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THE RELATIONSHIP OF HYDROGEN ION, PHOSPHATE, AND LIGHT TO THE RESTING MEMBRANE POTENTIAL IN *NITELLA CLAVATA*

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

As partial fulfillment
of the requirements for the degree of
Master of Science

By

Rosemarie K. Rent

April, 1971

THESIS APPROVAL

The Relationship of Hydrogen Ion, Phosphate and Light to the Resting Membrane Potential in *Nitella clavata*

by

Rosemarie K. Rent

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INTRODUCTION

Nitella clavata, a member of the Characeae (Chlorophycophyta) has a thallus consisting of an erect axis differentiated into nodes, each bearing a whorl of several cells, and internodes, each a single plant cell. Due to their excitable properties, single internodal cells of the Characeae have been the subject of much research over the past fifty years. The ability of the membrane to generate an action potential suggests many aspects of its membrane physiology similar to neurons. As a result, many of the techniques commonly used in neurophysiological investigations are employed in this research.

One of the most thoroughly studied aspects of membrane physiologyin the Characeae is the resting potential, the electrical potential difference between the vacuolar sap and the external bathing medium. It is easily demonstrated that by changing the ionic composition of the bathing medium, the resting potential will change. The nature of such changes and their relation to the magnitude of the resting potential have been the subject of much investigation. To date, no fully satisfactory explanation of the origin of the resting potential has been offered.

The earliest theories of the origin of the resting potential considered it to be the result of ionic concentration gradients across the cell membrane. The ions considered to control the magnitude of the potential were potassium, sodium, and chloride (Hope and Walker, 1961; Briggs, 1961) due to their predominance (80-90%) among the ions in the cell (Barr, 1965). In the simplest situation, considering the cell membrane to be permeable to only

one ion and having no active transport, the resting membrane potential, E_m, is given by the Nernst equation:

$$E_m = \frac{RT}{zF} \ln \frac{C_i}{C_o}$$

where R = universal gas constant, 1.99 cal/mole degree

T = absolute temperature

z = charge on the ion

F = Faraday constant, 23,060 cal/mole volt

C_i, C_o = concentration of the ion inside and outside the membrane

This equation requires that the ion be at electrochemical equilibrium, that is, net flux of the ion is zero. In a more complicated situation, when the membrane is permeable to more than one ion, the expression becomes more complex and additional activity and permeability terms must be considered (Briggs, Hope, and Robertson, 1961):

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

where P = permeability of each ion

K_o, K_i, etc. = internal and external activities of each ion.

If the equilibrium potential of an ion is equal to or close to the resting membrane potential, E_m, it is possible that the passive movement of this ion is controlling the potential due to its high permeability as compared to the other ions. The frequency with which the resting potential of both animal and plant cells approximate the K^+ equilibrium potential, led many investigators to assume the resting potential to be a K^+ diffusion potential. This could be tested by varying the external K^+ concentration. Studies of this type on Chara (Hope and Walker, 1961) demonstrated that such passive ionic control of the potential is possible only in the absence of Ca (Hope and Walker, 1961; Spanswick, et. al., 1967),

a condition under which cells survive only a few days at most. Under more natrual conditions, i.e. in the presence of Ca, E_m is quite unresponsive to changes in external ionic conditions.

The origin of the resting potential then remained an enigma for some time, especially since the membrane conductance was roughly ten times that which could be accounted for by the sum of the K, Na, and Cl fluxes (Williams, Johnston, and Dainty, 1964). Although studies involving metabolic inhibitors and light have suggested that K^+ influx, Na^+ efflux, and Cl^- influx in a variety of members of the Characeae are active (Barr, 1965; Hope, et al, 1966; MacRobbie, 1966; Coster and Hope, 1967; Findlay et al, 1969). This in no way pointed to any solution because the totality of fluxes was simply too small. In essence the problem hinged on finding the identity of the predominant flux or fluxes across the membrane. It appeared almost incredible that H^+ would be involved, since an external solution of pH 7.0 would be such a poor source of H^+. There also lingered some doubt about the reliability of membrane conductance measurements.

It was at this point that Kitasato (1968) found evidence for a very high H^+ permeability in _Nitella Clavata_; this led him to propose an active H^+ extrusion pump electrically balanced by passive H^+ influx. The demonstration of alternating acid and alkaline zones along the length of the _N. Clavata_ cell by Spear et al (1969) offered visual evidences and support for H^+ extrusion. If the extrusion is electrogenic, i.e., net charge in the form of H^+ is transferred, the level of resting potential becomes increasingly displaced from E_H as the magnitude of H^+ extrusion increases.

Kitasato (1968) estimated the passive H^+ influx at 40×10^{-12} moles $cm^{-2}sec^{-1}$ and this balanced almost entirely by H^+ extrusion (with a small passive component). Kitasato recognized that the stability of the intracellular pH depends on this balance, and this, in fact, was the actual basis for his proposal of the H^+ pump.

The relationship of light to maintenance of the membrane potential has been investigated with conflicting results (Table 1); no doubt this is partly due to the variety of experimental conditions utilized. Nishizaki (1968) found that during light-dark transitions, the time course of E_m changes cannot be explained by changes in permeability alone. This, along with loss of selectivity to K and Na while retaining sensitivity to light and, secondly, the lack of direct correlation of the effect of bicarbonate on the light reaction and O_2 evolution, suggest that light-induced change in E_m is mediated through some products of carbon fixation. The effects of light and this analysis are especially interesting in that Spear, et al (1969) have found that visibly detectable H^+ extrusion requires light.

Another interesting relationship is that between Cl^- influx and H^+ extrusion. Spear, et al (1969) found that Cl^- influx occurs almost entirely in the acid-extruding regions of the cell. Although this suggests a dependence of Cl^- influx on H^+ extrusion, Pitman's (1970) work on barley roots indicates that H^+ extrusion is not dependent on external Cl. Therefore, active Cl^- movement can tentatively be eliminated as an important factor in H^+ extrusion; consistent with this is the relatively small magnitude of Cl^- influx.

