Localization of Hydrogen Ion and Chloride Ion Fluxes in *Nitella*

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ABSTRACT Alternating bands of acid and base formation have been detected along the length of the internodal cell of *Nitella clavata* when it is illuminated, while in the dark this phenomenon is minimal. Chloride influx occurs only or largely in the acid-extruding regions, and this is also a light-dependent ion movement. Chloride efflux is slightly dependent on illumination and is not localized as are H^+ efflux and Cl^- influx. The results obtained support Kitasato's (1968) proposal that a large passive H^+ influx is balanced by an active efflux of this ion. Transport mechanisms suggested by the correlations of Cl^- and HCO_3^- influxes with H^+ extrusion are discussed.

INTRODUCTION

Since the early work of Hoagland and associates (1927), it has been known that the uptake of chloride by *Nitella* is promoted by light. MacRobbie and Dainty (1958) and Gaffey and Mullins (1958) first drew attention to the relatively large difference in electrochemical potential and thus the correspondingly high energy requirement (ca. 5 kcal per mole) for net chloride transfer into characean cells. Such a condition made their proposal of a chloride pump readily defensible and also suggested the possibility of a rather direct link of chloride transport with a specific aspect of metabolism. The first clue as to the nature of such a link came from the coordinated experiments of MacRobbie (1965) and Smith (1965) which were summarized by MacRobbie (1966): chloride uptake and CO_2 fixation proceed at proportional rates, both dependent on the intensity of red light (wavelength less than 705 nm). These results are consistent with, but not limited to, a mechanism whereby a photosynthetically generated reductant can travel from the chloroplasts to the cell membrane and there provide the energy for chloride pumping via a redox reaction.

Further work by MacRobbie (1966) on *Nitella* and Raven's (1967 *b*) critical experiments on *Hydrodictyon* indicated that oxygen is necessary for chloride transport whereas CO_2 is not. This information served to characterize more

definitely the chloride-linked substance as a soluble electron carrier such as NADPH rather than a carbon compound newly synthesized in light. However, as NADPH does not pass through the chloroplast envelope readily (Robinson and Stocking, 1968; Heber and Santarius, 1965), it presumably cannot serve as the direct link to the cell membrane.

The only other evidence suggestive of a redox system capable of shuttling between chloroplast and cell membrane is that of triose phosphate and phosphoglycerate; Latzko and Gibbs (1969) suggested the extrachloroplastal oxidation of triose phosphate to account for an unexpectedly high concentration of phosphoglycerate in their isolated chloroplast preparations. This possibility appears to be worth looking into since there is good evidence for the rapid movement of phosphoglycerate into intact chloroplasts (Robinson and Stocking, 1968; Heber and Santarius, 1965; Urbach et al., 1965). The studies of MacRobbie (1966), Raven (1967 *b*), and Smith (1968 *b*) involving both the inhibitor specificity and spectral specificity of chloride transport appear to have ruled out ATP as the energy link to the transport system; at least, the evidence is against ATP as the only substance involved.

The present study on *Nitella clavata* represents a different approach to the light dependence of chloride uptake. We were aware of the deposition of $CaCO_3$ on the surface of *Nitella* cells, generally attributed to a precipitation brought about by the alkalinization of the medium by the cells (Blinks, 1951). These $CaCO_3$ encrustations often take the form of circular bands which alternate with bare areas along the lengths of the cylindrical internodal cells. The influx of chloride might be related to the excretion of OH^-, e.g., via an anion exchange pump, in which case the electrical work of chloride transport would be eliminated and the over-all energy requirement would be reduced by about one-half. After the inception of this work, Kitasato's (1968) paper appeared, presenting information which can most simply be interpreted as evidence for a very high H^+ permeability of the *Nitella* membrane and net passive H^+ influx. The alternative explanation, that the alkalinity results from net passive OH^- efflux, has not been rigorously excluded; however, this seems less probable since the anion permeability of *Nitella* is generally much lower than the cation permeability.

We then followed Kitasato in attributing the external alkalinity to a net passive H^+ influx and thus rejected the idea of a Cl^-/OH^- exchange pump. An alternative mechanism, the coupled movement of Cl^- and H^+ into the cell, would, however, behave in an identical manner. In both cases the electrical work would be obviated and Cl^- influx would be limited to the alkaline regions. To test this hypothesis it was necessary to compartmentalize the external solution in contact with one alkaline region and one bare region. It was possible to accomplish this by means of thin rubber partitions around the cells, positioned at the borders between encrusted and bare regions.

A remaining problem recognized by Kitasato (1968) was that in the steady state net H^+ influx must be balanced by H^+ extrusion. As the results below will show, the bare regions of the cell are externally acidic. We therefore refer to them as acid or acid-extruding or bare, and refer to the regions of external base accumulation as alkaline or encrusted.

MATERIALS

Internodal cells of *Nitella clavata*, averaging 4 cm long and 0.074 cm in diameter, were separated from intact plants as described previously (Barr and Broyer, 1964). The organism was cultured in open 10 gal plastic containers under illumination of about 100 ft-c, measured at the solution surface. Light was provided by equal numbers of Sylvania Gro-lux and Cool White fluorescent lamps, with 16 hr of light alternating with 8 hr of darkness.

