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Intracellular Perfusion and Cell Centrifugation Studies on Plasmalemma Transport Processes in *Chara corallina*

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Summary. The OH^- transport system of *Chara corallina* was studied using the techniques of intracellular perfusion and cell centrifugation. Application of silk ligatures to internodal cells had quite a perturbative effect on the OH^- transport activity. Approximately 12 hr were required before normal pH profiles were reestablished.

Tonoplast-free internodal cells developed rather weak, uniform alkalinity along the cell surface. Redevelopment of control pH patterns was never observed in these experiments. Surface pH profiles similar to those observed on tonoplast-free cells could also be obtained by subjecting cells to mild centrifugation ($180 \times g$). Organelles of the streaming cytoplasm were contained within the centrifugal cell segment by applying a ligature near the cell center. Normal pH profiles were observed along the centrifugal segment, while the centripetal segment developed weak, rather uniform alkaline profiles. Upon redistribution of the cytoplasmic organelles, normal pH profiles were established along the entire cell length.

These results indicate that an organelle within the streaming cytoplasmic phase is responsible for the spatial location and control over OH^- transport. This explains the absence of control pH profiles in tonoplast-free cells, since during the disintegration of the tonoplast, most of the streaming cytoplasm coagulates at one end of the cell.

Parallel pH mapping and electrophysiological studies indicated that the plasmalemma of this species contains an ATP-dependent electrogenic H^+ transport system. Also, experiments conducted in the presence and absence of cellular ATP demonstrated that OH^- efflux can be driven passively by the membrane potential. Whether OH^- transport is strictly a passive process in normal cells remains to be resolved.

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Perfusion of the plant vacuole with an artificial sap was first attempted by Blinks (1935); he used the marine alga *Halicystis*. The giant internodal cells of the Characeae were also amenable to this type of experimental manipulation. Kamiya and Kuroda (1957) and later Tazawa (1964) developed the technique for perfusing the vacuole of Characean cells. Using this procedure Tazawa and his collaborators were able to examine the influence of vacuolar ionic composition adjustment (Nakagawa, Kataoka and Tazawa, 1974; Tazawa, 1975), the resting membrane potential, and membrane excitability (Tazawa & Kishimoto, 1964).

The tonoplast in these perfused cells remained intact, and as such it imposed a barrier to the movement of certain chemical agents between the artificial vacuole and the cytoplasm. This limited the usefulness of the vacuolar perfusion technique. Tazawa, Kikuyama and Shimmen (1976) overcame this problem by incorporating 5 mM EGTA into the perfusion medium; the presence of this Ca^{2+}-chelator caused the disruption of the tonoplast. Williamson (1975) reported that the tonoplast could also be removed by rapid perfusion using a medium containing 50 mM EGTA.

Extensive use is now being made of this tonoplast-free *Chara* cell system. Thus far the technique has been used to demonstrate the ATP and Mg^{2+} dependency of cyclosis (Williamson, 1975; Tazawa et al. 1976; Shimmen, 1978). Progress has also been made towards understanding the physiological requirements necessary for normal maintenance of membrane potential. Shimmen and Tazawa (1977) demonstrated that approximately one half the resting membrane potential is supported by Mg-ATP. A similar dependence of membrane excitability has also been established (Shimmen, Kikuyama & Tazawa, 1976). Recent progress using this technique has been reviewed by Tazawa and Shimmen (1980*a*, *b*).

In some situations, results have been obtained on

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perfused cells that are atypical of those found using normal intact cells. Cessation of cytoplasmic streaming, which occurs in normal Characean cells upon membrane excitation (Tazawa & Kishimoto, 1968), is not observed in tonoplast-free cells (Tazawa et al., 1976). In his investigation on the effect of internal Cl^- concentration on Cl^- influx into *Chara corallina*, Sanders (1980*a*) cautioned that the membrane potential of his perfused cells was low (−95 to −110 mV). Of equal importance was the pH insensitivity of the membrane potential in these perfused cells. Walker (1980) also presented current-voltage curves, obtained using perfused *Chara* cells, which indicated that the addition of ATP caused a hyperpolarizing current, but did not generally restore the original membrane conductance. These results suggest that the perfusion procedure may perturb certain aspects of membrane function. Since the number of laboratories employing this technique is increasing, it seemed essential to determine whether or not the procedure was seriously perturbative.

The plasmalemma-bound HCO_3^- and OH^- (alkaline band) transport systems of *C. corallina* have been extensively characterized (Lucas & Dainty, 1977*a*, Lucas, 1980). The purpose of the present study was, therefore, to determine whether tonoplast-free perfused *Chara* cells could develop normal alkaline bands, under conditions of HCO_3^- assimilation.

Materials and Methods

Culture Material

Culture material of *Chara corallina* Klein ex Willd., em. R.D.W. (=*C.australis* R.Br.) was grown in the laboratory in 120-liter containers in solutions containing (mM): 3.0 Na^+, 0.2 K^+, 0.2 Ca^{2+}, 1.0 Cl^-, and approximately 2.0 HCO_3^- (pH 9.0). Artificial fluorescent lighting was employed; the intensity at the solution surface was 17–20 W m^{-2}. *Chara* internodal cells were cut from the culture at least 24 hr prior to being used in experiments. During this pretreatment period cells were bathed in a modified pond water (CPW/B) containing (mM) 1.0 NaCl, 0.2 KCl, 0.2 $CaSO_4$, and 1.0 $NaHCO_3$ (pH 8.0).

Experimental Solutions

All experimental solutions were prepared from Analar Reagent grade chemicals using glass distilled water (Corning Mega-Pure automatic still, model 3513). Composition and pH (pH_i) of the various perfusion media (PM) is detailed in Table 1. Buffers used in these media were prepared and stored as 0.5-M stock solutions. Stock solutions of glucose (100 mM), sorbitol (1 M), EGTA (100 mM), $MgCl_2$ (100 mM) and K_2SO_4 (100 mM) were also stored under refrigeration. ATP (purchased as the dipotassium salt from Sigma, St. Louis, Mo.) was prepared as a stock solution immediately before daily PM preparation. Hexokinase (Sigma, Type III) was preweighed to give 1 mg/ml of PM, and was added to the PM immediately prior to cell perfusion. Ficoll (Sigma, Type 70) solution (approx. 30% wt/vol) was dialyzed (benzolated tubing) at 4 °C for two days against glass distilled water (changed daily). An accurate stock solution (20% wt/vol) was then prepared and stored at 4 °C.