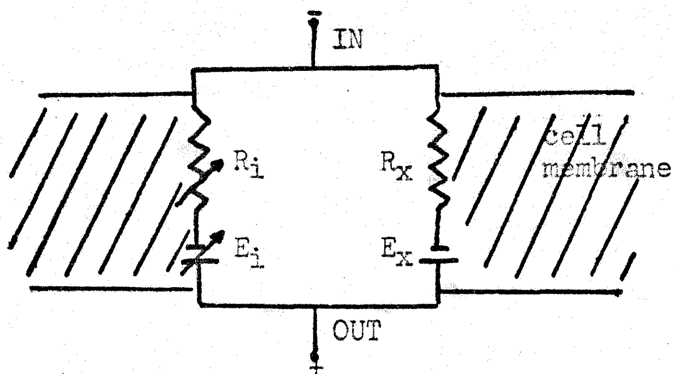
Slayman's (1965) model for an H^+ extrusion pump in Neurospora may

TABLE 1

ORGANISM (pretreatment)	SOLUTION mM	LIGHT, lux (source)	EFFECT OF LIGHT on E_m, in mV	PERMANENT E_m change? (duration)	OTHER COMMENTS	REFERENCE
Chara braunii (24 hr. dark)	0.5 KCl 0.5 $CaCl_2$ 0.2 NaCl	4000 (incandes.)	HYPERPOL. -136 dark -190 light	No (70 min.)	Membrane resistance in light much reduced	Nishizaki (1968)
Chara braunii (light adapted)	0.5 KCl 0.5 $CaCl_2$ 0.2 $NaHCO_3$	4000 (incandes.)	DEPOL. -165 dark -135 light	No (20 min.)	cf. Hope (1965)	Nishizaki (1968)
Chara australis (light adapted)	0.1 KCl 0.5 NaCl 0.5 $NaHCO_3$ 0.1 $CaCl_2$	? (microscope and room light)	HYPERPOL. -165 dark -215 light	No (20 min.) cf. Smith (1968)	Membrane resistance much reduced in light	Hope (1965)
Chara braunii (24 hr. dark)	0.05 KNO_3 0.2 $NaNO_3$ 0.4 $CaCl_2$ 0.1 $MgSO_4$ 0.1 Na_2HPO_4	7000 (incandes.)	HYPERPOL. -175 dark -205 light	No (30 min.)	Follows transient depol. pH probably above 8	Nishizaki (1963)
Chara braunii (light adapted)	same as above	2400 (incandes.)	HYPERPOL. -260 dark -305 light	No (8 min.)	pH probably above 8	Nishizaki (1963)
Chara corallina (light adapted?)	2.0 NaCl 0.2 KCl 0.05 $CaCl_2$	not given	HYPERPOL. -137 light -130 dark	No (120 min.)	SO_4 substituted for Cl - same results. Resistance oscillatory	Findlay, et al (1969)

TABLE 1, continued

Hydrodictyon verticulatum (not given)	0.5 NaCl 0.37 KH_2PO_4 0.29 K_3PO_4 0.2 $CaCl_2$ 0.05 $MgSO_4$ 0.55 KNO_3	not given	HYPERPOL. - 90 dark -150 light	? (10-20 min. trials)	pH probably above 8	Metlicka and Rybova (1967)
Nitella flexilis (1 hr. dark)	0.1 KCl	2000 (incandes)	DEPOL. -158 dark -135 light	? (12 min. trial)		Adrianov, et al, (1965)
Nitella flexilis (several hrs. dark)	0.1 KCl	2000 (incandes.)	HYPERPOL. -143 dark -171 light	Yes (24 Hrs.)		Nagai and Tazawa (1962)
same as above	0.1 NaCl	same as above	HYPERPOL -140 dark -203 light	Yes (24 hrs.)		Nagai and Tazawa (1962)
Nitella flexilis (not given)	0.9 KCl 0.8 KNO_3 0.09 NaH_2PO_4 1.4 Na_2SO_4 1.2 $Ca(NO_3)_2$ 3.0 $MgSO_4$ micronutrients	4000 (incandes.)	HYPERPOL. -100 dark -138 light	No (85 min.)	pH probably below 5	Volkov (1964)
Nitella translucens (dark adapted)	1.0 KCl 0.1 NaCl 0.1 $CaCl_2$	17.6 (676 nm.) 10.5 (676 nm.) 12.3 (676 nm.)	HYPERPOL. -140 d, -150 l -130 d, -135 l -148 d, -161 l	No (30-60 min) same same	Variety of wavelengths and efficiency of response	Vredenberg (1969)



from Slayman, 1965

Figure 1

be applied to Nitella in discussing H movements across the membrane. (Figure 1) He suggests that if we consider ionic and active emf's to be connected in parallel, the model requires only that R_i behave like the integral resistance across a K^+specific membrane in order that the internal potential should vary with log $[K^+]$ along a slope somewhat approximating the Nernst slope. The effect of Ca discussed earlier in decreasing K^+sensitivity of the membrane potential would be accounted for if Ca^{++}simply increased R_i. He has shown that raising the $Ca^{++}$from 0 to 1 mM appears to increase R_i/R_x sevenfold and has implied that R_x can be considered constant.

Refering to Slayman's diagram, we can consider what might happen to the membrane potential in certain situations. It is convenient to consider the cell in a high Ca solution so that R_i is very high and E_i is insignificant. Therefore, the potential we are considering is E_x, that due to the activity of the pump, and factors affecting it should produce changes in the potential.

Spear, et al (1969) have demonstrated that visibily detectable H extrusion requires light. Thus, factors affecting the H^+ pump could be those affecting photosynthesis: photosynthetic efficiency of the cell, light intensity incident on the cell, and availability of CO_2. If the intrinsic pump is strong and all factors are at high levels, we may expect a high E_m. In situations where one of the factors affecting the pump is limiting, we might expect a potential difference equivalent to E_H because of a non-functioning pump. At levels between these two extremes, any combination of factors might provide a complete spectrum of membrane potentials. In yet another case, if H^+ extrusion can be enhanced by light, perhaps by the formation of a redox substrate, as suggested by Spear, et al (1969), there may be no change in E_m if an increase in membrane conductance allows the extruded ions to flow more easily back into the cell at the time. It may be, in fact that the permeability decreases and resistance measurements do not take into account the high H^+ at the membrane. This is suggested by Kitasato's data which indicates a fivefold decrease in permeability for each tenfold increase in H^+. Therefore, the overall effect is a twofold increase in conductance. In respect to Slayman's model, all four terms change; each of the two terms in the half circuit add up to the same previous value and no change in E_m is observed. Thus, a lack of response to light does not necessarily mean that changes in H^+ transport are not occuring.

The object of this study is to further test the Kitasato hypothesis of active H^+ extrusion balanced by passive H^+ influx. We will attempt to measure net H^+ current indirectly by means of

titration and pH measurements of the bathing medium. Evaluation of E_x, the intrinsic EMF of the H^+ pump, will be attempted by reducing the return H^+ current to a low value through use of a high Ca^{++} medium.

MATERIALS

Nitella Clavata was cultured as previously discribed by Spear, et al. (1969). The composition of the nutrient solution is given in Table 2. The culture vessels were illuminated 16 hours by Sylvania Gro-lux and cool white flourescent lamps alternating with 8 hours of darkness.