The nutrient solution consisted of the following substances, with concentrations expressed as micromoles per liter: 4.0 NaH_2PO_4, 200 KNO_3, 200 KCl, 1000 $MgSO_4$, 3000 $CaCl_2$, 2000 $NaHCO_3$, 3.6 $FeSO_4$, 1.0 $MnCl_2$, 0.05 $CuCl_2$, 0.80 $ZnSO_4$, 0.20 $CoCl_2$, 4.5 1,2-cyclohexanediamine tetraacetate, 1.25 ethylenediaminetetraacetate, and 0.375 each of glycine, serine, aspartic acid, glutamic acid, histidine, and glycylglycine. The pH was 8.3. After completion of this work, it was found that tris(hydroxymethyl)aminomethane at 1–4 mM stimulated growth to some extent.

METHODS

The main experimental solution, Kb, contained 1.0 mM KCl and 0.1 mM each of $KHCO_3$, NaCl, $CaCl_2$, and $MgCl_2$, with the bicarbonate acting as a weak buffer at pH 6.9. After separation from the plant the internodal cells were kept in the above solution at 22°C under illumination of 2000 ergs/cm²sec (25 ft-c) from Sylvania Gro-lux lamps. These conditions were also in force during flux measurements except where otherwise indicated. For the measurement of membrane potentials the conditions were approximately the same, with light provided by the overhead Cool White ceiling lamps. The chloride concentration of vacuolar sap was determined by a coulometric method (Barr and Broyer, 1964).

Whole Cell Chloride Fluxes

Unidirectional chloride influx measurements were made with ^{36}Cl at a specific activity of about 0.6 mc/mole. Exposure of the cells to the tracer solution was usually for 24 hr or longer, after which external ^{36}Cl was removed with a three phase, 30 min rinse using nontracer Kb solution. Radioassay consisted of measurement of the radioactivity in the living cell which, for this purpose, was placed in a special holder directly beneath a Geiger-Muller tube. Appropriate corrections were made for geometry and self-absorption. In some cases small corrections for the back flux of ^{36}Cl during the uptake period were necessary.

Unidirectional Cl efflux was measured by collecting the ^{36}Cl lost by each cell during 24 hr in 5.0 ml of nontracer solution. Of this, 0.5 ml was prepared for radioassay by depositing it on a planchette, adding one drop of 10% polyvinyl alcohol (Elvanol

51-05), and bringing to dryness on a hot plate. Radioassay was made with a Nuclear-Chicago low background, gas flow beta counter.

Fluxes are expressed as picomoles (10^{-12} mole) per cm^2sec, abbreviated as pmoles/cm^2sec.

Flux Measurements on Insulated Cell Regions

For the measurement of chloride influxes and effluxes of the alkaline and acid regions of the cell surface, one alkaline region and one acid region were insulated from each other and from the other parts of the cell by means of thin rubber partitions. The partitions consisted of dental dam impregnated with petrolatum, the impregnation being done by immersing the material in melted petrolatum. Holes slightly smaller than the cross-sectional size of the cell were made by burning them into the rubber with a sewing needle, heated with a Bunsen burner. The cell was then inserted into the hole of each of three partitions (with slight stretching of the material), and the partitions were then positioned at the borders between alkaline and acid regions. The ensemble was then mounted in a Plexiglass trough with the partitions sealed to the bottom and sides of the trough with petrolatum. Checks for leaks between the compartments were made with ^{36}Cl.

Chloride influx measurements for alkaline and acid zones were made by adding ^{36}Cl solution to one of the compartments on alternate days and measuring the radioactivity of the cell while still in the trough. To ensure complete removal of external ^{36}Cl, the apparatus was repeatedly rinsed until the recorded radioactivity became constant; for the radioassay all solution was removed except for the surface film on the cell. Since the ^{36}Cl uptake was, of necessity, cumulative, accurate measurements of the daily uptake of a given cell could be made for only about 4 days. ^{36}Cl efflux measurements were made by first installing the cell in the compartmented trough, loading it with ^{36}Cl, collecting efflux solution, and measuring on planchettes as above. For flux calculations measurements of cell diameter and the lengths of the alkaline and acid regions were required. Each region was usually 0.5–0.8 mm long, representing about 15% of the cell length. Efflux was also collected from the two ends of the cell, thus permitting efflux calculations for the entire cell surface under these conditions.

Membrane Potentials

Membrane potentials were measured with Ag/AgCl glass microcapillary electrodes connected to a General Radio Co. (West Concord, Mass.) 1230A electrometer and recorder. Both the measuring electrode and the reference electrode were of the same type. In the measurement, the cell was placed in a small Plexiglass trough through which fresh solution slowly passed. Potentials required from a few minutes to 1 hr to stabilize, with the variability presumably due to the rate of sealing of cell membrane to the glass. Apparently protoplasm always enters the microcapillary tip (O.D. 5–10 μ) upon insertion and greatly impedes water movement; the net movement into the capillary predicted on the basis of the 5 atmospheres turgor pressure of the cell only occurs in capillaries with tips much larger than these.

The 100 mM KCl microcapillary salt bridge solution was so chosen as to simulate

the vacuolar sap composition in an attempt to reduce diffusion potentials which might arise at the tip. Only electrode pairs having "tip" potentials of less than 20 mv were used. The microelectrode tip was inserted well into the vacuole and thus the potential measured is not strictly that of the cell membrane, but consists of the potential across the cell wall, cell membrane, and vacuolar membrane in series; for convenience the observed values are referred to as membrane potentials. These are appropriate for use in calculating the electrochemical potential difference of chloride between the external bulk solution and the vacuole.