Bathing solutions used for electrophysiology experiments contained CPW/B and were buffered (2 mM) at pH 7.0 with NaOH-HEPES, pH 8.0 and 8.25 with NaOH-Tricine, and pH 8.5 and 9.0 with NaOH-TAPS.

Intracellular Perfusion

The perfusion technique employed was similar to that of Tazawa, Kikuyama and Shimmen (1976). For the present experiments long internodal cells were employed (8 to 12 cm in length and approximately 0.7 mm in diameter). Surface water was blotted from the cell wall before it was placed on a Plexiglas block. One of the nodal complexes was then immediately irrigated with a small

Table 1. Perfusion media composition

Component	pH of ATP/ATP-free (H.K.) media											
	7.0(ATP)	7.0(HK)	7.5(ATP)	7.5(HK)	8.0(ATP)	8.0(HK)	8.25(ATP)	8.25(HK)	8.5(ATP)	8.5(HK)	9.0(ATP)	9.0(HK)
Buffer (100 mM)	HEPES	HEPES	HEPES	HEPES	Tricine	Tricine	Tricine	Tricine	TAPS	TAPS	TAPS	TAPS
ATP (mM)	2	–	2	–	2	–	2	–	2	–	2	–
Glucose (mM)	–	5	–	5	–	5	–	5	–	5	–	5
Hexokinase(mg/ml)	–	1	–	1	–	1	–	1	–	1	–	1
K^+ (mM)	45	41	78	73	75	71	100	88	89	79	105	101
$MgCl_2$ (mM)	6	6	6	6	6	6	6	6	6	6	6	6
EGTA (mM)	5	5	5	5	5	5	5	5	5	5	5	5
Sorbitol (mM)	60	60	50	35	20	20	10	30	35	35	10	0
Ficoll-70 (wt/vol%)	5	5	5	5	5	5	5	5	5	5	5	5

Sorbitol was used to adjust perfusion media osmotic pressure to 545 kPa (220 mOsM)

amount of perfusion medium. Loss of water vapor from the internodal cell wall eventually reduced the turgor to zero. During this drying process six silk threads (Kanagawa Co. Ltd., Kanagawa, Japan, Type: Orizuru 9 and 50) were positioned at the required places along the cell surface and loosely tied. It was essential to complete this operation before the cell became flaccid.

Upon wilting, the PM-bathed nodal complex was removed by carefully cutting the internodal cell wall with a pair of fine-pointed scissors. The opposite nodal complex was then irrigated and removed in the same way. A reservoir (approx. 0.5 ml) of PM was then placed over this open cell end prior to raising the end of the Plexiglas block. (A gradient of 1 in 40 mm was generally applied). An absorbent paper "wick" was used to draw PM through the cell until the vacuolar volume had been replaced at least three times. This step took between 30 to 60 sec, depending on the cell length. Post-perfusion cell ligation was performed in the following manner. An initial set of ligatures was placed approximately 1 cm in from each cut end of the cell. The cell was then irrigated with a CPW/B solution containing 180 mM sorbitol. A second set of ligatures was then applied to the turgid section of the cell. After a period of 2 min, a single ligature was applied at one end of the cell, and, after a further 2 min, the final ligature was applied at the opposite end. The cell wall was then cut between the second and third sets of ligatures using fine-pointed scissors. The spacing between the three sets of ligatures was adjusted such that the final perfused cell segment was approximately 2 cm in length, for cells used in pH mapping experiments, and 3 cm in length for electrophysiological studies. Full turgor was restored by irrigating the ligated cell segment with CPW/B.

Cyclosis and the ligatured ends of the cell were examined using a binocular microscope (100 to 400× magnification). The areas of ligation were checked both for leakage and symmetry. Cells with nonsymmetrically ligatured walls were discarded, as these had a much greater tendency to either leak or rupture during the course of an experiment. (It is perhaps worth mentioning that the greatest frequency of symmetrical ligatures were obtained by applying an half-reef knot. If applied with sufficient tension, this silk knot does not loosen.) Inclusion of 5 mM EGTA in the PM (*see* Table 1 for composition of various perfusion media employed) caused the destruction of the tonoplast (Tazawa et al., 1976). Establishment of the tonoplast-free state was easily identified under the microscope. A normal cyclosis pattern was always observed until the tonoplast disintegrated, then, upon its disruption it collected, along with other cytoplasmic organelles, near one end of the perfused cell. In general, the tonoplast was destroyed within 5 min of PM entry into the vacuole. When ATP was included in the PM, cyclosis continued at the control rate after EGTA-induced membrane coagulation; however, only small "granular" particles remained in the streaming phase. Depletion of endogenous ATP by perfusion with glucose and hexokinase (Shimmen & Tazawa, 1977; Kikuyama, et al., 1979) resulted in complete stoppage of streaming within 5 to 10 min of perfusion.

At the end of each experiment cells were re-examined for chloroplast appearance and cyclosis. At this stage a general check was also made on cell turgor. This was done by observing the cell's response to a bending force. Cells that deformed under this force had lost the ability to maintain turgor. Results obtained on cells in which either streaming, or normal turgidity was not observed were discarded.

Cell Centrifugation

Distribution of streaming cytoplasm was manipulated using a bench-top centrifuge (International Clinical Centrifuge, model CL). A silk thread was attached to one end of the experimental cell and this thread was used to suspend the cell in a test tube containing CPW/B. An ice bath was used to lower the temperature of the centrifugation medium. The reduced temperature slowed the rate at which cytoplasmic streaming redistributed the centrifuged cell contents. After four minutes of centrifugation ($180 \times g$) cells were transferred to the Plexiglas perfusion block and irrigated with CPW/B containing 180 mM sorbitol (4 °C). A single ligature was then applied to the cell center. For these experiments, ligatures were tied rather weakly so that the plasmalemma on opposite sides of the cell did not fuse. Cyclosis was then observed in CPW/B. In successful experiments, the centrifuged half of the cell contained the larger organelles of the streaming cytoplasm, while the streaming phase of the centripetal segment contained only small "granules".

pH Measurement Along the Chara Cell Wall

The pH profile along the cell surface of *C. corallina* internodal cells was measured using the technique developed by Lucas and Smith (1973). Agar blocks, that we used as embedding media for experimental cells, were prepared in the manner described by Lucas and Dainty (1977*b*). Perfused cells were found to be much more sensitive to mechanical manipulation during insertion into the agar blocks. To overcome this problem, we cut wider channnels (3–4 mm) in the agar block, and, in addition, a new procedure was used to position the cell in the diffusion channel. This involved the following sequence: (i) the diffusion channel was filled with CPW/B, (ii) the perfused cell was floated on top of the block, (iii) one end of the block was then raised to allow the CPW/B to drain; this pulled the cell down into the diffusion channel, and (iv) the agar block was then positioned into the experimental chamber. All experiments were performed in CPW/B media at 25 °C under a light intensity of 20 W m^{-2}.