Internodal cells, usually the second to the fifth from the growing tip, were separated from the intact plant by cutting away the neighboring and nodal cells at both ends. The cells ranged from 2 to 5 cm. in length. After harvesting, the cells were preconditioned in standard bathing medium, Kb (in mM, 1.0 KCl, 0.1 $KHCO_3$, 0.1 NaCl, o.1 $CaCl_2$, and 0.1 $MgCl_2$). Cells were kept in glass culture dishes in an incubator at 22-23°C and approximately 50 foot-candles cool white flourescent light. Length of preconditioning period (cell age) varied from 0 to 35 days.

TABLE 2

COMPOSITION OF *NITELLA* CULTURE SOLUTION

3.0 mM $CaCl_2$
1.0 mM $MgCl_2$
0.2 mM KCl
2.0 mM $NaHCO_3$
0.2 mM KNO_3
0.004 mM NaH_2PO_4
4.0 mM tris-(hydroxymethyl)-aminoethane neutralized with HCl to pH 7.7
1 ml/liter micronutrient stock solution

Micronutrient stock solution: 50 g. Brockport brown soil boiled 30 min. in 1.0 liter of 6.0 mM ethylenediamine tetraacetate at pH 8.0.

METHODS

pH Change

In order to measure apparent H^+ movement in *Nitella*, nine cells were placed in 5 ml. K solution (in millimoles, 1.0 KCl, o.1 NaCl, 0.1 $CaCl_2$, 0.1 $MgCl_2$), pH 4.7, at room temperature and in flourescent room light. The pH change of the bathing solution was measured every 5 minutes to 30 minutes, then every 10 minutes to 60 minutes. Additional readings were taken at 2 and 3 hours. a control of K solution with no cells was measured at the same time intervals to detect changes in pH due to the solution itself. After the initial trial, cells were screened for H^+ extrusion by placing them in phenol red (Spear, et al, 1969) before beginning the experiment. Only those cells which developed well defined acid and base areas were chosen. The experiment was repeated using these cells in light and again in the dark to eliminate the effects of light on H^+ movement. All cells were observed in phenol red following the procedure. In order to evaluate the effects of the cell wall on the observed pH changes, several cells were dried until the cytoplasm had disintegrated, leaving the intact cell wall. The same pH measurements were then performed on the isolated cells walls.

pH Titration

Twenty cells were pretreated for an hour in K solution, pH 4.5. At ten minute intervals the entire volume of solution was replaced to assure maintenance of the low pH. After the initial acid treatment, the cells were placed in K solution, pH 6.0 for 2 hours with the entire volume of solution again replaced at 10 minute

intervals. This low pH procedure was developed to eliminate any immediate buffering effect of the wall. The cell was then placed in pH 6.0 to bring it back to a more "normal" state. At the end of this period, the cells were washed with pH 4.5 K and placed in a plexiglass partitioned trough in 3 ml. K solution, pH 4.5. The solution in the trough could circulate freely between the two compartments. At 10 minute intervals the pH was measured. Due to the partition, the trough could be tilted and electrodes inserted in the cell-free compartment for the pH readings, thus avoiding interference of or damage to the cells. A control K solution, pH 4.5, without cells, was measured at the same time intervals. After each reading, the pH was adjusted back to the initial value with 5mM H_2SO_4 and the quantity of acid used was recorded. Readings were taken for 4 hours. At 1 hour intervals Cl concentration was measured in the bathing medium with an Orion Ionalyzer halide electrode. At the termination of the experiment, solution was collected and K^+ analysis was carried out by atomic absorption spectroscopy.

Membrane Potential Measurements

Membrane potentials were measured with Ag/AgCl electrodes connected to a General Radio Company 1230A electrometer and an Esterline Angus A6016 ink recorder. The reference electrode was a Coleman reservoir, fiber type, placed in the solution downstream from the cell in order to prevent leaking K^+ from affecting the cell. The measuring electrode was a glass microcapillary type pulled to an outside diameter of 5-10 (Narashige PN-3 glass microelectrode puller). Both reference and measuring electrodes were filled with

artificial cell sap, ACS (in mM, 80 KCl, 20 NaCl, 5 $CaCl_2$) chosen to stimulate the vacuolar sap composition in order to reduce diffusion potentials which might arise at the tip. Electrode potentials were measured with the tip of the measuring electrode in a reservoir filled with ACS. Only those microelectrodes having potentials less than 35 mV were used. Electrode potentials were measured before and after every experiment.

During experimentation, the internodal cell was held in a plexiglass trough. Experimental solutions were delivered to the trough through polyethylene tubing from a reservoir at a regualted rate. Drip rates were standard; a fast rate of 20 drops/min., moderate rate of 8 drops/min., and slow rate of 2 drops/min. A dissecting microscope was mounted directly above the trough to aid insertion of the microelectrode and was removed for the remainder of the experiment. The tip of the measuring electrode was inserted well into the vacuole of the cell, thus the measured potential is not strictly that of the cell membrane, but consists of the potential across the cell wall, cell membrane and vacuolar membrane in series. The observed values were referred to as "membrane" potentials and are a measure of the potential difference between the vacuole and the external bathing medium. After insertion of the microelectrode, rate of stabilization of the potential varies depending on the rate of sealing of the cell membrane to the glass.

Light during these experiments was provided by both flourescent and incandescent sources. Light intensity ranged from 100-200 foot-candles, half flourescent, half incandescent, to 30-100 foot-candles flourescent only. Composition of the experimental solutions is

given in Table 3. All solutions were generally used at pH 6.9-7.1. Change in potential with variation in external bathing solution and light intensity was measured.

TABLE 3

COMPOSITION OF EXPERIMENTAL SOLUTIONS (mM)

Component	Solution					
	Kb	E_2	Ca	Ca-K	Ca-Mg	Ca-Na
pH	6.9-7.0	7.05	7.0	7.0	7.0	7.0
KCl	1.0	0.75		2.0		
NaCl	0.1	0.15				2.0
$CaCl_2$	0.1	0.15	5.0	4.0	4.0	4.0
$MgCl_2$	0.1	0.15			2.0	
$KHCO_3$	0.1	0.1				
$K_{1.6}H_{1.4}PO_4$		0.5				

RESULTS

pH Experiments

Figure 2 shows the time course of the rise in pH of the bathing medium brought about by the cells placed in a low pH solution. This is consistent with Kitasato's hypothesis which predicts a net H^+ influx under these conditions. The initial net flux values for the trials A and B are 40 and 47 pmoles/cm^2sec. In trial A, unscreened cells were used and the rise in pH was observed. In relating these results to the visible H^+ extrusion, cells of group B were screened for visual evidence of H^+ extrusion using phenol red as described by Spear, et al (1969). Only cells having well defined acid and base regions were used. These cells, when subjected to the same treatment as the cells above, exhibited the same time course of pH change (Figure 2, curve B). At the conclusion of the experiment, the cells were again placed in the phenol red solution. At 30 minutes all cells showed base formation, but less than 50% showed any acid zonation at all. Approximately 3 hours were required for development of acid-base zonation similar to that observed previous to the experimentation. A third group of cells, C, was screened in the same manner, but were bathed in K solution in darkness. Curve C is the result of dark experiment and the time course of pH change is very similar to curve B.