RESULTS

Chloride Influx, Entire Cell Surface

Fig. 1 shows that internodal cells separated from the plant and placed under the experimental conditions have a high initial chloride influx which greatly decreases during the next few days. During this time the unidirectional efflux is relatively constant, which would seem to indicate that the outward movement of chloride is relatively independent of the active transport of chloride into the cell. The vacuolar Cl^- concentration, accounting for about 90% of the cellular chloride, changes with time corresponding well with the cumulative net flux as calculated from influx and efflux values.

We made no attempt to ascertain how the magnitude of Cl^- influx is related to the factors which might influence it. Among these are the composition of the experimental solution itself (designated Kb because of the predominance of potassium among the cations and the 0.1 mM bicarbonate present); the time dependence of the influence of this solution on cell functioning (median postharvest life-span about 40 days); and the increased light absorption under the experimental conditions.

Chloride Electrochemical Gradient

The chloride electrochemical gradient between the external solution and the vacuole did not change markedly over the period of the flux measurements and thus cannot be a significant factor in the influx changes observed. The membrane potentials of newly harvested and older cells averaged −83 mv with a standard deviation of 10 mv. The alternation of light and darkness had very little effect on the membrane potential.

Localization of H^+ and Cl^- Fluxes in Nitella

Fig. 2 shows that only under illumination do acid and base accumulate near the surface of *Nitella* cells, as detected with phenol red. Following the 20 min dark pretreatment, a definite lag period occurs for acid excretion after the onset of illumination. The almost immediate appearance of base is probably attributable to two factors: (*a*) the rapid transient uptake of H^+ by chloroplasts upon illumination (Jagendorf and Hind, 1963) which would create a

steeper gradient for H^+ inward movement, and (*b*) a light-induced increase in membrane permeability (Hope, 1965). When the cells are placed in darkness, the observable excretion of acid continues for at least 5 min but subsides within 20 min. Base formation is observable even after 20 min of darkness although the rate is much reduced.

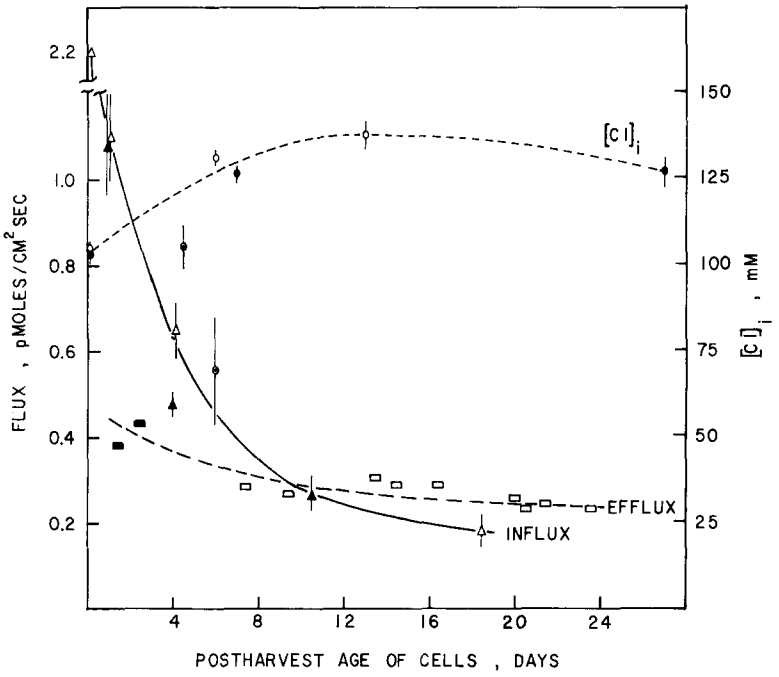

FIGURE 1. Unidirectional chloride fluxes and vacuolar chloride concentration of internodal cells of *Nitella clavata* kept in the Kb solution under constant conditions (see Methods) after harvest. Each point is the mean for 6 to 12 cells. Variation is expressed as the standard error of the mean; for effluxes the SEM was 0.02–0.04 pmole/cm² sec throughout. The different symbols represent results from different experiments.

Such behavior would be consistent with Kitasato's (1968) hypothesis that the passive influx of H^+ balances the active H^+ extrusion. Although transient changes in the membrane potential of a few millivolts are observed for light-dark transitions, the high degree of stability of the potential suggests that H^+ extrusion and H^+ permeability are equally affected by light.

