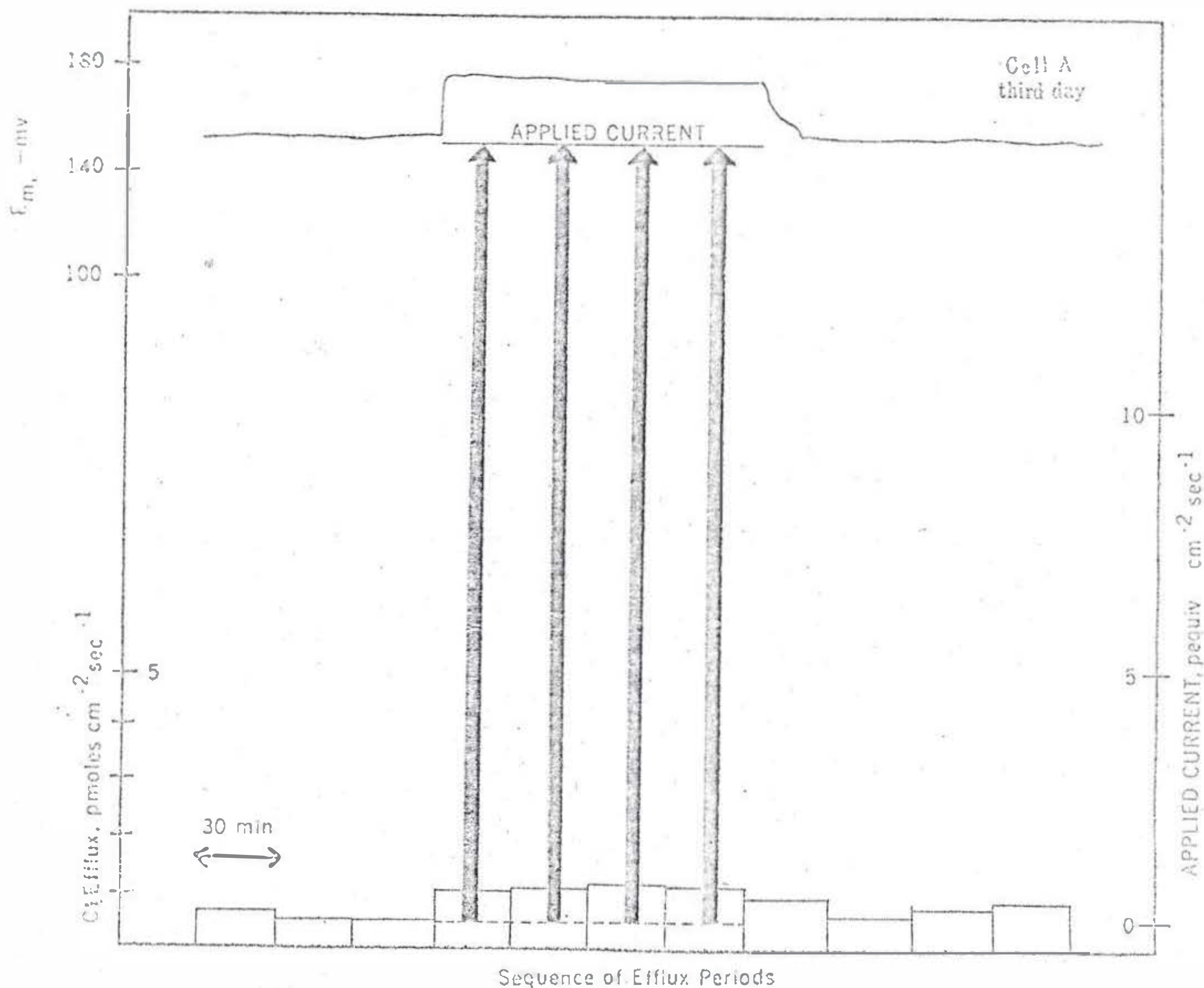


Figure 22. Chloride efflux of the same cell used in Figs. 20 and 21 under similar experimental conditions. These measurements were made one day after those of Fig. 21, with the cell now 10 days old.