The pH measurements were made using a miniature pH electrode (Microelectrodes, Londondery, N.H., model MI-405) in conjunction with a Beckman model 4500 digital pH meter. A micromanipulator (Leitz) was used to position the electrode on the cell wall. In experiments using perfused cells, the electrode was positioned approximately 5 μm away from the cell wall to prevent possible mechanical disturbance. From the values obtained the pH pattern established at the cell surface was mapped. Details relating to individual scanning experiments will be specified in the text.

Electrophysiological Measurements

Membrane potential and resistance measurements were made in a similar manner to that reported by Lucas, Spanswick and Dainty (1978). The potential between the vacuole and the bathing solution (or across the plasmalemma in tonoplast-free cells) was measured (WPI model 750 amplifiers; WPI Instruments, New Haven, Conn.) using an inserted microelectrode (1 μm tip diameter) and an external reference electrode (10–20 μm tip diameter). Microelectrodes were filled with 3 M KCl, adjusted to pH 2 to reduce tip potentials (Okada & Inouye, 1975). Membrane resistance was calculated from the deviation of the membrane potential that occurred when a square current pulse (0.5 to 1.0 μA, 1 sec duration) was injected into the cell (*see* Lucas et al., 1978, for full details).

Results

Influence of Ligation and Perfusion on OH^- Band Pattern

Potentially, any one of the steps or conditions necessary to obtain a tonoplast-free perfused cell could

perturb the OH^- (and/or HCO_3^-) transport system. The first aspect to be examined was the procedure of cell ligation. Experimental cells were illuminated (10 W m^{-2}) in CPW/B containing phenol red (acid/base indicator: 5 mg/liter) for 30 min to ensure that normal HCO_3^- assimilation was occurring, as evidenced by the presence of strong alkaline bands. Cells were then dried down and ligated in the manner detailed in the Materials and Methods, except that the nodal complexes were not removed and the vacuole was not replaced. Ligated cells were either returned to CPW/B (plus phenol red) or positioned in agar blocks for pH mapping. The alkaline banding pattern response to this treatment is typified by the results presented in Fig. 1. Immediately after ligation (30–60 min) only very weak OH^- efflux activity was observed. Transport activity then increased gradually with time, and within 10 to 14 hr normal pH banding patterns were recorded. Complete agreement was found between the pH mapping data and the visual observations made on cells placed in phenol red solutions. Once recovered, these cells exhibited stable alkaline bands for weeks.

Hydroxyl transport perturbation occurred whether or not the nodal complexes were cut from the ligated cell (data not shown). This indicated that the perturbation was not caused by injury resulting from cutting, *per se*. Recent studies (J.M. Alexander and W.J. Lucas, *unpublished results*) have also shown that the procedure adopted to reduce and then return cell turgor does not significantly affect the OH^- transport system.

Experiments on cells in which the vacuolar sap had been replaced by a simple PM (110 mM KCl and 5 mM $CaCl_2$) gave OH^- transport perturbation and recovery profiles identical to those observed for ligation-only experiments (data not shown). However, long term pH scan experiments, conducted on cells in which the tonoplast had been destroyed, gave quite different results. In the initial period immediately after perfusion and ligation, the entire cell surface became alkalinized, exhibiting rather uniform pH profiles (Fig. 2, 30–60 min scan). Generally, these pH profiles varied little with time during the first 3 to 4 hr after perfusion. With prolonged periods (4 to 6 hr after perfusion) the profiles often became irregular, with some regions on the cell surface becoming slightly more acidic than the background (*see* Fig. 2). However, the alkaline regions always remained rather broad and were relatively weak in OH^- transport activity. Indeed, in these tonoplast-free experiments, redevelopment towards the control pH pattern was never observed. Measurement of cyclosis, at the conclusion of each experiment, indicated that the streaming mechanism was still functioning; rates were always above the critical level necessary for maintenance of normal alkaline bands (Lucas & Dainty, 1977*b*).

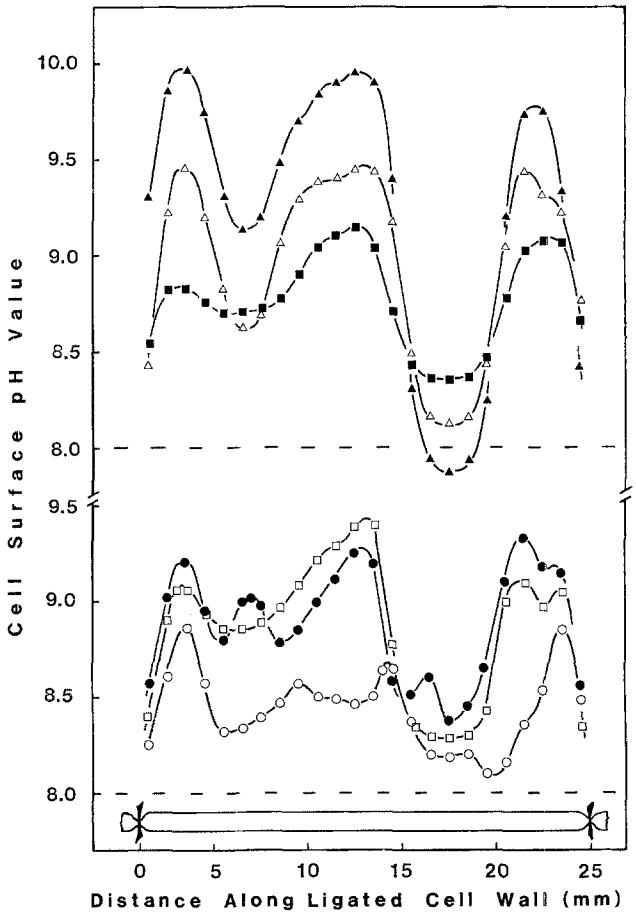

Fig. 1. Time-dependent recovery of OH^- efflux activity following cell ligation. Silk ligatures were applied (*see* Materials and Methods), and then, in this particular experiment, the nodal complexes were cut away. The vacuole was not replaced by perfusion. Cell surface pH scans were conducted at 0.5 (○), 1.5 (●), 3.0 (□), 5.5 (■), 8.0 (△) and 12 (▲) hr after cell ligation. (Horizontal broken lines represent background pH value.)