Chloride influx occurs either entirely or almost entirely in the acid-extruding regions of internodal cells, as indicated in Fig. 3. The mean value of 0.26 pmole/cm² sec for the insulated acid regions of these four cells is appreciably lower than the 0.6 predicted from Fig. 1 (taking into account the age of the cells and the fact that the acid regions account for about one-half the cell surface). This difference can probably be accounted for by the lower light

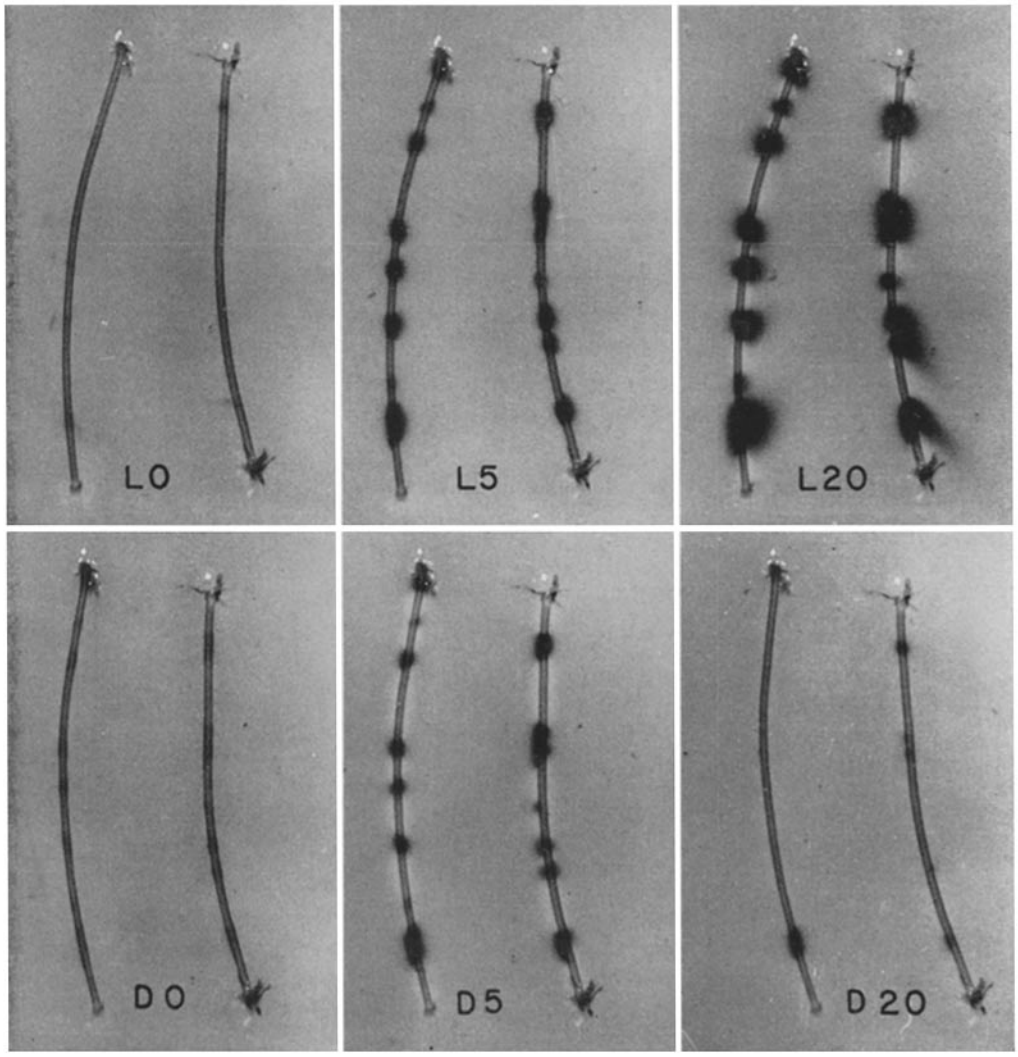

FIGURE 2. Upper sequence: accumulation of base (darkened areas) and acid (light areas) in *Nitella clavata* for zero, 5, and 20 min in light, after 20 min of dark pretreatment. Lower sequence: decreasing rates of base and acid formation in darkness immediately after the 20 min exposure to light, above. Each picture shows the amount of base and acid produced for the 5 min interval preceding the picture (base and acid were dispersed after each 5 min). For detection of base and acid 0.1 mM K salt of phenol red was used in the Kb solution, pH 6.9. Lighting was incandescent, 70 ft-c equivalent to 4400 ergs/cm^2 sec for the wavelength interval 400–700 nm. Temperature, 25°C. The internodal cells, harvested 1 day before the experiment, were recently matured cells which had not as yet developed encrustations; thus accumulated base in the form of $CaCO_3$ was not present. Cell lengths, 3.9 and 3.6 cm. Photographs taken with Kodak green filter.

intensity incident upon the cell when the insulation technique is used; for the two experimental situations, chloride influx and light intensity are proportional. The high Cl^- influxes in cells 1 or 2 days after harvest are correlated with high rates of acid and base formation in these young cells; in older cells acid and base formation are much reduced.