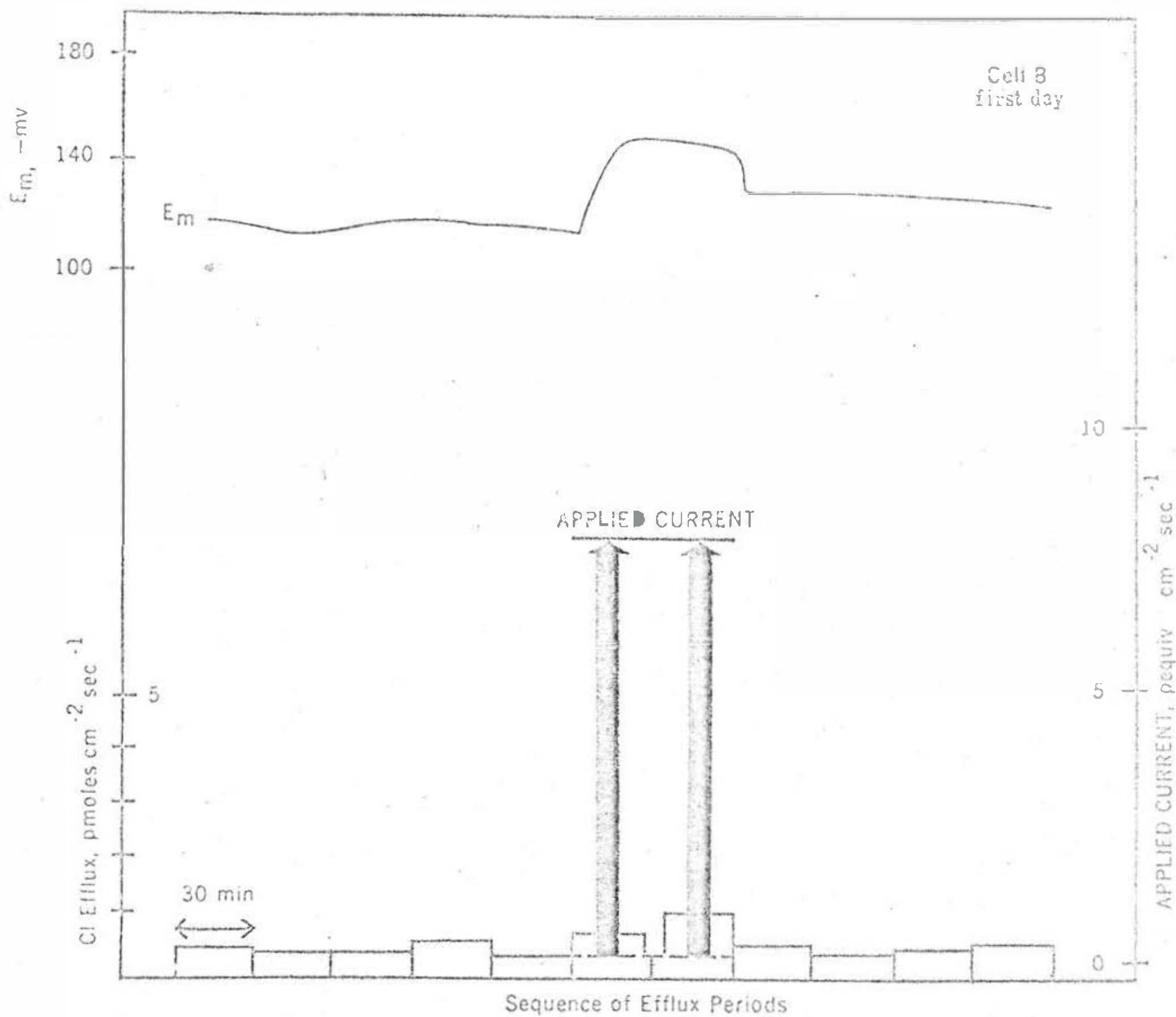


Figure 23. Chloride efflux of a 8 day old Nitella cell at rest and during application of a hyperpolarizing current. See Fig. 20 for further details.

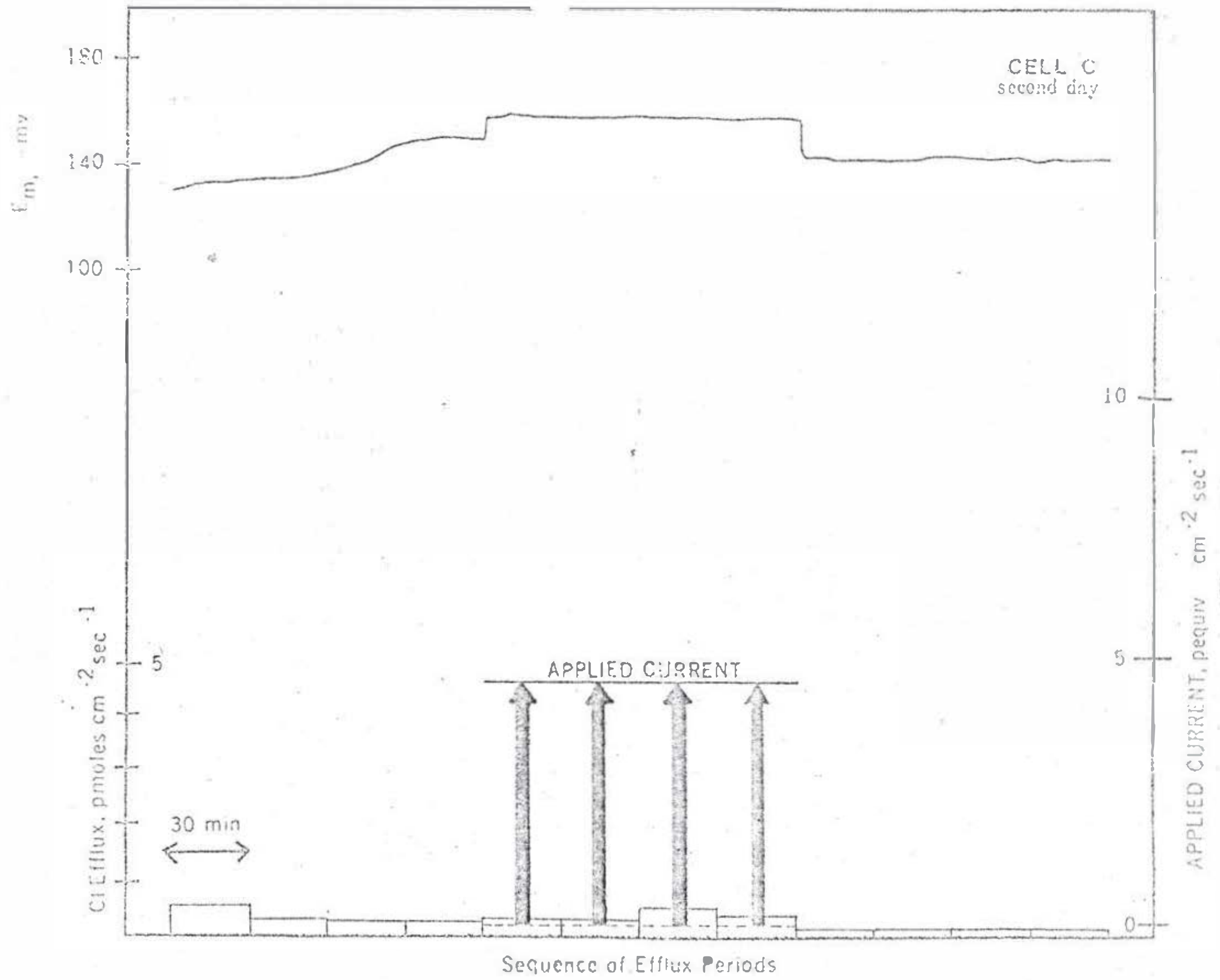


Figure 24. Chloride efflux of a 7 day old Nitella cell at rest and during application of a hyperpolarizing current. Effluxes were measured the day following insertion of the microelectrodes. See Fig. 20 for further details.