Centrifugation of Organelles within Streaming Cytoplasm

Removal of the tonoplast may have impaired, in same way, the characean cellular mechanism which is believed to control and synchronize the spatially separated HCO_3^- and OH^- transport systems (Lucas, 1980). Certainly, including 5 mM EGTA in the PM caused significant ultrastructural changes to the streaming phase of the cytoplasm (*see* Materials and Methods for details). Recent electrophysiological (Ogata & Kishimoto, 1976) and plasmalemma transport studies (Lucas & Nuccitelli, 1980) on the Chara-

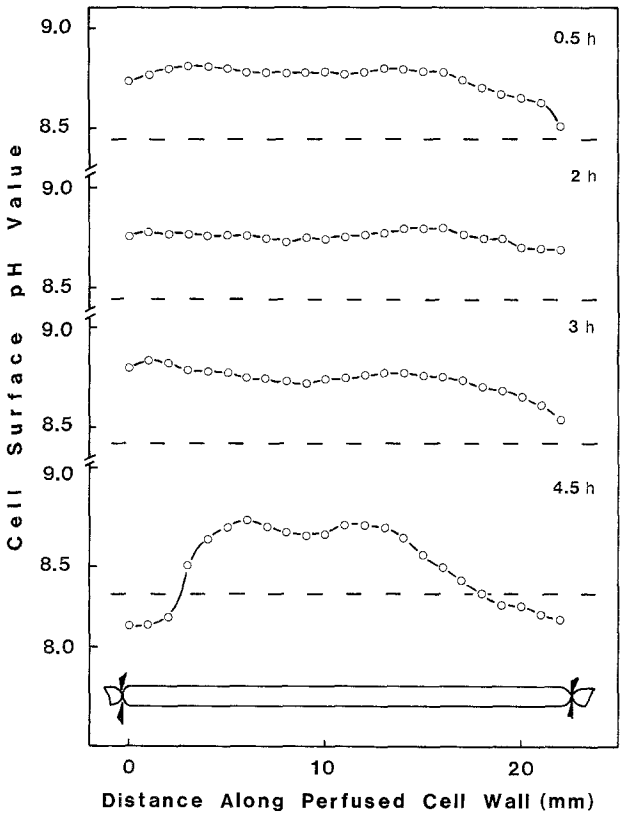

Fig. 2. pH profiles developed at various periods after establishing the tonoplast-free condition. Scans were commenced at the indicated times and each took approximately 30 min to complete. The perfusion medium used in this experiment contained 2 mM ATP and its pH_i was 8.5 (*see* Table 1 for full details). At the conclusion of the experiment the cell pH_i had decreased to 8.05. (Background pH values are indicated by horizontal broken lines.)

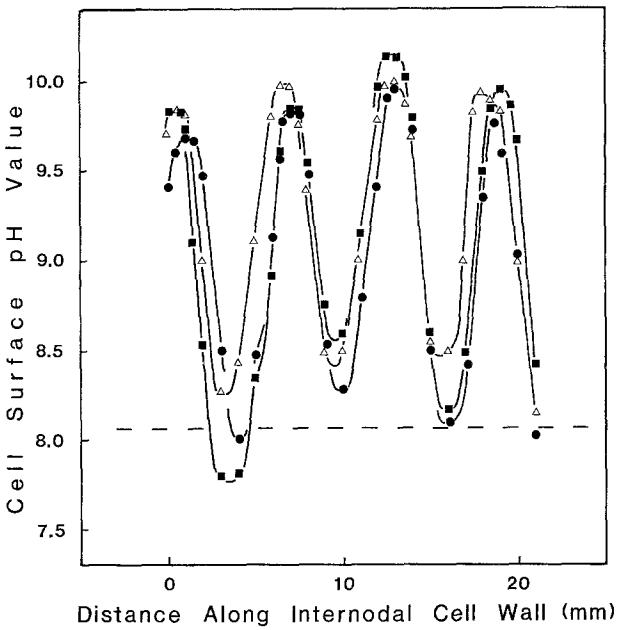

Fig. 3. Effect of cell centrifugation on the spatial arrangement of the OH^- efflux bands. A control pH scan (△) was conduced (2 hr preillumination, 20 W m^{-2}) prior to cell centrifugation. Recovery profiles were mapped 0.5 hr (●) and 2.5 hr (■) after the cell had been centrifuged for 4 min at $180 \times g$

ceae have indicated that a membrane-associated control signal(s) (feedback) may orginate from within the streaming cytoplasm. If the control signal(s) that regulates OH^- and HCO_3^- transport is produced by an organelle within the streaming phase, 5 mM EGTA may also destroy it, along with the tonoplast.

This possibility was examined using the technique of cell centrifugation. By employing the correct combination of centrifugation time and speed (*g*-force), we were able to concentrate in the centrifuged end of the cell all the large organelles of the streaming phase. When the organelles were allowed to redistribute throughout the cell, it was found that the centrifugation treatment had little overall effect on the OH^- transport pattern. Within 30 min after centrifugation there were only slight differences between the pre- and post-centrifugation pH profiles (Fig. 3). By 2.5 hr, the only remaining differences involved a slight positional rearrangement of the bands and minor reallocation of OH^- transport activity (e.g., *see* the central bands of Fig. 3).

An entirely different result was obtained when the redistribution of the organelles was restricted to the centrifuged half of the cell (Fig. 4). A normal pH banding pattern was always observed along the centrifugal segment, but the centripetal segment in which the cytoplasmic streaming phase contained only small "granules", developed rather weak diffuse regions of alkalinity (Fig. 4*A*). These profiles were very similar to those obtained from perfused, tonoplast-free cells (*cf.* Figs. 2 and 5). An important feature of the ligation technique used to partition the cytoplasm of the centrifuged cell was that the imposed blockage was reversible with time. In general, cells took 8 to 24 hr to remove the blockage. After ligation, the streaming within the two segments was monitored. It was found that when the blockage was removed, the large organelles of the centrifugal segment became redistributed throughout the cell. Although the time at which the blockage was removed was extremely variable, once it had occurred the redistribution of organelles and the recommencement of normal alkaline banding along the centripetal segment was rapid (approximately 5 to 10 min). Figure 4*B* indicates that when the larger cytoplasmic organelles were again present in both cell segments, normal alkaline bands were distributed along the entire cell length. These results clearly indicate that a cytoplasmic organelle (yet to be identified) is involved in regulating OH^- (and possibly HCO_3^-) transport