The data from screening procedures seems to indicate a non-functional H^+ extrusion mechanism when the cells are placed in a low pH medium. Since H^+ extrusion does not occur in the dark, (Spear, et al, 1969), the observation of a similar time course

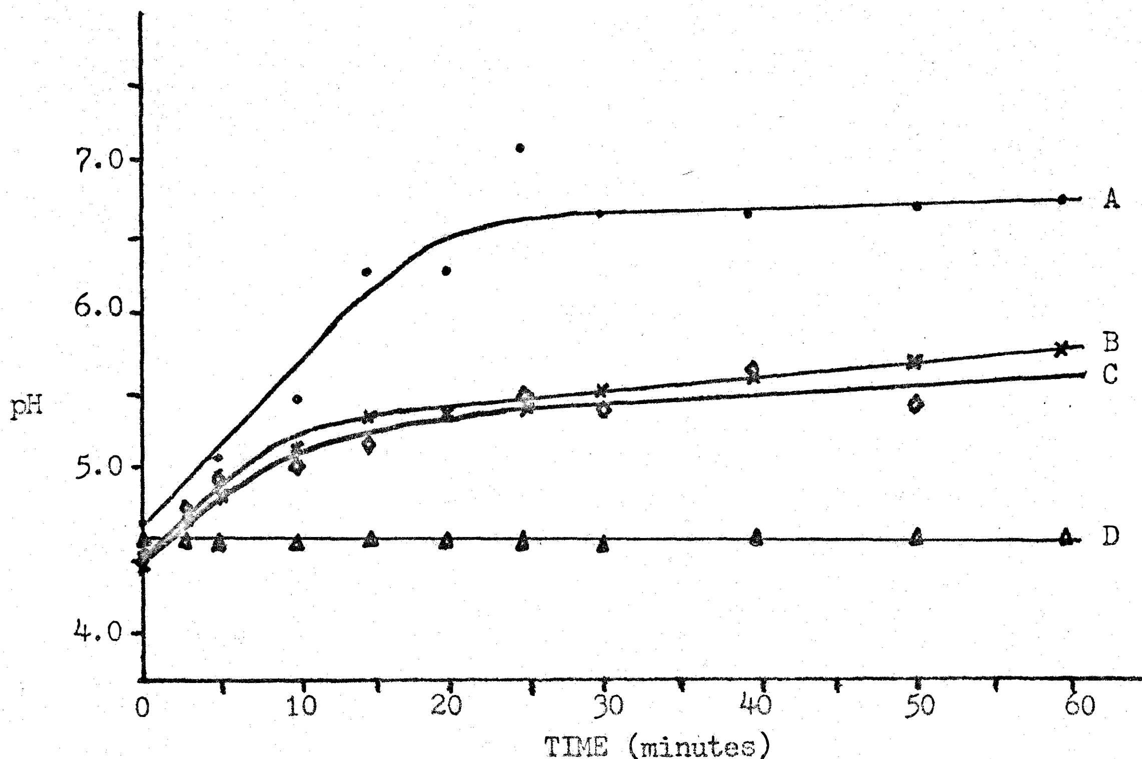


FIGURE 2. pH CHANGE IN STAGNANT K^+ SOLUTION, LOW pH

(A) Nine cells with total surface area of 11.5 cm^2 in 5 ml K^+ solution and 3 Kerg/cm^2sec cool white flourescent light. Initial H^+ flux is approximately 40 pmole/cm^2sec.

(B) Ten cells screened for high rates of acid and base production in 5 ml K^+ solution and 15 Kerg/cm^2sec cool white flourescent and incandescent light. The cells were not measured. Approximate initial flux is 47 pmoles/cm^2sec.

(C) Ten cells were screened for high rates of acid and base production in 5 ml K^+ solution and darkness. Cells were not measured. Initial flux is approximately the same as curve B, 47 pmoles/cm^2sec.

(D) Eighteen isolated cell walls with total surface area of 10.4 cm^2 in 5 ml K^+ solution and 15 Kerg/cm^2sec cool white flourescent and incandescent light.

In all cases the solution bathing the living cells reached a final pH of 7.6. This value seems to be a steady state pH and may take several hours to reach.

in the dark would also indicate a non-functional mechanism for extrusion. In order to be certain of the situation, it is necessary to consider the effect of the ion exchange properties of the cell wall (Dainty, Hope, and Denby, 1960) on pH change observed. Dried and isolated cell walls were subjected to the low pH medium with no change in pH over the same period of time. Thus, the effect of the cell wall can be eliminated in assesing H^+ influx in these experiments.

Where pH change experiments yielded data for a range of pH over a period of time, titration experiments were designed to assess the long term effects of low pH. At timed intervals, the pH was adjusted so that for the four hours duration of experimentation, themean pH was 4.7. Figure 3 indicates the time course of H^+ addition and calculated net H^+ influx. The cells were pretreated in pH 4.5 K for 1 hour, then pH 6.0 for 2 hours in these experiments to eliminate some of the expected effects of the cell wall, although as indicated in Figure 2, the cell wall does not seem to affect H^+ movement. For these data, and pH change data, apparent permeabilities were calculated (Figure 4). Permeability calculations were made according to the equation:

$$P = \frac{\bar{J}}{\frac{zF_E}{RT} \cdot \frac{[H_o] - [H_i] \exp(zFE/RT)}{1 - \exp(zFE/RT)}}$$

where $\bar{J}$ = net H^+ influx during the pH interval determined by change in $[H_o]$ and cell surface area.
E = the estimated potential at the midpoint of a changing pH interval for the pH change experiments or mean pH in the titration experiments determined from Figure 5.