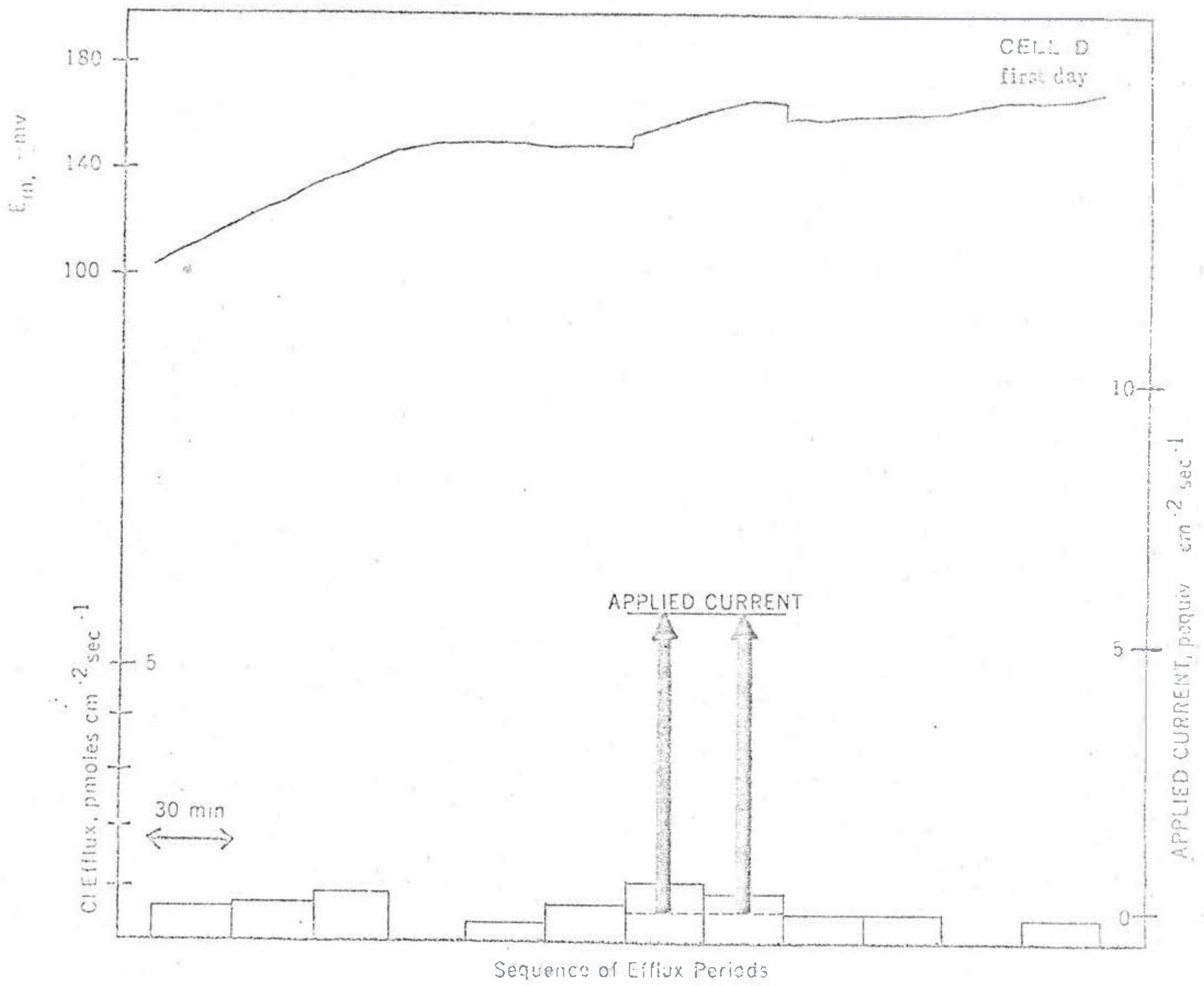


Figure 25. Chloride efflux of a 8 day old Nitella cell at rest and during application of a hyperpolarizing current. See Fig. 20 for further details.

DISCUSSION

The electrogenic H^+ efflux pump was first proposed by Kitasato in 1968 in order to have something to balance an apparently very high passive H^+ influx. Without H^+ extrusion to control internal pH the cell interior would soon become too acid for metabolic processes. The H^+ influx in turn seemed an inescapable conclusion coming from the finding that an increase in clamping current was needed to hold the potential at the resting level when the pH of the external solution was lowered.

Further evidence for a large passive H^+ influx is the rise in pH of the external solution observed by Rent et al. (1972); however, they point out that the membrane potential depolarizes much less quickly than expected from the large net H^+ influx. They suggest the H^+ missing from solution is adsorbed on the outer surface of the membrane, or balanced by some other ionic current. Recent work in this laboratory (Barr, unpublished) indicates the balancing current is not extrusion of amino acids or organic acids. This was confirmed by the fact that the external solution had no buffering capacity after 69 cells with a total surface area of 50.5 cm^2 had removed 3.4×10^{-6} moles of H^+ from 10 ml of solution kept at a constant pH of 4.7.