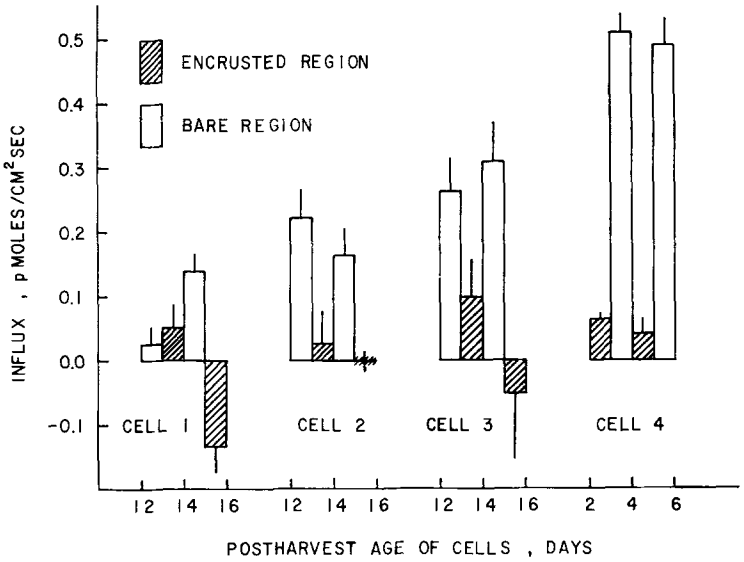

FIGURE 3. Chloride influxes for a bare (acid) and an encrusted (alkaline) region of each of four internodal cells of *Nitella clavata*, in the Kb solution for alternating 1 day flux periods. Influx was calculated from the increment of ^{36}Cl taken up during the period, with back flux being negligible. The length of the vertical line appended to each value is the estimated standard deviation attributable to randomness of the radioactive decay process; since the standard deviation is that of a difference, and the total number of counts is small, the relative error is rather large. The error (on the high side) for the 3rd day of cell 1 appears to be considerable; this is reflected in a large apparent negative influx for the 4th day. The light intensity was 900 ± 100 ergs/cm² sec as compared to the usual 2000 (Figs. 1 and 5). The mean influx values were 0.26 ± 0.06 pmole/cm² sec for the acid regions and 0.01 ± 0.2 for the alkaline (eight determinations each, variation expressed as SEM).

Fig. 4 shows that Cl^- efflux is about three times higher for the insulated acid regions as compared to the alkaline (encrusted). To what extent the $CaCO_3$ deposits within the interstices of the cell wall interfere with the outward movement of chloride cannot be evaluated. This result is important, however, in that it demonstrates that some chloride movement in this region does occur and thus the absence of Cl^- influx here cannot be attributed to a complete impregnation of the cell wall with $CaCO_3$.

The mean efflux value of 0.35 pmole/cm² sec for the entire surface of these

four cells is in reasonable agreement with the value of 0.24 obtained by averaging the acid and alkaline zones, and with the 0.28 of the six control cells (without partitions). The lower light intensity for partitioned cells should have only a slightly depressing effect on chloride efflux since the effect of total darkness is not great (see Fig. 5).

Measurable pH changes of the solution in the insulated compartments

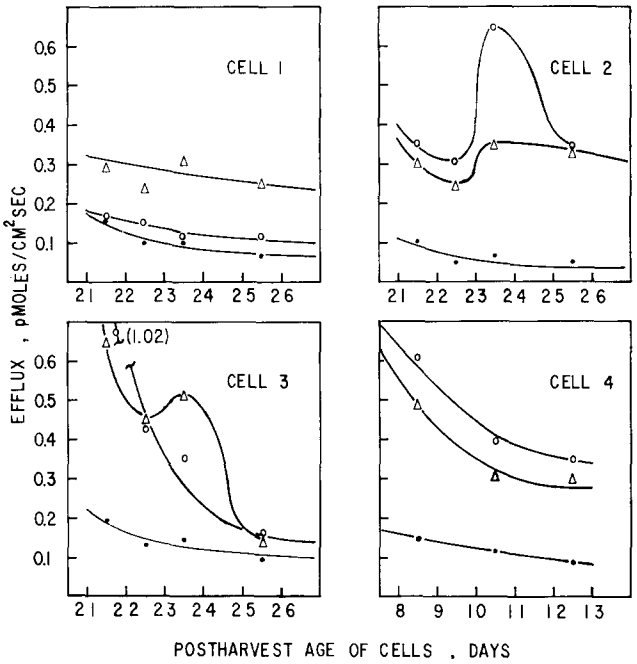

FIGURE 4. Chloride effluxes for an acid region (open circles), an alkaline region (dots), and the entire cell surface (triangles) for each of four cells of *Nitella clavata* in Kb solution. In most cases the efflux period was 1 day. The light intensity was 900 ± 100 ergs/cm² sec as compared to the usual 2000 (Figs. 1 and 5). The mean efflux values were 0.37 ± 0.03 pmole/cm² sec for the acid regions, 0.11 ± 0.02 for the alkaline regions, and 0.35 ± 0.03 for entire cell surfaces (15 determinations each, variation expressed as SEM). Six control cells (without partitions, in glass containers) had a mean efflux of 0.28 ± 0.04.

occurred during the course of the 1 day flux periods. Our preliminary results indicate that the initial pH of 6.9 changed to about 6.2 in the acid compartments and to 8.0 in alkaline compartments. These changes took place within a few hours after which the pH remained steady.

Although no flux measurements were conducted under conditions of controlled pH, the results for partitioned cells can be compared with entire cell fluxes inasmuch as a pH gradient develops outside the cell surface, without partitions, as demonstrated in Fig. 2. The validity of a direct comparison

depends upon the absence of convection during the experimental period, a factor which is difficult to evaluate. Some information on the influence of the placing of partitions around the cell, with the attendant changes of pH, on the membrane potential and the magnitude of the chloride influx is presented below under Influence of pH, etc.