Permeabilities calculated from pH change data indicate permeabilities

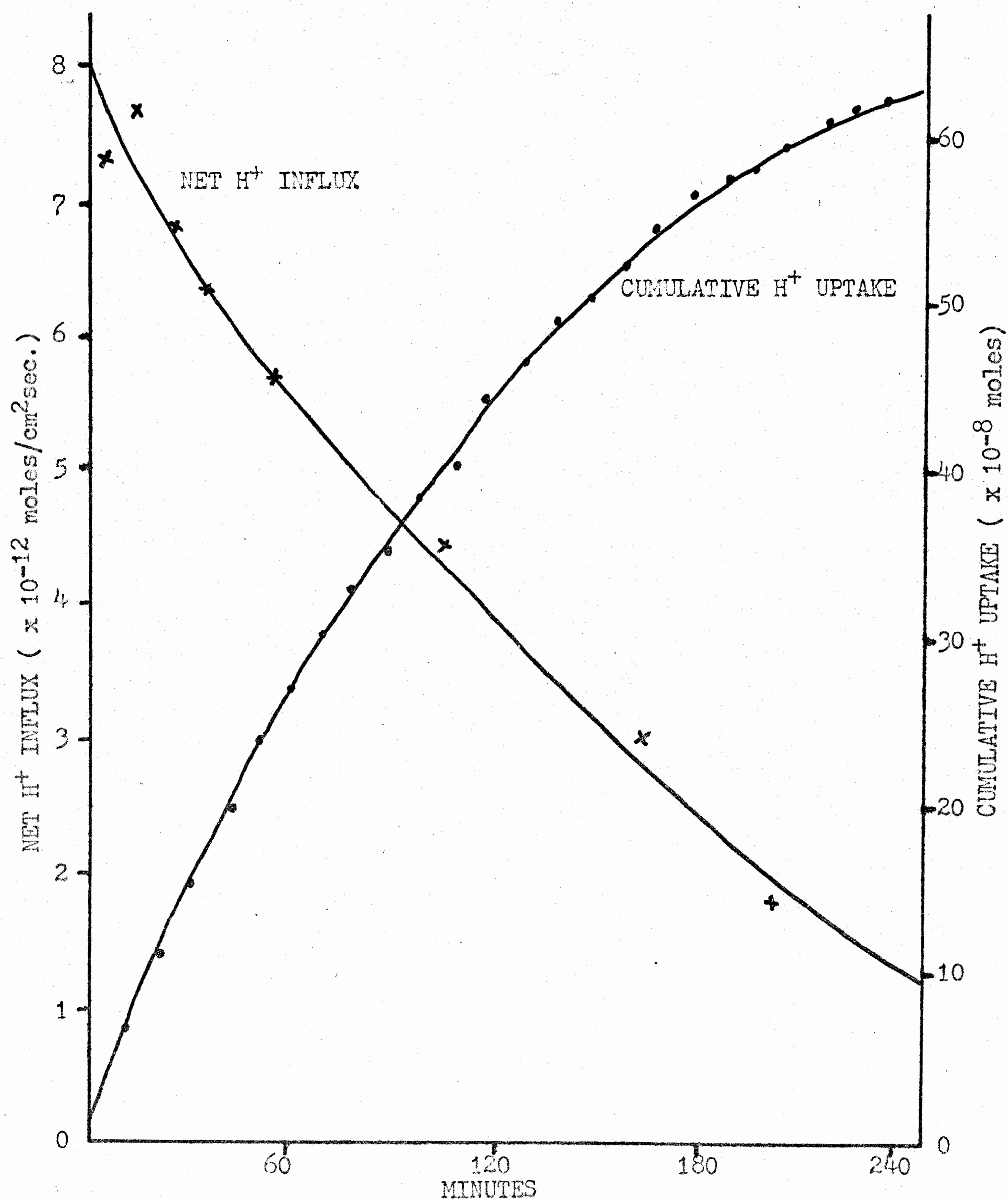


FIGURE 3. pH TITRATION EXPERIMENTS

Cumulative H^+ uptake (o) and net H^+ influx (x) for 20 Nitella cells with total surface area of 10.0 cm^2. Light intensity was approximately 200 foot-candles, incandescent and cool white flourscent.

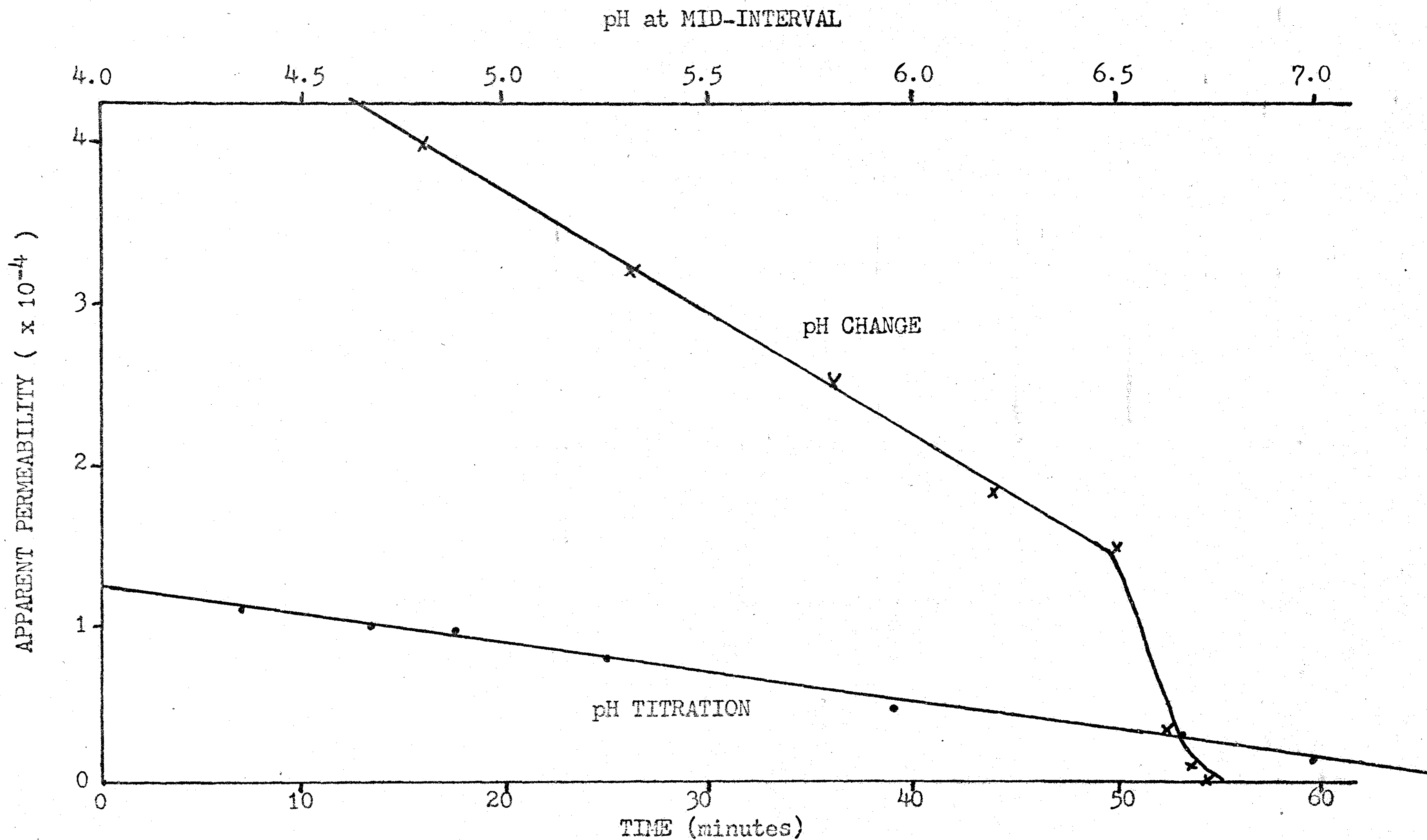


FIGURE 4. Variation in Apparent Permeability in pH Change (x) and Titration (o) Experiments