It appears that most of the H^+ missing from solution is adsorbed on the outer surface of the membrane and perhaps enters the cell only very slowly. Further evidence against passive H^+ permeability makes this alternative more attractive:

(a) Changes in external $[H^+]$ were able to control the membrane potential only in the range pH 4 - 6 (Kitasato, 1968); the present work shows changes in P_{Cl} could account for this. (b) Very little change in membrane resistance was observed when $[H^+]_o$ was changed (Spanswick, 1972; Brown et al., 1973). (c) An applied current has no effect on the pH of the external solution (see RESULTS; Walker and Hope, 1969). It is possible that some H^+ does get into the cell at this unnatural pH (4.7); however, at higher pH (5.7) CO_2 uptake could cause the observed changes in pH.

The results of the present work make it unnecessary to postulate a large H^+ permeability. The net Cl^- efflux observed at low pH could cause the depolarizations observed by Kitasato (1968), and, at least under the present conditions, K^+ rather than H^+ has been found to carry much of the current passed through the membrane via an external circuit. These results still necessitate a H^+ extrusion pump on the basis of the very large negative resting potentials and in order to have something to balance the inward positive current carried by K^+ in the resting state. The visual evidence of Spear et al. (1969) is still the only concrete evidence that H^+ extrusion exists. As mentioned previously recent work of Barr (unpublished data) has shown the acidification of the external solution is not due to an organic acid efflux. The large K^+ transference numbers obtained in this work also makes it unnecessary to hypothesize that currents passed through the membrane cross the membrane as changes in the rate of H^+ pumping (Spanswick, 1972). It will be shown below that the large

applied current carried by K^+ also does not relate in any way to the Goldman model of Passive ionic movement.

The Goldman flux equations for determining the passive fluxes of an ion, i, are:

$$J_{i \rightarrow in} = -P_i \frac{Z F E_m / R T}{1 - \exp Z F E_m / R T} [i]_{out} \tag{9}$$

$$J_{i \rightarrow out} = P_i \frac{Z F E_m / R T}{1 - \exp Z F E_m / R T} [i]_{in} \exp Z F E_m / R T \tag{10}$$

where $J_{i \rightarrow in}$ = Passive influx of i
$J_{i \rightarrow out}$ = Passive efflux of i
Z^i = Valence of i

Since K^+ efflux is thought to be entirely passive the experimental efflux value obtained in this work may be used to calculate P_K from Equation (10). This value is 1.14×10^{-7} cm sec^{-1}. Allowing that E_m is more negative than E_K, only a part of the K^+ influx is attributable to passive movement along the electrical gradient. Using Equation (9) the calculated passive K^+ influx for typical cells at the mean resting potential of -152 mv is 0.68 pmoles cm^{-2}sec^{-1}, and the calculated net passive influx is 0.53 pmoles cm^{-2}sec^{-1}. The actual net K^+ influx from tracer experiments and vacuolar sap analysis is about 1.3 pmoles cm^{-2} sec^{-1}. These calculations indicate about one-half of the K^+ influx can be explained as passive movement; the other half must be attributed to an active K^+ transport mechanism. If the K^+ transport mechanism is separate from H^+ extrusion it would be anti-electrogenic. This suggests H^+ may be exchanged for K^+ by the cell.

Tracer experiments also show that low pH essentially eliminates all of the active K^+ influx since the average influx at low pH is equal to the passive portion as calculated from

Equation (9) when $E_m = E_K$. Brown et al. (1973) have found that E_m is near E_K at pH 4.7. The constancy of P_K at low pH as determined by the magnitude of K efflux is consistant with the idea that low external pH only affects the passive K fluxes through its effect on E_m. Kitasato (1968) came to the same conclusion. This does not mean the observed potential is a K diffusion potential. It could mean that the electrogenic H^+ pump is still operating, at a rate equal to the net Cl^- efflux observed at low pH. The loss of K^+ transport may possibly be due to H^+ competition for sites on a transport mechanism which can exchange H^+ for either K^+ or H^+. The loss of K^+ active transport was confirmed by the K^+ influx experiments with DNP in the external solution.

The component of K^+ influx which is passive and the expected passive increase in K^+ influx when the hyperpolarizing current was applied to the cells shown in Table 8 was also calculated from Equation (9). These calculated values along with the actual increments in K^+ influx caused by the applied current are listed on Table 11. The actual increments shown on Table 11 are 10 to 128 times the values expected from the passive model. The greatest deviations from the expected passive values were observed with 0.44 mM K solution present externally. The average in 1.0 mM K solution was 40 times the expected value. For a total of 9 cells the expected increase in passive K^+ influx was 0.09 pmoles $cm^{-2}sec^{-1}$ while the actual average increase in K^+ influx was 4.0 pmoles $cm^{-2}sec^{-1}$. Not one of the cells followed what is predicted by the Goldman model.

If these arguments are correct, one must conclude that the passive channels for K^+ movement can account for only a very small fraction of the K^+ current drawn through the membrane by means of an external circuit. The results of Walker and Hope (1969) agree with this idea. They found both K^+ influx and efflux were effected to a much greater extent than predicted by the passive model. It follows that the membrane resistance bears very little relation to the passive properties of the membrane as classical interpretation suggests. This makes it unnecessary to hypothesize very large H^+ fluxes as Kitasato (1968) and Brown et al. (1973) have done to account for the discrepancy between electrically measured conductance and conductance calculated from passive fluxes of ions. In fact it is arguable that K^+ actually accounts for almost all of the applied current: if it is assumed that the applied current interferes with the normal operation of the active channel and none of the K^+ going into the cell is active during this time, the average transference number for cells in solution containing 0.86 to 1.0 mM K^+ is 0.9.Fluxes of Na^+ and Cl^- could presumably account for the rest of the applied current.