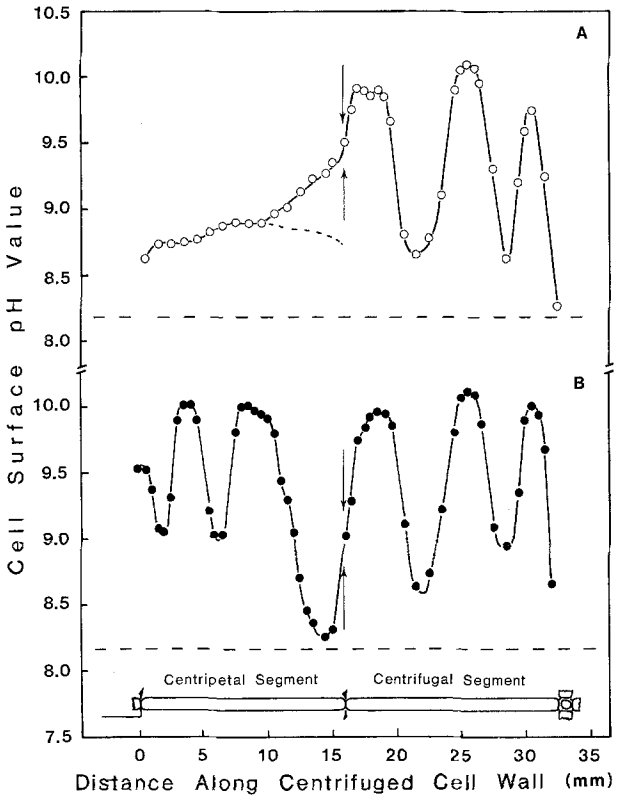

Fig. 4. Influence of streaming cytoplasmic organelle distribution on the alkaline banding phenomenon. Following centrifugation a weakly applied silk ligature was used to partition the cell into centrifugal (containing all large organelles present in streaming phase) and centripetal (containing only small "granules") segments. Banding pattern established in presence of asymmetric organelle distribution (Fig. 4*A*) and after the blockage had been removed and the organelles were streaming in both cell segments (Fig. 4*B*)

at the plasmalemma. We feel that the absence of the OH^- banding pattern in perfused cells is due, therefore, to the EGTA-induced destruction of that organelle, rather than a direct effect of the PM on the transport systems of the plasmalemma.

ATP and Hexokinase Perfused Cells

Experiments were performed to investigate the effect of the presence and absence of ATP on the pH profile established at the surface of perfused, tonoplast-free *Chara* cells. An alkalinization of the entire cell surface of ATP-free (depleted) cells was observed over a pH_i range of 7.0 to 9.0 (Fig. 5, ○). A much more complex situation was observed when the perfused cells contained 2 mM ATP. At pH_i values of 8.5 and 9.0, no significant differences were observed between the pH profiles established by ATP and ATP-free cells. However, for pH_i values ranging from 7.0 to 8.25, the cells containing ATP developed more acidic (less alkaline) pH profiles than ATP-free cells having the same pH_i value. This was particularly significant at pH_i values of 7.5 and 8.0; here the cell surface pH values were depressed below the background medium (Fig. 5). The difference between ATP and ATP-free pH profiles presumably reflects the operation of an ATP-dependent acidification processes (proton pump?).

The situation seemed to be even more complex when the pH_i was 7.0. In the presence of ATP we expected the cell surface to go more acidic than the 7.5 and 8.0 pH_i conditions. An indication that greater membrane transport complexity was being introduced at pH_i values near neutrality was also obtained from light/dark studies. Figure 6*A* and *B* illustrate the pH profiles established in the light and dark, respectively (30 min after perfusion, $pH_i = 7.0$, 2 mM ATP). Dark treated cells developed slightly acidic wall profiles, while those of the illuminated cells were weakly alkaline. Acidic profiles, established in the dark, could be reduced appreciably by 30 min of illumination (Fig. 6*C*). This light/dark sensitivity was not observed when pH_i values of 8.5 and 9.0 were employed (data not shown).

Parallel experiments, identical to those illustrated

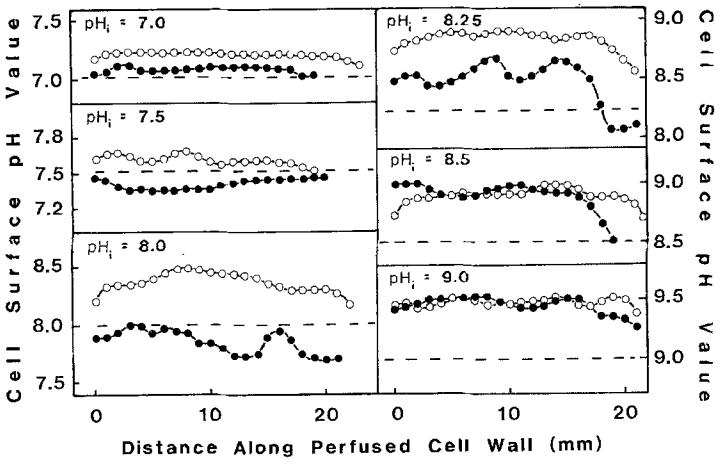

Fig. 5. Development of pH profiles by perfused cells in which either 2 mM ATP (●) or 1 mg/ml hexokinase plus 5 mM glucose (○) was included in the PM. A range of pH_i values was investigated, and, in each situation, the pH of the external medium (pH_o, indicated by horizontal broken line) was adjusted to the same value as the PM pH_i using either 100 mM NaOH or 100 mM H_2SO_4. Cells were illuminated (20 W m^{-2}) in CPW/B (pH_o as indicated) for 30 min before scanning. Between 4 and 5 perfused cells were scanned for each treatment; representative pH profiles are presented