for constantly changing pH intervals. Permeabilities from titration data indicate changing permeabilities for the same pH interval over a period of time. Initial permeabilities for the two experiments were quite different, although of the same order of magnitude. These two values are not strictly comparable because of the discrepancy in procedures of the two experiments, i.e., pretreatment, number and size of cells in relation to volume of solution, and light intensity.

Along with pH measurements, Cl^- and K^+ determinations were made in the titration experiments. Results of the net flux determinations over a 4 hour period are given in Table 4. The death of one cell makes interpretation of these data difficult. For example, we may assume that the ions from the cell sap are released into the media upon death of the cell and this would considerably alter flux calculations in comparison to those made considering that all twenty cells were living. In any event, the results indicate that there is no good evidence yet for the large participation of K^+ and Cl^- fluxes at low pH, but H^+ influx remains strikingly unbalanced.

Potential measurements at low pH produced very consistent results (Table 5). Statistically significant depolatizations were produced at pH 5.0 or lower in light or dark. Depolarizations in most cases were rapid and quickly reached a less negative stable E_m. Return to neutral pH produced a rapid rise in E_m, most often with an overshoot which returns in time to a level equal to or slightly higher than the previous neutral E_m. From this data, Figure 5 shows variation in E_m with change of pH.

TABLE 4. NET ION FLUXES IN LOW pH K SOLUTION

Twenty Nitella cells, surface area 10.0 cm^2 were placed in K solution, pH 4.5 - 4.9, for 4 hours. Results below show net ion fluxes for two situations, based on twenty living cells, and with corrections for one dead cell. The + indicates influx of the ion and the - indicates efflux.

	CORRECTION FOR DEAD CELL*	20 LIVING CELLS
Ion	Net Flux pmoles/cm^2sec	Net Flux pmoles/cm^2sec
Cl	+ 1.39	- 3.3
K	+ 0.15	- 3.05
H	+ 4.5	+ 4.3

* based on cell volume and concentrations of ions in cell sap

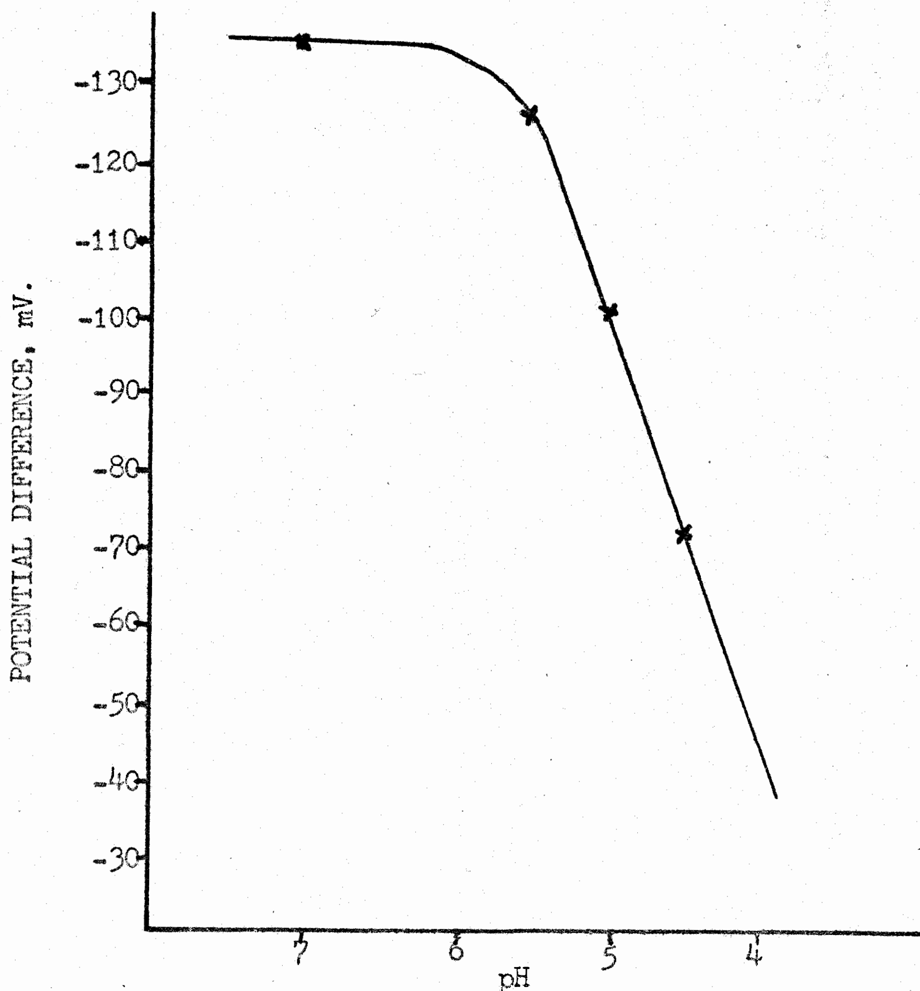


FIGURE 5. VARIATION IN E_m WITH CHANGE IN pH OF Kb SOLUTION

The curve is normalized to a mean E_m of -135 mV for 36 cells in Kb at pH 7.0 (Table 5). The E_m at lower pH was determined by subtracting mean depolarizations (Table 5) from the mean E_m at pH 7.0. In Ca and K solutions the relative depolarization is about the same as in Kb, approximately 40 - 45% (Johnson, 1968).

TABLE 5

CHANGE IN MEMBRANE POTENTIAL DIFFERENCE WITH VARIATION IN pH

Number of cells	Solution sequence	pH sequence	$\bar{E}_m$ ± SEM	% depol.	Statistical significance
4	Kb " "	7.0 5.5 7.0	157 ± 16 147 ± 11 170 ± 16	6.4	---
2	Kb " "	7.0 5.0 7.0	118 ± 5 83 ± 1 122 ± 3	29.7	****
19	Kb " "	7.0 4.3 7.0	123 ± 6 59 ± 5 142 ± 9	52.1	****
8	Kb " "	7.0 9.3 7.0	144 ± 10 128 ± 6 113 ± 5	11.1	**
16	E_2 " "	7.0 5.0 7.0	175 ± 5 133 ± 8 173 ± 6	23.6	****
3	Kb (dark) " "	7.0 4.3 7.0	155 ± 9 53 ± 8 158 ± 9	65.8	****

**** significant at 0.001 level
** significant at 0.01 level

Change in E_m is 2 mV between pH 6 and 7, 33 mV between pH 5 and 6, and 59 mV between pH 4 and 5. If a passive ionic movement is considered, a 59 mV change is expected for each unit change in pH. This data may suggest that an active H^+ extrusion is at least partially functional above pH 5.0, but non-functional below pH 5.0.