If the large K^+ current cannot pass through the passive channels of the membrane one must conclude that either (a) the membrane is drastically altered by the applied current or (b) the current passes through the active channels as Spanswick (1972) has proposed H^+ does. Again using Equation (9) and the average E_m obtained in K solution plus 0.05 mM DNP, a comparison of the calculated influx with the experimental value

indicates that the K^+ influx during DNP treatment is equal to the passive component; the presumptive evidence then is that DNP blocks the active K^+ influx. Under these conditions where resistance is high the transference number of K^+ is probably as large as in the resting state; actual measurements are difficult due to backflux of tracer when cells are in DNP solution. The high measured resistance under these conditions indicates K^+ has much more difficulty crossing the membrane than under normal conditions. These results suggest the channel through which K^+ passes during an applied current appears to be part of the active transport mechanism.

P_{Cl} calculated from Equation (10) and the average resting Cl^- efflux is 1.25 cm sec^{-1}. When a hyperpolarizing current was applied to <u>Nitella</u> cells the average increase in Cl^- efflux was 0.5 pmoles $cm^{-2}sec^{-1}$. The expected increase in passive Cl^- efflux calculated from Equation (9) is 0.6 pmoles $cm^{-2}sec^{-1}$. Agreement between the two constitutes good evidence that Cl^- efflux is purely passive, consistant with the Goldman equation. The lack of effect when Cl^- is removed from the external solution also supports this conclusion. These results also show that applying a current does not drastically change the properties of the membrane.

This study solidly demonstrates that H^+ is not the main current carrier during resistance measurements either passively as Kitasato (1968) suggests or as changes in the rate of H^+ pumping as suggested by Spanswick (1972). It has also been shown that the K^+ passing into the cell when a current is applied bears little relation to the passive properties of

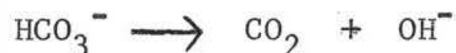
the membrane; the Cl^- fluxes under applied current conditions show the membrane is not radically changed at least as far as passive Cl^- movement is concerned.

In the normal resting state the H^+ extrusion pump must must be working at a rate which balances the positive inward charge carried by K^+ since E_m is steady and Cl^- fluxes are balanced. At low pH (4.7) there is no net K^+ influx; however, at this pH there is a net Cl^- efflux equal to about one-half the resting net K^+ influx at normal pH (5.7). If increased external H^+ concentration has no effect on the rate of H^+ pumping the potential should hyperpolarize, assuming the membrane is not passively permeable to H^+. The fact that depolarizations were observed by Brown et al. (1973) indicates increasing external $[H^+]$ reduces the rate of electrogenic H^+ pumping, while also stopping active K^+ influx. This suggests these two fluxes may be related or at least both affected by low pH. The effects of DNP on H^+ efflux and K^+ influx were similar to those outlined above for low pH, again suggesting active H^+ efflux and K^+ influx are related. The fact that both DNP and low pH increased Cl^- influx indicates another similarity between the two and also that this active ionic movement is independent of the active H^+ and K^+ fluxes.

Poole (1973) has proposed a H^+/H^+ or H^+/K^+ exchange pump in the membrane of red beet storage cells. Nobel (1969) has also proposed K^+ influx into chopped pea leaves is balanced by H^+ release into the bathing solution. He suggests the possibility of an exchange mechanism. These models fit well with the data presented here. The pump will be electrogenic if the

sum of inwardly moving H^+ and K^+ is less than the amount of extruded H^+. The effect of low pH on E_m can therefore be looked at as making the pump more of a H^+ for H^+ exchange pump which is less electrogenic. Low pH should then also decrease the capacity for H^+/K^+ exchange which is what was observed. The data of Spanswick (1972) also supports the idea of an exchange pump. When he increased external K^+ above 10 mM electrogenicity was lost and E_m followed an E_K potential.

A problem with this interpretation is that the net K^+ influx requires net H^+ extrusion. In *Nitella* net H^+ extrusion is observed only in the acid regions while the net flux for the entire cell surface makes the external solution alkaline. It is well known that the Characeae develop $CaCO_3$ incrustations on their outer surfaces. Smith (1970) suggested an OH^- efflux was responsible for the external alkalinity and subsequent precipitation of $CaCO_3$. He proposed the alkaline regions were due to absorption of HCO_3^- with OH^- being released to the external solution by the reaction:



$$HCO_3^- \longrightarrow CO_2 + OH^-$$

Lucas and Smith's (1973) estimates of HCO_3^- uptake from the pH of the alkaline regions and also from previous $H^{14}CO_3^-$ tracer work (Smith, 1968) allow the possibility that an OH^- efflux could mask the net H^+ extrusion which has been proposed as necessary for K^+ influx.

Carbon fixation may also play a role in making H^+ available to the cell for exchange with K^+. The results of Koh (1973) indicate the rate of CO_2 fixation may be fast enough

to provide the necessary amount of H^+ if CO_2 is fixed as a carboxylic acid. Stiller (1962) has proposed that a C_2 fragment, possibly glycolic acid arises de novo by an independent carboxylation reaction. CO_2 uptake could also raise the pH of the external solution. Further experimentation into the kinetics of HCO_3^- and CO_2 assimilation are necessary before pH changes are characterized as fluxes of H^+. If H^+ extrusion does exist it is possible that a reverse or anti-electrogenic pump also operates in the membrane of *Nitella*. If this is true active OH^- efflux or H^+ influx could be responsible for external pH changes.

If these assumptions are correct the simplest way of accounting for the large K^+ current is to suppose that it travels through the pump. The fact that the membrane potential during an applied current is not too much different from the resting potential and yet K influx is several times greater than normal suggests this movement is completely different from normal ionic movement. The inhibitory effects of DNP on H^+ extrusion and K^+ influx coupled with the increase in resistance also suggests the applied current travels through the pump. Perhaps the applied current stops the pump and converts it to a low resistance channel through which K^+ and perhaps to some extent H^+ can move depending on the external pH. This is equivalent to saying that maintainence of the resting potential is taken over by the external circuit. The level of the potential would thus be determined by the decrease in electrogenicity caused by the applied current and the constant applied current in combination with the low resistance

of the modified active channels.