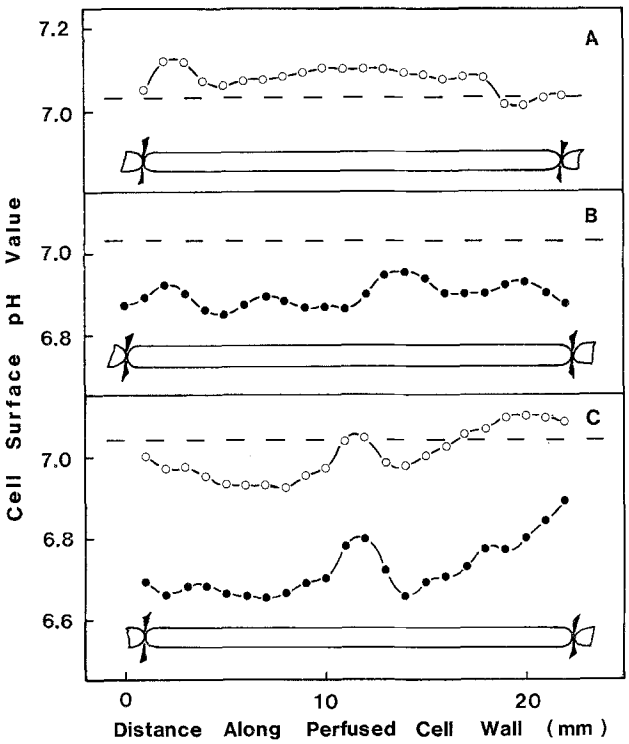

Fig. 6. Comparison between pH profiles obtained under light and dark conditions for cells in which the ATP perfusion medium pH_i was 7.0. (*A*): Profile obtained after 30 min illumination (20 W m^{-2}); (*B*): pH profile after 30 min dark treatment; (*C*): •, 30 min dark treatment, cell then illuminated for 30 min (○)

in Fig. 5, were conducted in which the pH_i values were measured after 2 hr illumination. The perfusion medium was withdrawn from the cell using the technique of Fujii, Shimmen and Tazawa (1979), and the pH_i was measured with a micro combination pH electrode (Microelectrodes Inc., model 410). For cells containing ATP, the changes in pH_i followed a simple trend; values higher than 8.0 were reduced, while lower values were increased (Table 2). This response suggests that a pH-stat mechanism is operating in these perfused cells. In ATP-free (depleted) cells the general trend was a reduction in pH_i, with the change being greatest at the highest pH_i values. This trend would be consistent with a reduction in passive OH^- efflux which would be due to diminished OH^- availability at lower pH_i values, provided the membrane potential remained constant over the pH_i range examined.

Included in Table 2 are the changes in pH_i that occurred during a 2-hr dark treatment. Cells containing ATP raised their pH_i in the dark, while the pH_i of ATP-free cells did not appear to change. The relationship between these data and the results presented in Fig. 6 will be discussed in a later section.

Electrophysiological Properties of Perfused Cells

The influence of pH_i on the membrane potential of perfused *Chara* cells is illustrated in Fig. 7. Illuminated cells that contained ATP exhibited highly negative potentials when the pH_i was 7.0 to 8.0. Indeed, these values were quite similar to normal control cells (-210 ± 4.9 mV). Figure 7 also indicates that within this pH_i region the potential responded to light/dark changes. At pH_i values above 8.0, the potential depolarized to a level similar to that observed for ATP-free cells. In this depolarized state, as with the ATP-free situation, the potential was no longer light-responsive. Related membrane resistance values which were measured under these various perfusion conditions, are presented in Fig. 8. Again, as with the membrane potential, the resistance values for ATP- and ATP-free cells converged at pH_i values above 8.0, and, under these conditions, sensitivity to light was also lost.

The response of the membrane potential to changes in the pH of the external medium (pH_o) was

Table 2. Tonoplast-free *Chara* cell pH_i values measured after 2 hr light pretreatment

Medium	Initial perfusion medium pH_i values					
	7.0	7.5	8.0	8.25	8.5	9.0
ATP (2 mM)	$7.14 \pm 0.14(3)$ $\Delta pH = +0.14$ $[7.18 \pm 0.02(3)]$	$7.60 \pm 0.06(5)$ $\Delta pH = +0.10$	$7.83 \pm 0.06(4)$ $\Delta pH = -0.17$	$8.14 \pm 0.08(7)$ $\Delta pH = -0.11$	$8.22 \pm 0.12(5)$ $\Delta pH = -0.27$	$8.75 \pm 0.07(3)$ $\Delta pH = -0.25$
Hexokinase (1 mg/ml)	$7.07 \pm 0.02(3)$ $\Delta pH = +0.07$ $[7.01 \pm 0.13(4)]$	$7.43 \pm 0.06(4)$ $\Delta pH = -0.07$	$7.83 \pm 0.11(4)$ $\Delta pH = -0.17$	$7.99 \pm 0.01(3)$ $\Delta pH = -0.26$	$8.18 \pm 0.09(8)$ $\Delta pH = -0.32$	$8.68 \pm 0.12(3)$ $\Delta pH = -0.32$

Perfused cells were pretreated for 2 hr in CPW/B; pH_o adjusted to particular pH_i value. Full details of the respective perfusion medium composition for each pH_i value is given in Table 1. Mean ± SEM, number of duplicates indicated in parenthesis. [ ], values obtained on cells given 2 hr dark treatment.

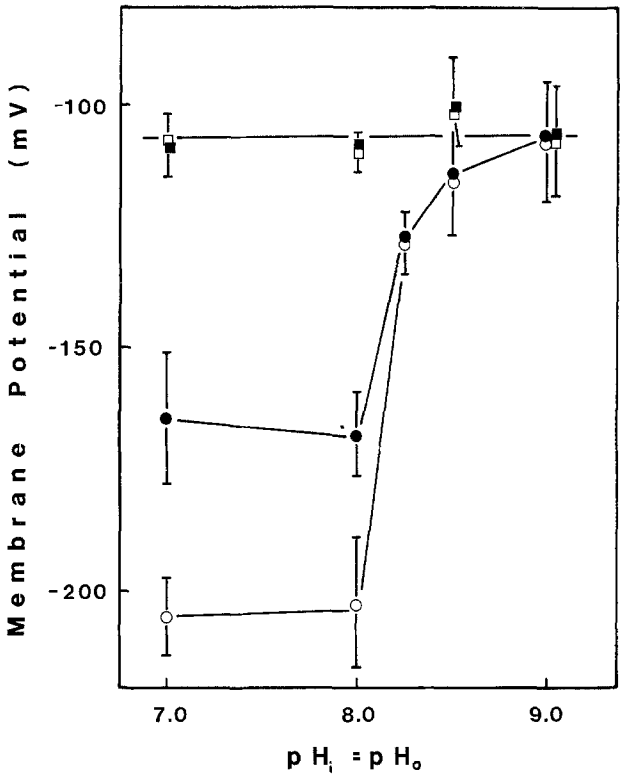

Fig. 7. Membrane potential of perfused, tonoplast-free *Chara* cells. In individual experiments, the pH of the external medium (pH_o) was adjusted to the perfusion medium value (pH_i). Stable values in the light (open symbols, 20 W m^{-2}) and the dark (filled-symbols) were obtained on each perfused cell: ○, ATP-perfused; □, hexokinase-perfused cells. (Experimental duration was usually 2 hr)