Magnitude of H^+ Extrusion

From Fig. 2 (20 min light) it is possible to make an estimate of the H^+ extrusion rate. It is reasonably certain that the rate is at least 5 pmoles/cm² sec,

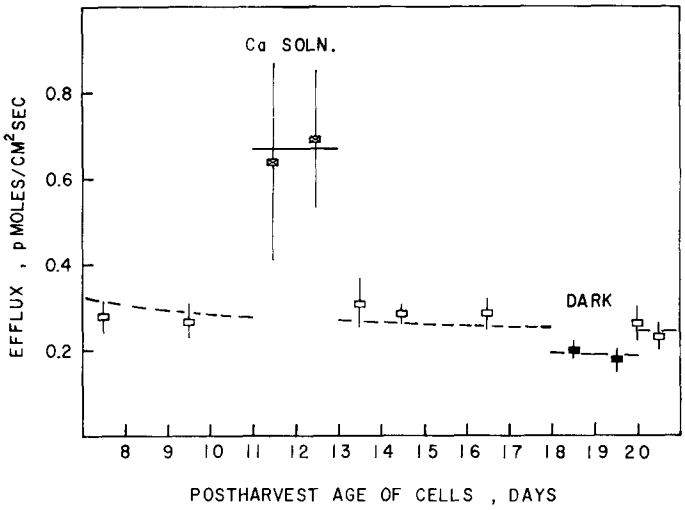

FIGURE 5. Unidirectional chloride effluxes of internodal cells of *Nitella clavata* kept in the Kb solution under constant conditions (see Methods) after harvest, except where indicated. For cells in the Ca solution (4.0 mM $CaSO_4$, 1.0 mM $MgCl_2$; pH about 6.0) the mean membrane potential was −180 mv as compared to −83 for the Kb solution.

and it is possibly higher than 20 pmoles/cm² sec (based on entire cell surface). For this calculation the following information was used: (*a*) volume of the acidic (light) regions, (*b*) knowledge that the pH of these regions is at least as low as 6.4, (*c*) the buffering capacity of the HCO_3^- initially present in these regions, (*d*) the diffusion of HCO_3^- from the bulk solution into the acid regions assuming a diffusion coefficient of 10^{-5} cm²/sec, and (*e*) the acid regions constitute very close to 50% of the cell surface. Not included in the calculation of the minimum value above was an estimate of H^+ lost to the neighboring alkaline regions.

Of possible relevance here is the oxygen consumption rate of *Nitella* since the idea of a redox-generated H^+ secretion (Conway and Brady, 1948) now has some experimental support (Mitchell, 1966). According to the simplest

redox mechanism, four H^+ are produced for each O_2 molecule consumed. Some years ago we made measurements on 100 internodal cells of *Nitella clavata* in a large Warburg vessel in darkness at 27°C. The initial O_2 uptake was 10.5 pmoles/cm² sec; this decreased exponentially during the next 6 days with a half-time of 76 hr (solution renewed periodically). Since H^+ extrusion in *Nitella* occurs only in light, its oxygen dependence would have to be related to an increment of O_2 uptake associated with photometabolism. The O_2 consumption rate in darkness is of the appropriate magnitude to allow for this possibility; for example, an additional O_2 uptake of 2.5 pmoles/cm² sec in light would correspond to a H^+ extrusion rate of 10 pmoles/cm² sec according to the above mechanism. Experiments with N_2 (free of CO_2 and O_2) are needed to determine whether H^+ secretion is oxygen-dependent.

Influence of pH on Membrane Potential and Chloride Influx

Although Kitasato (1968) showed that the membrane potential of *Nitella clavata* can be depolarized by lowering the pH of the ambient solution to below 5.0, the localization of the net H^+ influx causing the depolarization has not been investigated. In work of a preliminary nature we used the insulation technique and alternately applied a slowly flowing solution of pH 4.0 to an acid zone and an alkaline zone. Acidification of an acid zone caused a depolarization of 15 ± 5 mv as compared to 19 ± 4 for an alkaline zone (10 and 7 determinations, respectively, on the same 4 cells). These results were, however, rather unsatisfactory because of the unaccountably high membrane potentials, averaging −130 mv, which is 40–50 mv more negative than those of cells without partitions. Although the 0.1 mM $KHCO_3$ was left out of both control solution (pH 7.1) and the depolarizing solution (pH 4.0) so that the release of CO_2 would not be a factor here, it does not appear that this is related to the high potential.

A partial explanation for this puzzling behavior was obtained through an experiment with nonpartitioned cells in Kb solution. When cells several days old were subjected to a pH change sequence of 7.0 to 5.9 to 7.0, a substantial hyperpolarization occurred in three of four cells; the mean potential rose sharply from −103 to −149 mv upon return to pH 7.0. All cells were initially depolarized about 4 mv by the pH 5.9 treatment, but only cells having a post-harvest age of more than 4 days responded in the above manner. There was no effect of a pH change sequence of 7.0 to 8.1 to 7.0, either temporary or permanent. Presumably the hyperpolarization, which lasts at least 24 hr, results from a decrease in H^+ permeability, with H^+ extrusion being unaffected.

The simplest conclusion from the equal depolarizations of acid and alkaline zones by pH 4.0 solution is that both zones are equally permeable to H^+, at least for the hyperpolarized state. Further work is needed to clarify

the nature of the hyperpolarized state and its relation to the permeability characteristics of the membrane in its normal state.

It is not certain whether the partitioned cells in the ^{36}Cl flux localization study were hyperpolarized, since the alkaline regions were never exposed to a solution of pH lower than 7.0. If present, the hyperpolarization would increase the energy requirement for Cl^- transport from about 5 kcal/mole to about 6. How this would affect the transport rate is yet to be ascertained. The limited evidence available (Fig. 3) suggests that Cl^- influx for partitioned cells did not depart significantly from the value predicted on the basis of entire cell influxes. The possibility that the pH outside a given region of a cell would be the predominant factor in the localization of chloride influx is taken up under Discussion and Conclusions.