Although the idea of H^+ extrusion proposed by Kitasato (1968) appears to be correct this work indicates the very high H^+ fluxes calculated by him from applied current data are greatly overestimated.

SUMMARY

The results of this study indicate membrane resistance of Nitella cannot be related to passive fluxes of ions as classical interpretation suggests. When a hyperpolarizing current of about 0.6 μamp cm^{-2} was passed through the membrane, with ^{42}K present in the external solution, it was found that 50% of the current was carried by K^+. The increase in K^+ influx was 40 times the value predicted by the Goldman passive model for the observed changes in potential. The large transference number for K^+ is not in agreement with Kitasato's conclusion that 95% of the cationic influx is due to H^+.

Membrane potential measurements and tracer studies of the major ions necessitate proposal of an active electrogenic cation efflux. Previous work suggests this is H^+ extrusion. The depolarizing effects of increased external $[H^+]$ and DNP are explained as decreases in the rate of electrogenic H^+ extrusion. Evidence against passive H^+ fluxes is presented.

Tracer studies also indicate a component of K^+ transport which in most cases acts differently from a simple passive mechanism for K^+ uptake. The depressing effects of increased external $[H^+]$ and DNP on the rate of active H^+ extrusion and active K^+ influx suggest the H^+ pump can act partially as a H^+/H^+ or H^+/K^+ exchange pump. The simplest way of accounting for the large K^+ transference number would be that the K^+ current travels through the electrogenic mechanism.

LITERATURE CITED

Barr, C. E. 1965. Na and K fluxes in *Nitella clavata*. *J. Gen. Physio*. 49: 181-197.

Brown, D. F., T. E. Ryan and C. E. Barr. 1973. Calculated H^+ Fluxes in *Nitella clavata*. *Proc. Liverpool Workshop on Ion Transport in Plants* (W. P. Anderson Ed.) Academic Press, London. (In Press).

Dainty, J., A. B. Hope and C. Denby. 1960. Ionic relations of cells of Chara australis. II. The indiffusable anions of the cell wall. *Aust. J. Biol. Sci.* 13: 267.

DiGregorio, J. A., C. E. Barr, A. G. Endress, J. R. Sherwin and R.P. Mannhardt. 1973. Phosphorus Status and Cl Transport in *Nitella*. Submitted to *Plant Physiol*.

Finkelstein, A. 1964. Carrier Model for Active Transport of Ions Across a Mosaic Membrane. *Biophys. J.* 4: 421.

Hirakawa, S. and H. Yoshimura. 1964. Measurements of intracellular pH in a single cell of *Nitella flexilis* by means of micro-glass pH electrodes. *Jap. J. Physiol.* 14: 45-55.

Hogg, J., E. J. Williams and R. J. Johnston. 1969. Light Intensity and the Membrane Parameters of *Nitella translucens*. *Biochim. Biophys. Acta*. 173: 564-566.

Hope, A. B. and N. A. Walker. 1960. Ionic Relations of Cells of *Chara australis*. III. Vacuolar fluxes of Sodium. *Aust. J. Biol. Sci.* 13: 277-291.

Hope, A. B. and N. A. Walker. 1961. Ionic Relations of Cells of *Chara australis*. IV. Membrane Potential Differences and Resistances. *Aust. J. Biol. Sci.* 14: 26-44.

Hope, A. B. 1963. Ionic Relations of Cells of *Chara australis*. VI. Fluxes of Potassium. *Aust. J. Biol. Sci.* 16: 429-441.

Hope, A. B. 1965. Ionic Relations of Cells of *Chara australis*. X. Effects of Bicarbonate Ions on Electrical Properties. *Aust. J. Biol. Sci.* 18: 789-801.

Hope, A. B., A. Simpson and N. A. Walker. 1966. The Efflux of Cl^- from Cells of *Nitella* and *Chara*. *Aust. J. Biol. Sci.* 19: 355-362.

Hope, A. B. and P. A. Aschlerger. 1970. Effects of Temperature on Membrane Permeability to Ions. *Aust. J. Biol. Sci.* 23: 1047-1060.

Hope, A. B. 1971. Ion Transport and Membranes - A Biophysical Outline. Butterworth and Co. LTD. London.

Kitasato, H. 1968. The Influence of H^+ on the Membrane Potential and Ion Fluxes of Nitella. J. Gen. Physio. 52: 60-87.

Lannoye, R. J., S. E. Tarr and J. Dainty. 1970. The effects of pH on the ionic and electrical properties of the internodal cells of Chara australis. J. Exp. Bot. 21: 543-551.

Lucas, W. J. and F. A. Smith. 1973. The formation of Alkaline and Acid Regions at the Surface of Chara corallina cells. J. Exp. Bot. 24: 1-14.

MacRobbie, E. A. C. 1970. The Active transport of ions in plant cells. Quart. Rev. Biophys. 3: 251-294.

MacRobbie, E. A. C. 1971. Fluxes and compartmentation in plant cells. Annual Rev. Plant Physiol. 22: 75-96.

Paszewski. A., J. Stolarek and T. Gebal. 1968. Ionic relations and electrophysiology of single cells of Characeae. Part I. Investigations on electric potentials and resistance in cells of Nitellopsis obtuse. Acta Societatis Botanicorum Poloniae. 37: nr 2.

Poole, R. J. 1973. The H^+ pump in Red Beet. Proc. Liverpool Workshop on Ion Transport in Plants. (W. P. Anderson Ed.) Academic Press (In Press).

Raven, J. A. and F. A. Smith. 1973. The regulation of intracellular pH as a fundamental Biological Process. Proc. Liverpool Workshop on Ion Transport in Plants. (W. P. Anderson Ed.). Academic Press, London. (In Press).

Rent, R. K., R. A. Johnson and C. E. Barr. 1972. Net H^+ influx in Nitella clavata. J. Membrane Biol. 7: 321-244.