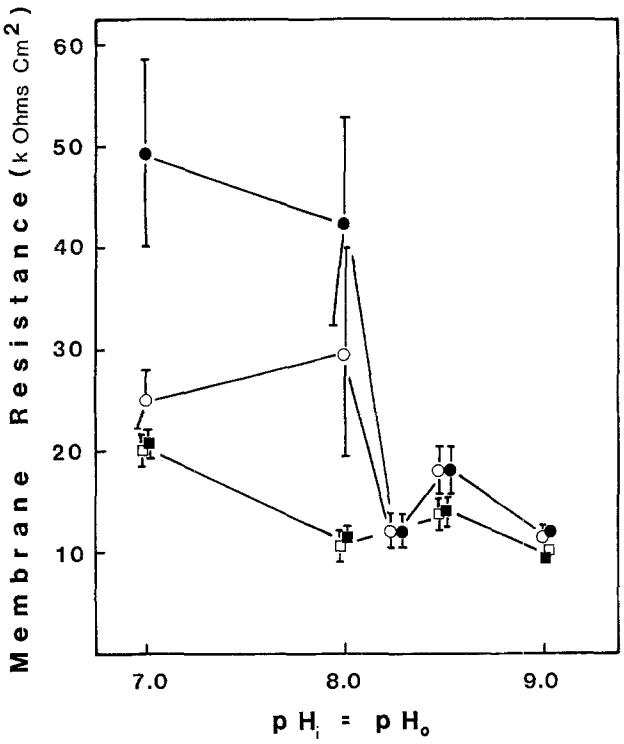

Fig. 8. Membrane resistance of perfused tonoplast-free *Chara* cells. Details are as in Fig. 7

also investigated. Normal sensitivity to pH_o was observed when the perfused cells contained ATP and the pH_i was in the 7.0 to 8.0 region (*see* Fig. 9). These data also serve to illustrate the observed stability of the membrane potential in these perfused cells. The present results on the effects of pH_i and pH_o on the membrane potential and the membrane resistance were similar to those obtained previously in perfused cells of *Chara australis* (Fujii et al., 1979, Kawamura et al., 1980).

Discussion

Regulation of OH^- Transport by a Cytoplasmic Organelle

One of the most important features of the present study was the clear demonstration that control over the OH^- transport system originates within the streaming phase of the cytoplasm. In these giant cells, the chloroplasts are fixed in position and uniformly distributed along the inner boundary of the gel layer. Also, the mitochondria, endoplasmic reticula, and Golgi are rather uniformly distributed in the gel matrix (Franceschi & Lucas, 1980). Hence, unless there are functionally different types of these organelles within the cell, they cannot be directly involved in the transport regulation processes. The main organelles that are most probably removed by either EGTA-perfusion or centrifugation are the nuclei (multinucleate cells), microbodies, spheresomes, and mini-vacuoles. At this point it would be pure speculation to suggest which of these organelles is involved. However, ultrastructural studies are presently being conducted, on centrifuged cells, in an attempt to identify the "control" organelle(s) whose redistribution into the centripetal segment is necessary for banding to reoccur. Organelle isolation and purification, using continuous sucrose density gradient centrifugation, will also be employed in an attempt to identify the organelle involved. We may be able to reinstate control OH^- transport function by perfusing the purified organelle(s) back into tonoplast-free cells.

Certainly the removal (destruction) of this organelle in tonoplast-free cells would offer a reasonable explanation for their observed pH profiles. However, the origin of the ligation-induced perturbation of the

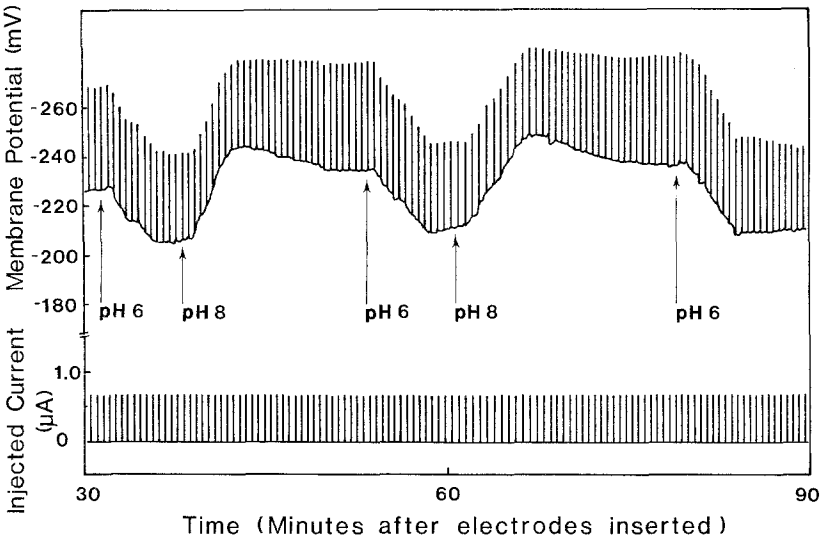

Fig. 9. Membrane potential response to changes in pH_o. Perfused cell (2 mM ATP, $pH_i = 7.0$) was allowed a 30-min recovery period after electrodes were inserted. The pH_o was then changed from its initial value of 7.0 to 6.0 and 8.0

OH^- efflux pattern remains unknown. Mechanical damage to a local region of the membrane may impair transmission of the control signal. This could be either via a direct effect transmitted along the entire membrane surface, or indirectly through a perturbation of cellular metabolism. Whatever the mechanism, the peturbation is long-lasting, and its existence must serve as a cautionary note to those using the perfusion technique to study ion transport processes.

Energy Relations of OH^- Transport

Although tonoplast-free *Chara* cells did not establish normal OH^- transport bands they did, however, alkalinize the cell surface. This rather weak alkalinization appeared to be ATP-independent. The membrane potential of hexokinase perfused cells was approx. −106 mV (Fig. 7), and this could easily act as the sole driving force for the passive efflux of OH^-. Presumably the OH^- ions cross the plasmalemma by carrier-mediated facilitated diffusion. However, it should be stressed that in normal cells, where the OH^- transport control system is functioning, much higher OH^- efflux values are obtained. Extrapolation of the ATP-independence of OH^- transport to the control cell situation should be performed with caution.