Another aspect of the complexity introduced by the partitions is how the local circuit, carried mainly by H^+, might be affected. Although we were unable to detect any significant potential differences between the external compartments, there is little doubt that the distribution of the local current would be affected.

The low Cl^- influxes observed for old, nonpartitioned cells (Fig. 1) are not attributable to the hyperpolarized state, as this was not detected in any of the cells under these conditions.

Chloride Efflux, Entire Cell Surface

Fig. 5 includes information which has been excerpted from the same set of experiments as presented in Fig. 1. The magnitude of the efflux observed for the Ca solution is, within the experimental error, proportional to the membrane potential. The high membrane potential (-180 mv) for the Ca solution would suggest that the passive permeability of the membrane is much reduced, but this general condition does not apply to the chloride ion. Presumably the effect is mainly on the H^+ permeability. Cl^- influx for the Ca solution (pH about 6.0) averaged about 0.6 pmole/cm^2 sec for the older cells. Although this mean value is somewhat higher than the Cl^- influx for cells of comparable age in the Kb solution, the variability was very large. It appears that with regard to Cl^- influx some factor other than the magnitude of resting potential was predominant under these conditions.

The modest decrease in Cl^- efflux brought about by darkness is in sharp contrast with the results of Hope et al. (1966) who found that darkness caused a marked increase in efflux in *Chara australis* under somewhat different conditions. In this characteristic *Nitella clavata* is quite similar to *Hydrodictyon africanum* (Raven, 1967 *a*).

DISCUSSION AND CONCLUSION

H^+ Extrusion

The simplest conclusion that can be drawn from the present study is that H^+ extrusion in *Nitella* is localized in discrete bands along the cell length and that passive H^+ influx occurs uniformly over the entire cell surface. These findings offer detailed support for Kitasato's (1968) hypothesis that the appreciable passive H^+ influx in *Nitella* must be balanced by H^+ extrusion. It is more difficult to defend the alternative conclusion that OH^- is pumped inwardly. The high electrical conductance of the characean cell membrane is also consistent with a high H^+ permeability (Kitasato, 1968; cf. Williams et al., 1964).

One can calculate that the minimum energy requirement for H^+ extrusion into a medium of pH 7.0 is only 0.25 kcal/mole if an internal pH of 5.5 (Hirakawa and Yoshimura, 1964) and a membrane potential of -100 mv are assumed. The low energy requirement makes more acceptable the idea of a rapid cycling of H^+ across the membrane, but the role of this process in membrane functioning needs clarification. Although visibly detectable H^+ extrusion requires light, the membrane potential was found to be independent of light under the conditions of this work. Under other conditions, however, the membrane potential of characean cells is known to be influenced by light (see Nishizaki, 1968): Here Nishizaki discusses some of the aspects of what appears to be a very complex relationship between light and the level of the resting potential. The light-induced increase in membrane conductance in *Chara* when bicarbonate is present points to one factor which must be considered (Hope, 1965).

It is perhaps premature at this time to conclude that H^+ extrusion is electrogenic under all conditions. Worth looking into are the possibility of a K^+/H^+ exchange pump and the possibility that a permeability barrier may be involved in the over-all process of H^+ extrusion.

Relation of Cl^- Influx to H^+ Extrusion

The spatial and light dependency correlations between Cl^- influx and H^+ extrusion suggest a possible mechanistic link between the two. This topic, however, cannot be discussed without bringing into the picture the quite distinct relationship between Cl^- influx and HCO_3^- influx as found by Raven (1968) and Smith (1965, 1968 *a*). In *Hydrodictyon* (and presumably *Nitella*) Cl^- and HCO_3^- influxes have the same light requirement, i.e. both are promoted by wavelengths below 705 nm, being influenced to a lesser degree by longer wavelengths (Raven, 1968). In both *Nitella* and *Hydrodictyon* HCO_3^-

inhibits Cl^- influx (Smith, 1965, 1968 *a*; Raven, 1968). Since the presence of CO_2 has no effect on Cl^- influx (Raven, 1967 *a*, *b*), it may be concluded that the inhibition is due to bicarbonate as an anion rather than as a carbon source. The inhibition of Cl^- influx by HCO_3^- may then be interpreted in a general way in terms of competition for an anion transport mechanism.

For *Nitella* it must be true that in the acidic regions outside the cell surface the bicarbonate is converted to H_2CO_3, which then can enter the cell much more readily as an uncharged entity. This is the simplest explanation for the light-dependent transport of HCO_3^- found by Raven (1968) for *Hydrodictyon*. Although we are not aware of specific evidence for H^+ extrusion in this organism, the many similarities between *Hydrodictyon* and characean species in their transport characteristics suggest this extrapolation. It is, of course, possible that both this phenomenon and a more specific type of HCO_3^- transport mechanism are operative. At this time the simplest explanation for the inhibition of Cl^- influx by HCO_3^- is that it removes H^+ which is somehow necessary for Cl^- entry.