Slaymen, C. L. 1965. Electrical Properties of Neurospora crassa. Respiration and intracellular potential. J. Gen. Physiol. 49: 93.

Smith, F. A. 1967. Rates of Photosynthesis in Characean Cells. I. Photosynthetic $^{14}CO_2$ fixation by Nitella translucens. J. Exp. Bot. 18: 509-517.

Smith, F. A. 1968. Rates of Photosynthesis in Characean Cells. II. Photosynthetic $^{14}CO_2$ fixation and ^{14}C-bicarbonate uptake by Characean cells. J. Exp. Bot. 19: 442-451.

Smith, F. A. 1970. The mechanism of chloride transport in Characean cells. New Phytol. 69: 903-917.

Spanswick, R. M., J. Stolarek and E. J. Williams. 1967. The membrane potential of Nitella translucens. J. Exp. Bot. 18: 1-16.

Spanswick, R. M. 1970. Electrophysiological techniques and the magnitudes of the membrane potentials and resistances of Nitella translucens. J. Exp. Bot. 21: 617-627.

Spanswick, R. M. 1972. Evidence for an electrogenic ion pump in Nitella translucens. I. The effects of pH, K^+, Na^+, light and temperature on the membrane potential and resistance. Biochim. Biophys. Acta. 288: 73-89.

Spanswick, R. M. 1973. Electrogenesis in Photosynthetic Tissues. Proc. Liverpool Workshop on Ion Transport in Plants. (W. P. Anderson Ed.) Academic Press, London. (In Press).

Spear, D. G., J. K. Barr and C. E. Barr. 1969. Localization of hydrogen ion fluxes in Nitella. J. Gen. Physiol. 54: 397.

Stiller, M. 1962. The path of Carbon in Photosynthesis. Ann. Rev. Plant Physiol. 13: 151.

Walker, N. A. and A. B. Hope. 1969. Membrane fluxes and electric conductance in Characean cells. Aust. J. Biol. Sci. 22: 1179-95.

Williams, E. J., R. J. Johnston and J. Dainty. 1964. The electrical resistance and capacitance of membranes of Nitella translucens. J. Exp. Bot. 15: 1-14.

Williams, E. J. and J. Hogg. 1970. The (K^+ - Na^+)-dependence of the membrane parameters of Nitella translucens. Biochim. Biophys. Acta. 203: 170-172.

Zarlengo, M. H. and S. G. Schultz. 1966. Cation transport and metabolism in Streptococcus fecalis. Biochim. Biophys. Acta. 126: 308-320.

Koh, M. S. W. 1973. The relation of the membrane potential of Nitella to photosynthetic processes and external conditions. M. S. Thesis. S. U. C. Brockport, Brockport, New York.

APPENDIX

Geometry Correction

A special holder was designed to count radioactivity in live cells. This consisted of a section of plastic ruler mounted on a piece of plexiglass. The cell was placed on filter paper in the groove on the ruler and then the whole apparatus was placed under a GM tube. For comparison with standards a number of cells of different lengths were read live then broken up in 1.0 ml of "Killing solution" (6 parts by volume 1.0 M $NaHCO_3$, 1 part H_2O and 1part EtOH) and read wet in 1 inch diameter aluminum planchettes. The standards were 1ml of "Killing solution" containing a known amount of the ^{42}K K solution. These were counted in the same manner as the wet cells. This enabled live cells to be corrected to wet for comparison with the standards. This correction factor, which varies with cell length, may be obtained from Figures 1A and 2A for ^{42}K and ^{36}Cl, respectively.