<u>Potential Measurements in High Ca Solutions</u>

Due to the effect of calcium on the membrane implicated by the Slayman model, a series of experiments was carried out to determine the effect of a high external Ca^{++} concentration on the membrane and also the effect of this high concentration on the influence of K, Na, and Mg in controlling the resting potential. Compositions of the high Ca^{++} solutions ar given in Table 3. Consideration of the mean E_m for the different solutions (Table 6) immediately indicates that the potential is not affected by the presence of any of the major ionic species, with the possible exception of potassium. The rise in potential going from Kb to Ca may be considered the influence of Ca on the wall and membrane (Dainty and Hope, 1959; Spanswick Stolarek, and Williams, 1967). The failure of the membrane to return to the initial potential after the experiment may still be considered the effect of Ca^{++}. The most pronounced effects were found in the CaK solution, where the influence of the K ion is demonstrated. Although this cannot be called a K^+ equilibrium potential, the shift of the potential in that direction is definite. This result suggests three possible conclusions: (1) K^+ goes into the cell at an appreciable rate; (2) the presence of K^+ facilitates greater H^+ influx; and (3) a possible H^+ out, K^+ in exchange pump. Na and Mg did not produce

TABLE 6. VARIATION IN MEMBRANE RESTING POTENTIAL WITH VARIATION IN SOLUTION COMPOSITION, pH 7.0

SOLUTION SEQUENCE (downward)	E_m of nine different cells varying in postharvest age up to 7 days									$\bar{E}_m \pm$ SEM
	1 (0 da.)	2 (1 da.)	3 (2 da.)	4 (3 da.)	5 (4 da.)	6 (5 da.)	7 (6 da.)	8 (6 da.)	9 (7 da.)	
Kb	154	146	202	115	123	193	183	165	169	161 ± 9
Ca	156	162	167	193	150	213	206	222	177	181 ± 8
Ca-K	127	86	127	175	136	160	138	170	140	137 ± 10
Ca-Mg	144	156	181	205	140	203	200	214	181	180 ± 9
Ca-Na	154	168	178	210	155	210	198	230	174	186 ± 8
Ca	143	167	174	213	154	---	216	232	190	186 ± 11
Kb	150	202	157	218	180	188	180	212	161	183 ± 8
depol., mV Ca - Ca-K	29	76	40	18	14	53	86	52	37	44
% depol. $\frac{\text{Ca - Ca-K}}{\text{Ca}}$	19	47	24	9	9	25	32	23	21	24

this effect. Slayman has suggested that the resistance to ionic movement through the wall and membrane in high Ca^{++} solutions indicates that the bulk of the E_m is due to an active metabolic component. The implication here is that the high Ca^{++} concentration does not alter the active H^+ current. This is also suggested by our results.

Potential Measurements in Light and Dark with PO_4

Table 7 presents data from light-dark experiments. In Kb, light to dark transitions produced no permanent changes in E_m, although the cells are slightly hyperpolarized in the transition from dark to light. Mean E_m in E_2 overall is higher. For cells preconditioned in Kb and placed in E_2 for experimentation, transitions from light to dark produced a significant depolarization. However, cells preconditioned in E_2 and placed in E_2 for experimentation do not show a significant depolarization. As would be expected from the above data, transition from Kb in light to E_2 in dark also produced a significant depolarization. Preliminary experiments with another buffer, N-tris (hydroxymethyl) methyl-2-aminoethanesulphonic acid (TES) for phosphate in E_2 (see Table 3) produced very similar effects on the E_m.

TABLE 7

CHANGE IN MEMBRANE POTENTIAL DIFFERENCE WITH VARIATION IN LIGHT AND PO_4

Number of cells	Solution sequence	Light sequence	$E_m \pm$ SEM	% depol.	Statistical significance
24	Kb " "	L D L	127 ± 7 129 ± 8 136 ± 7	-----	none
40	E_2 (a) " "	L D L	163 ± 5 144 ± 6 170 ± 4	21.7%	****
27	E_2 (b) " "	L D L	173 ± 4 164 ± 7 177 ± 4	5.2%	none
20	Kb E_2 Kb	L D L	120 ± 8 101 ± 7 131 ± 8	25.8%	**

(a) cells preconditioned in Kb
(b) cells preconditioned in E_2

**** significant at 0.001 level
** significant at 0.01 level

DISCUSSION AND CONCLUSION

Through the experimants presented here, we hoped to obtain evidence to further substantiate and characterize the active H^+ extrusion mechanism proposed by Kitasato (1968) and Spear, et al (1969). Results of low pH experiments indicate that H^+ extrusion is probably non-functional below pH 5.0. This is evident from the observed 59 mV change in E_m (Figure 6) expected by the Nernst equation, as the pH changed from 5 to 4. Therefore, the discussion of these results will refer mainly to the passive circuit in Slayman's model (Figure 1) where $E_x = 0$ and E_i and R_i are variable. pH change experiments show that the apparent permeability decreases with increasing pH. If we consider that decreasing permeability mirrors increasing resistance, we may say that at low pH the relative resistance to passive H^+ movement is low and increases with increasing pH. Potential measurements with solution changes comparable to the time course of pH change, (that is, low pH to high pH bathing medium) show that as the pH is increased, the E_m becomes more negative. This would be expected if the resistance was also increasing.

At pH 4.0, according to the Nernst equation, expected E_m is +90 mV. According to Figure 5, the probable E_m is -47 mV. Solutions of pH lower than 4.3 were not used because of expected membrane damage. It is possible that some physiological factor would not permit the membrane to be depolarized any further than -40 mV except during an action potential. The discrepency between +90 mV and -47mV is interesting in light of another experimental finding. We observed in several cases that some cells exhibited

extremely steady E_m which was not affected by any of a number of experimental factors, including changes in light intensity and bathing medium composition. These cells had either a very low E_m, -50 ± 10 mV, or a very high E_m, -200 ± 10 mV. In the course of our experimentation, these cells were noted in our records, but no further investigation was made. Several investigators have suggested theories of discrete physiological states of the membrane (Oda, 1962; Findlay, et al, 1969). Oda (1962) has described a polarized or non-ionic equilibrium state in K-poor medium and a depolarized or ionic state in K-rich medium. Although our results are too fragmentary to contribute to the elaboration of this hypothesis, the indication is that triggering from one state to another is a definite possibility.