Certain critical experiments need to be done to ascertain whether the localization of chloride influx is related only to the pH at the surface of the cell or is more closely coupled to the H^+-generating mechanism. It should be noted that under natural conditions, the alkaline regions are probably never exposed to an acidic environment, and chloride entry would therefore be limited to the bare regions, as in the present study. Nevertheless, flux localization studies with controlled pH are required to ascertain whether chloride-transport capability is an intrinsic property of the bare regions or a general property of the membrane as a whole, with a very sensitive dependence on pH.

Although, like Cl^- and HCO_3^- influxes, H^+ extrusion also has a light requirement, the wavelength dependence is not known. Since the wavelength dependence of Cl^- influx and HCO_3^- influx is consistent with the photosynthetic formation of a reductant, and since some general evidence is available that H^+ secretion can be effected by redox mechanisms (Mitchell, 1966), one suspects that the spectral requirements may be the same for all three transport processes.

Possible Mechanisms for H^+-Dependent Anion Transport

It is of interest to briefly discuss mechanisms by which H^+ extrusion may implement Cl^- transport, or, perhaps in general, anion transport. It seems probable that the site of H^+ generation and initial accumulation is within the cell membrane itself. Surface organelles of the type found in *Nitella* and *Chara* by Crawley (1965) and Barton (1965) are perhaps involved; unfortunately the location of these organelles in relation to the acid and alkaline regions was not specified in these studies. If the site of H^+ formation is at the

very surface of the membrane, the types of transport mechanisms possible are more limited. Of the three mechanisms listed below only (3) would require that H^+ be produced within an intramembrane space.

Possible Mechanisms

1. A carrier mechanism in which a relatively high $[H^+]$ converts the carrier to its chloride-bearing form.
2. H^+ extrusion is incidental to Cl^- transport, the latter being mediated by a Lundegardh-type (1954) electron-anion exchange mechanism. Since H^+ extrusion and the equivalent electron flow are much greater than Cl influx, a one-for-one exchange would not be possible. A serious weakness here is that the alkalinity produced by the reduction of oxygen by the electrons at the membrane surface would remove most of the H^+ produced.
3. A selective movement of Cl^- (or HCO_3^-) into the intramembrane region of H^+ formation, followed by a partitioning of molecular HCl into the lipids of the membrane and diffusion inwardly. A necessary condition is that the outer portion of the cell membrane serve as a barrier to HCl movement outwardly; the asymmetric physiological properties of the *Nitella* membrane (Tazawa and Kishimoto, 1964; Sinyukhin and Ozolina, 1965; Konoshenko and Vorob'yev, 1965) suggest that a structural asymmetry may also exist. Specific channels for H^+ and Cl^- ionic movements between the intramembrane space and the external solution are also required; most of the H^+ would escape to the outside via the H^+ channel.

   This mechanism would effect net transfer of Cl^- only if the intramembrane $[H^+]$ $[Cl^-]$ product is higher than that of the vacuole. A pH of 3 and a $[Cl^-]$ of 1 mM apparently satisfy this condition: the concentration product of 10^{-6} M^2 is slightly higher than that of the vacuole, this being about $10^{-6.5}$ M^2 based on 100 mM Cl^- and a pH of 5.5 (Hirakawa and Yoshimura, 1964).

   That HCl would partition fast enough into the membrane lipids has some basis in that the mole fraction solubility of HCl in a variety of hydrophobic organic solvents is about 0.03 as compared to 0.27 for H_2O, at 1 atmosphere HCl (Linke, 1958). This solubility corresponds to approximately 2% of that of water on a volume basis. The standard free energy of solution of gaseous (molecular) HCl is only −8.58 kcal/mole (Rossini et al., 1952). One might conceive of HCl migrating through the membrane lipids in a way comparable to that of O_2 or CO_2. Work on artificial membranes now indicates that H_2O also migrates through the lipid portion of membranes by dissolving rather than by movement through aqueous pores (reviewed by Rothfield and Finkelstein, 1968);

this provides a firmer basis for the idea of membrane permeability of this type.

One point of serious inadequacy for all three mechanisms is that the partial dependence of K^+ and Na^+ influxes on the presence of Cl^- in the medium suggests a link between the two (Smith, 1967, 1968 *b*). This might possibly be related to a H^+/cation exchange pump or perhaps to some influence of chloride on the cation permeability of the cell membrane (cf. Gaffey and Mullins, 1958). Flux localization studies with cations would help clarify this point.

Chloride Efflux

The evidence presented that Cl^- efflux in the acid region is three times that of the alkaline region may, at least in part, be attributed to the retardation of movement by the $CaCO_3$ encrustations, as mentioned under Results. Another possible explanation is that of exchange diffusion in the acid region. Consistent with the latter is the observation of Hope et al. (1966) that Cl^- efflux in *Chara* sharply decreases when external chloride is replaced with sulfate or benzenesulfonate. It can be seen that exchange diffusion would be an intrinsic characteristic of mechanism (3) above.

The simplest explanation for the doubling of Cl^- efflux with the Ca solution is that the efflux is purely passive, and the Goldman (1943) relation is applicable, i.e., for ionic movement down an electrical gradient the rate is very close to proportional to the potential, providing the latter is greater than 60 mv. If some type of exchange diffusion is present, however, the relationship becomes more complex. If, for example, half the Cl^- efflux for the Kb solution is due to exchange diffusion, a 70% increase in Cl^- permeability would have to occur to account for the magnitude of Cl^- efflux for the Ca solution.

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