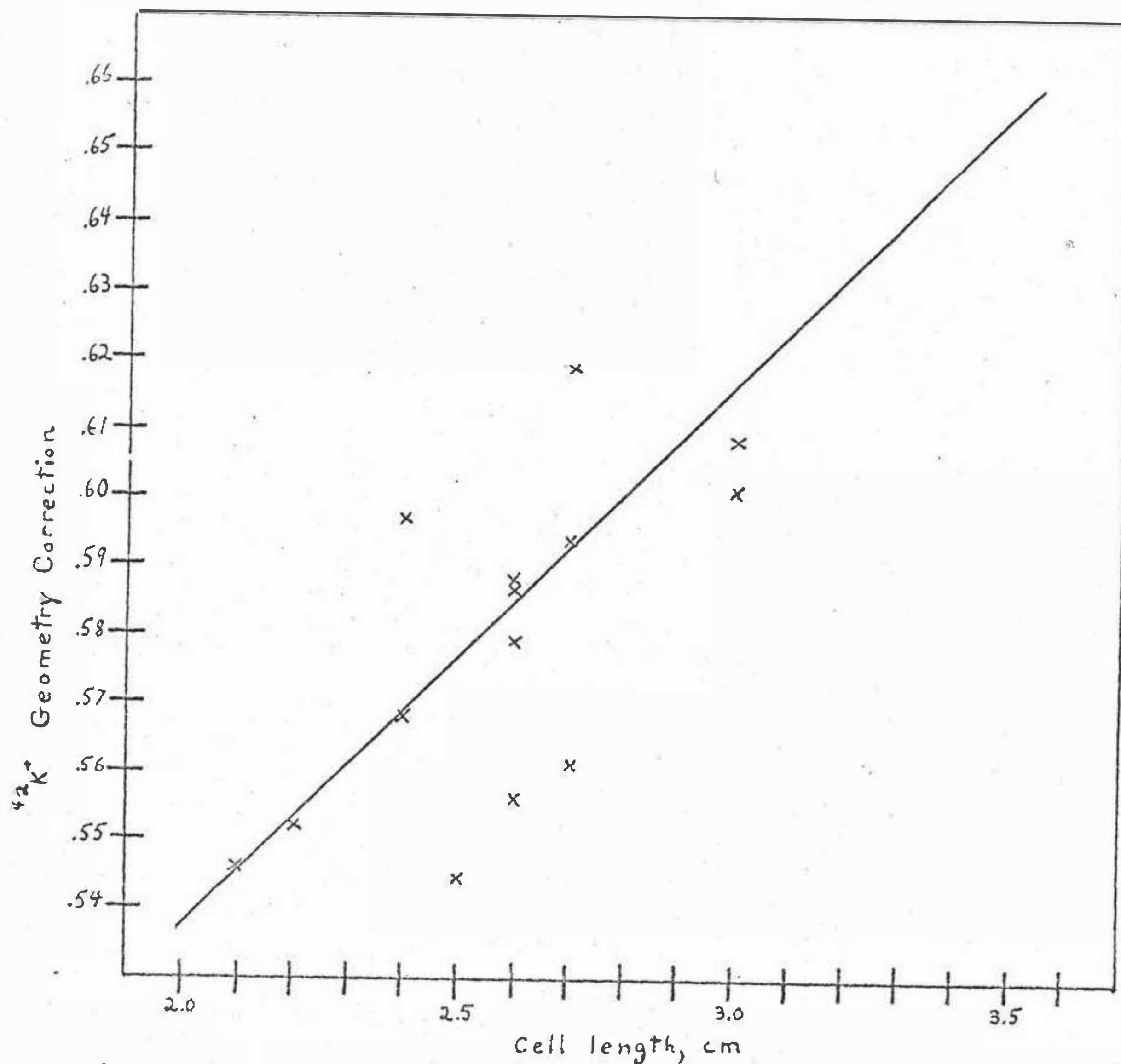


Figure 1A. ^{42}K geometry correction factor versus cell length for Nitella. Geometry correction factor = cpm of cell dispersed in 1.0 ml "killing solution"/ cpm in live cell.

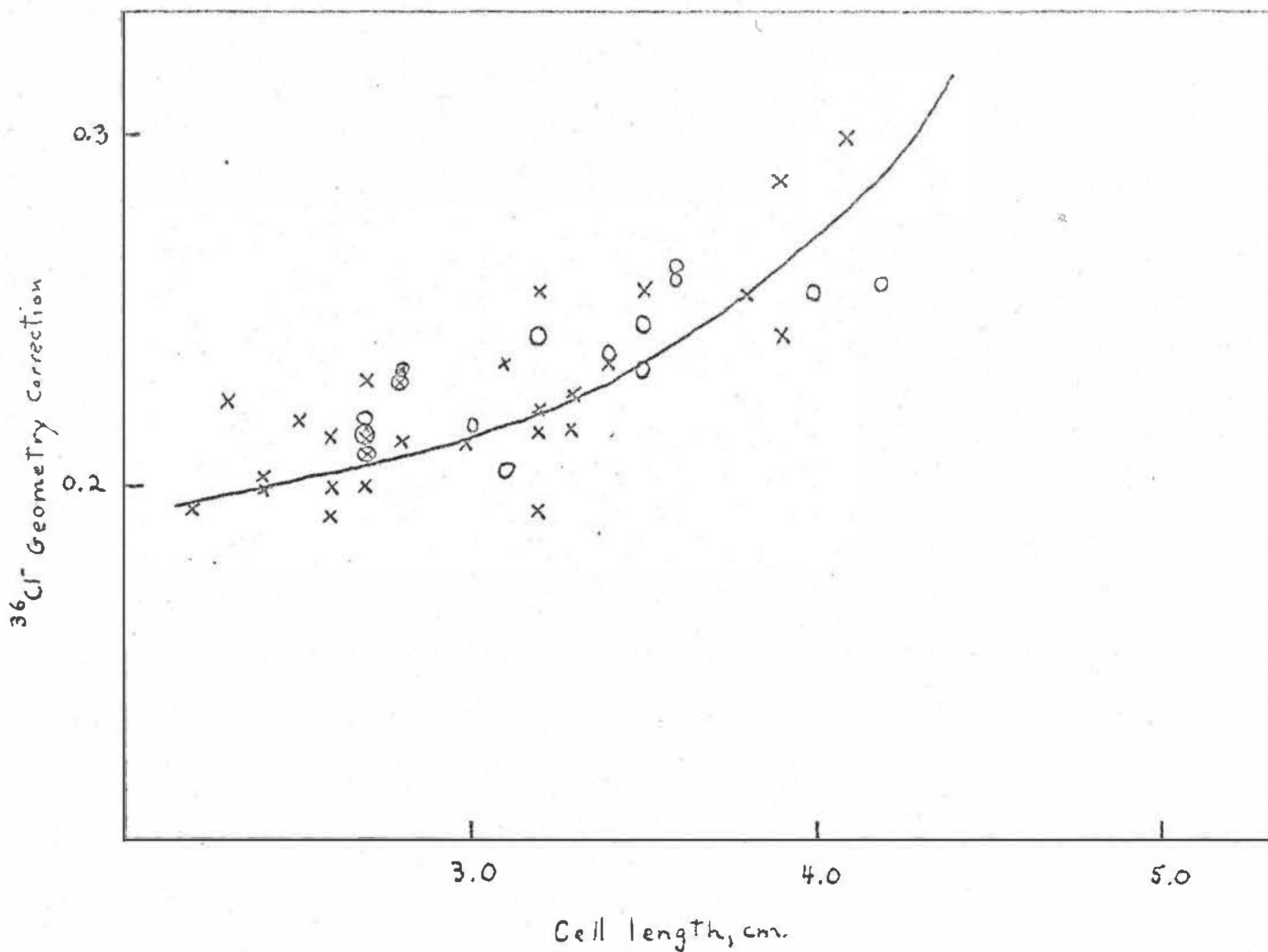


Figure 2A. Cl^- geometry correction factor versus cell length for Nitella. Geometry correction factor = cpm of cell dispersed in 1.0 ml "killing solution"/ cpm in live cell.