ATP-Dependency of Membrane Potential in Relation to External pH Profiles

Cells perfused with ATP-PM of pH_i between 7.5 and 8.25 had surface pH values that went below that of the background value (Fig. 5). This result differs significantly from that observed for ATP-free cells, where a rather simple alkalinization was observed. We suggest that the external pH differences between the ATP and ATP-free cells reflect the operation of an ATP-dependent external acidifying process. The most simple mechanism for this acidification would be an ATP-dependent H^+ transport system.

Based on this interpretation of the external pH profiles, one would predict that if the putative ATP-dependent H^+ efflux system was the dominant electrical component of the plasmalemma (Kitasato, 1968; Spanswick, 1972; Shimmen & Tazawa, 1977; Keifer & Spanswick, 1978, 1979), at pH_i values above 8.25 the membrane potential should be reduced, since no H^+ efflux activity could be detected under these conditions. The results presented in Figs. 5 and 7 indicate that an extremely close correlation was observed between the pH_i-induced changes in membrane potential and the external pH profiles. These results are consistent with the earlier reports that the membrane potential (Shimmen & Tazawa, 1977) is dependent on intracellular ATP. Collectively, these results provide extremely strong evidence in support of the electrogenic H^+ transport hypothesis (Kitasato, 1968).

pH-Stat Hypothesis

The data presented in Table 2 indicate that in the presence of ATP, the processes occurring within the cytoplasm and at the plasmalemma tend to regulate pH_i towards the region 7.7 to 7.8. This region falls within the range of measured (25 °C) cytoplasmic pH values for internodal cells of *C. corallina* (Raven & Smith, 1978). These data offer general support for the proposed biophysical pH-stat mechanism in *Chara* (*see* Smith & Raven, 1979). In the presence

of ATP, efflux of both H^+ and OH^- occurred simultaneously; however, in the absence of ATP, only OH^- efflux occurred. In this latter situation the cytoplasmic pH tended to decline. This may indicate that in normal cells only the efflux of H^+ is utilized to regulate the internal pH through some, as yet unidentified sensing mechanism. Alternatively, the removal of OH^- transport control in the tonoplast-free cell could prevent the operation of a cytoplasmic pH regulating signal. Whether two pH stat signals (H^+ *and* OH^-) exist must remain conjectural.

At, or above, pH_i 8.5 net H^+ efflux was not detectable (Fig. 5). Such a response could be due to pH_i inactivation of the ATP-dependent H^+ transport system. Alternatively, under these pH_i values the passive OH^- transport component may dominate the electrical properties of the plasmalemma. Since membrane potential and resistance both changed dramatically at slightly lower pH_i values (>8.0 and <8.25) both alternatives remain possible. With respect to this issue, long-term pH scans indicated that, although H^+ efflux was not observed during the first few hours after pH_i 8.5 perfusion, acid regions did eventually develop (Fig. 2). Presumably the passive OH^- efflux system lowered the pH_i value sufficiently to either reduce the OH^- contribution or reactivate the H^+ efflux system. In any event, this type of response indicates that the ATP-dependent H^+ efflux system is not permanently inactivated by high pH_i treatment.

Complex pH_i 7.0 Situation

The external pH profiles obtained for pH_i 7.0 (Figs. 5 and 6) suggest that a more complex transport situation may exist under these conditions. Since under these conditions the membrane potential has hyperpolarized at -206 ± 9 mV (2 mM ATP-PM 20 Wm^{-2}), the reduction in the acidifying trend (*cf.* pH_i 7.5 and 8 of Fig. 5) is presumably not due to an inactivation of H^+ transport activity. It is possible that other transport processes, which utilize H^+ as a co-substrate, may increase in activity at near neutral pH_o values. Sanders (1980*c*) has recently presented rather compelling evidence in support of a $Cl\text{-}2H^+$ cotransport system in *C. corallina*. Chloride transport in normal *Chara* cells has a pH_o optimum around 6.5; influx of this ion may therefore complicate the external pH profile at pH_o 7.5 to 7.0. Presumably Cl^- influx does not occur in the dark (Lucas & Smith, 1976; Sanders, 1980*b*) and this would explain both the more acidic profiles and the enhanced alkalinization of the cytoplasm observed in the dark. This aspect will be further investigated by manipulating external ionic composition (e.g., removal of external Cl^- may remove the light/dark effect on the pH profiles). It should also be noted that alkalization was also accelerated, to some extent, by light in hexokinase-perfused cells (pH_i 7.0) (data not shown).

Stability of the Membrane Potential

Membrane potential measurements indicated that stable potential and resistance values were obtained when the cells were in the hyperpolarized state. These values were stable over periods of more than two hours, and if the perfused cells were given light-to-dark and dark-to-light treatments, the membrane potential (hyperpolarized state only) and resistance responded in a regular manner. Given the acceptance that the pH and electrical data of the present study provide irrefutable evidence for the existence (and function) of an ATP-dependent electrogenic H^+ transport system, this stability in membrane potential must indicate that the control process(es), that regulates the H^+ efflux activity, has remained intact in the tonoplast-free cell system. This control process may not possess an EGTA-sensitive site. Alternatively, insufficient EGTA may have penetrated into the gel layer to prevent its operation. This aspect could be tested by perfusing cells with a range of EGTA concentrations.

Perhaps a more appealing explanation is that the control process is intimately associated with the inner surface of the membrane; it may form an integral component of the ATP-dependent H^+ transport system. This would suggest that quite different regulatory mechanisms exist for what may otherwise appear as closely related phenomena (i.e., H^+ efflux and OH^- efflux – which by some is considered to be H^+ influx).

Perfused Cells as a Physiological Tool

The results presented in the present study indicate that perfused cells can be used to elucidate important biophysical aspects of the characean cell. However, the perturbative effect of ligation, and the EGTA-induced destruction of at least one transport regulating system, indicates that extreme caution must be used when interpreting data obtained on tonoplast-free cells. We suggest that in future studies an hyperpolarized membrane potential (equivalent to control values) be used as an initial screening criterion for cell usefulness. Many secondary membrane transport processes (e.g., Cl^- influx) may be difficult to investigate using this technique, unless of course they are controlled and regulated in a much simpler manner than that evolved for the OH^- transport process.

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