Although we have recorded large fluxes of H at low pH, the discrepancy in expected and observed E_m indicates that passive H^+ movement alone is not controlling the potential. A situation similar to that expressed in equation 2 (Briggs, et al, 1961) with terms representing H, K and Cl will more fully describe the observed E_m. Ion flux studies (Table 4) did not produce conclusive evidence, but suggest that fluxes other than H^+ are, under certain conditions, important to maintenance of the E_m. We have no way of measuring accurately permeability changes in the dying cell, but it seems that death can probably be attributed to the leakage out of the cell of essential substances. The extreme conditions we have accounted for, in Table 4, either that the dead cell retained its intracellular electrolytes or completely released them into the medium, indicates that fluxes may be considerable.

In considering the effect of pH changes on the membrane, there is a complication that deserves some mention. The alkaline and acid regions demonstrated in phenol red are differently affected by external pH and the relation will vary depending on the absolute pH. For example, we may assume the normal pH of the region immediately outside the cell is 5 for acid zones and 9 for alkaline zones. A bathing solution of pH 6.0 would have little effect on the pH 5.0 acid zone, but since the alkaline zone is 10^{-5} in OH^-, if this mixes with an equal part of pH 6.0 solution, the pH will drop from 9 to about 8.7. This is a small change and would probably leave the E_m unaffected. If the external pH were 5.0, the acid zone again would remain the same, but if the alkaline zone mixes with an equal amount of pH 5.0 solution the final pH will be 7.0. If conductance increases twofold for each pH unit decrease, the pH 7.0 alkaline zones should add about 1/4 to the H^+ influx.

The suggestion that the proposed H^+ pump is non-functional in the dark or in acid media is subject to several questions, as yet unanswered. The fact that extrusion is not observable in darkness with a significant E_m change suggests a possible movement of H^+ within the cell wall but not outside of it. Also, we cannot be certain yet about internal changes in pH in darkness which might affect H^+ movement.

Potential measurements in high Ca solutions are thought to be particularly useful in assessing the metabolic component of the E_m (Slayman, 1965; Spanswick, et al, 1967). In reference to Slayman's model, in the presence of Ca^{++}, R_i is relatively very

large as compared to E_i. The E_m is therefore primarily that due to E_x. Referring to Table 7, E_x would correspond to 183 ± 9 mV (mean of means for Ca, Ca-Mg, and Ca-Na solutions). The magnitude of this E_m suggests that it is the hyperpolarized or non-ionic equilibrium state discussed above. The effect of K^+ in reducing this potential is confusing in light of a decreased K^+ influx (2-3 times) in as little as 0.25 mM Ca^{++}(Hope, 1963). Measurements of H^+, K^+, and Cl^- fluxes were not made in high Ca solutions, but this data would be especially enlightening in analyzing the effect of the Ca-K solution.

The effects of light and dark on the E_m were predictable only to a limited extent. For the Kb solution little or no response to light was found. In terms of Slayman's model, this may be an instance where changing one factor affecting the metabolic source of E_m also affects the passive permeability of the membrane. Hope (1965) found a definite increase in resistance for light to dark transitions. This, along with the fact that a H^+ extrusion rate high enough to be detected does not occur in the dark (Spear, et al, 1969) would seem to explain the lack of response in Kb. In E_2, $PO_4^{=}$ has been added to the basic Kb medium, keeping Cl^- and cation ratios the same. When E_2 is added at the beginning of a light-dark-light cycle, a definite depolarization occurs in the dark. In an attempt to determine if this was a buffering effect, another buffer, TES, was used with very similar results. Little or no response to light and dark was recorded in E_2 preconditioned cells.

Endress and Sherwin (personal communication) found a direct

ionic relationship between chloride and phosphate influx. Phosphate influx is enhanced in proportion to the amount of chloride in solution. In E_2, phosphate and chloride are both present. In the dark, with addition of the phosphate, a statistically significant depolarization is measured. Records also indicate a series of action potentials as the depolarization is taking place. This depolarization does not occur in phosphate-free media. In light of the known involvement of chloride with the action potential, it may be that phosphate has some relation to chloride efflux also, although there is no experimental basis for this assumption.

Involvement of H^+ in the light-dark response is suggested in view of the association of H^+ and Cl^- fluxes in the acid extruding region of the *Nitella* cell (Spear, et al, 1969). It has been shown that isolated acid regions of the cell adjust the pH of the bathing medium to pH 6.2. The buffering capacity of $PO_4^=$ would require more hydrogen ion to attain this pH than in Kb, and this may increase or prolong the activity of the H^+ pump in the light. This would be another explanation for greater Cl^- influx in this medium.

Investigation of the effects of specific wavelengths of light on the E_m would provide clues to the metabolic source of the light-dark reaction and have been initiated in this laboratory. However, as of this date, results are inconclusive. Ion fluxes would also be valuable to supplement potential measurements.

Perhaps the most evident conclusion resulting from this study is that each physiological state of the cell membrane is characterized

by a unique combination of metabolic and ionic factors. The extremes seem to be characterized by a high potential, greater than -180 mV, where a metabolic component, theoretically an electrogenic H^+ sets the level of the potential, and a low potential, less than -70 mV where a combination of passive fluxes of H^+, K^+, and Cl^- control E_m. Findlay, et al, (1969) have suggested a similar scheme with four physiological states based on K^+ and Cl^- fluxes and their relationship to light. More intensive study of ion fluxes coinciding with potential measurements are necessary in future investigation of the complicated control of membrane potential.

SUMMARY

This study further characterizes the active H^+ extrusion mechanism proposed by Kitasato (1968) and Spear, et al, (1969). Results are presented in terms of the Slayman (1965) model of a membrane circuit with metabolic and ionic components. Below pH 5.0 a 59 mV change in potential per ten-fold change in $[H^+]$ is observed indicating that ion movement is passive and the metabolic component is non-functional. Apparent permeability of the membrane to H^+ seems to increase as pH increases. In high Ca solutions, resistance to ionic movement is greatly increased and E_m is essentially equivalent to the metabolic component alone. E_m in high Ca solution in this study is approximately 183 mV. Dark and PO_4 have an effect on E_m similar to an increase in $[H^+]$, that is, a depolarization is observed. Due to the reported interaction between PO_4 and Cl influxes and the spacial limitation of Cl influx to H^+ extruding regions of the cell, some connection between H^+ extrusion and Cl influx is suggested to explain the dark depolarizations. Overall, a more thorough study of ion fluxes along with potential measurements is recommended.

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