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# Physiological Control of Chloride Transport in *Chara corallina*[1]

## I. EFFECTS OF LOW TEMPERATURE, CELL TURGOR PRESSURE, AND ANIONS

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### ABSTRACT

**The rate of $Cl^-$ transport at the plasma membrane of the freshwater alga *Chara corallina* is investigated with respect to possible *in vivo* controls acting in addition to the two well established ones of cytoplasmic $Cl^-$ and cytoplasmic pH. In contrast with results from many other plant tissues, halides appear to be the only anions capable of inhibiting $Cl^-$ transport, either from the outside or inside surfaces of the plasma membrane. Cell turgor pressure was also investigated. It was found that neither the influx of $Cl^-$ nor that of $K^+$ or $HCO_3^-$ is sensitive to turgor. Internal osmotic pressure is also insensitive to turgor, a situation contrasting with that in closely related brackish water charophytes.**

**After temperature downshift (from 20–4 C) $Cl^-$ transport displays a slow, time-dependent rise. Return of cells from 4 C to 20 C results in a large stimulation of $Cl^-$ influx in comparison with cells maintained at 20 C throughout. This stimulation persists for several hours and is also apparent (to a reduced extent) in cells which have had cytoplasmic composition controlled by intracellular perfusion. The stimulation therefore arises, in part, from a change in plasma membrane properties. The results are discussed with respect to recent work on membrane fluidity as a function of temperature.**

The $Cl^-$ transport system at the plasma membrane of *Chara* is now one of the best understood plant ion transport systems. For each $Cl^-$ ion transported, 2 $H^+$ ions are carried in by the transport system (2, 25). By using intracellularly perfused cells, kinetic studies have revealed the probable binding order, both externally and internally, of $Cl^-$ and $H^+$ to the transmembrane carrier (26); $Cl^-$ is the first ion to bind externally and the first to dissociate at the cytoplasmic surface. This simple mechanism, which is intrinsic to the operation of the transport system, allows high sensitivity of $Cl^-$ transport to both internal $Cl^-$ and $H^+$. As cytoplasmic $[Cl^-]$ or $[H^+]$ start to rise, $Cl^-$ influx is considerably reduced, a phenomenon known as transinhibition. Influx is, in a physiological sense at least, feedback-controlled, although this is attained simply through the intrinsic kinetics of the transport system.

Evidence for *in vivo* feedback control of $Cl^-$ transport by $[Cl^-]_c$[3] and $pH_c$ has been presented previously (24, 25). However, little is known of the way in which $Cl^-$ transport is more generally integrated into the physiology of the cell. For example, the nature of the intermediate step involved in the regulation of $Cl^-$ transport by light remains uncertain (23). It is possible that additional controls exist to regulate $Cl^-$ transport in the intact cell.

One factor controlling $Cl^-$ transport might be internal anions. In higher plants, transport of a variety of anions is sensitive to the internal concentration of chemically unrelated anions (7). The anionic specificity of the transport system is studied here, both with respect to transport of $Cl^-$ into the cell, and to the ability of $Cl_c^-$ to transinhibit transport. An anion which, when first applied inhibits $Cl^-$ influx, may well compete with $Cl^-$ for binding to the transport system. Similarly, $Cl^-$ starved cells, which normally show enhanced $Cl^-$ influx due to depletion of $Cl_c^-$ (24), may fail to show enhanced influx if another ion also binds to an internal $Cl^-$ binding site. According to the model for transinhibition of $Cl^-$ transport discussed above, transinhibition results from nothing more than the higher proportion of substrate ($Cl^-$)-loaded carrier under conditions of high $[Cl^-]_c$. If the model is correct, any ion able to inhibit transport from the external surface by competition with $Cl^-$ for a binding site, should also inhibit $Cl^-$ starvation-enhanced transport at the cytoplasmic surface, since the same binding site is involved in both cases.

In addition, the effect of turgor pressure on $Cl^-$ influx is studied. Previous work has shown that, unlike most plant cells (7), fresh water characean cells do not appear to control turgor (13, 34). However, these studies were performed on cells whose turgor had been lowered almost to zero with application of high sucrose concentrations externally. It is possible that such drastic lowering of turgor is injurious to the cells. Thus the situation is reinvestigated over a wider range of turgor pressure. The problem of whether characean cells control turgor is also considered in relation to the observation that $L_p$ apparently rises at low turgor (31, 40). An explanation proposed to account for a similar response of $L_p$ in the marine alga *Valonia* (39) was that water and solute flows are coupled (33). An observation which supports this hypothesis is that $K^+$ influx rises at low turgor in *Valonia* (10). The question of whether ion influxes similarly increase at low turgor in characeans is therefore of interest if the solute-water coupling hypothesis applies to fresh water algae.

Finally, the effects of low temperature are investigated. A high $Q_{10}$ has been reported for the fluxes of many ions in *Chara*, although for experiments conducted over long periods, a recovery of $Cl^-$ influx is seen at low temperature (21). This suggests that some kind of temperature-activated control system may act on $Cl^-$ influx.

## MATERIALS AND METHODS

**Biological Material.** Internodal cells of the alga *Chara corallina* Klein ex Willd., em. R.D.W. (= *C. australis* R.Br.) were used. They were cultured under standard conditions (23) at 14 to 18 C and removed from neighboring internodal cells the day before use. The cells were then stored overnight under continuous illu-

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[3] Abbreviations: $[Cl^-]_c$ cytoplasmic $Cl^-$ concentration; $pH_c$, cytoplasmic pH; $L_p$, membrane hydraulic conductivity; $\pi_i$, internal osmotic pressure; $\pi_o$, external osmotic pressure; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; APW, artificial pond water.

mination and at room temperature (19–21 C) unless otherwise specified.

**Solutions.** The composition of artificial pond water bathing medium (APW) was 1 mM NaCl, 0.2 mM $K_2SO_4$, 1 mM $CaSO_4$, 2 mM Mes-NaOH (pH 5.4–5.5). In experiments involving use of $HCO_3^-/CO_2$, solutions were made up immediately before use to avoid excessive exchange with atmospheric $CO_2$.

**Ion Fluxes in Intact Cells.** Ion fluxes in intact cells were measured in the light as detailed previously (23, 25). The light source was 2 × 40 w "Daylight" fluorescent tubes. Light intensity at the cell surface was 12 w $m^{-2}$. All fluxes reported are for unidirectional (tracer) influx. In experiments involving measurement of ion fluxes in the absence of an anion to which cells had previously been exposed, a short (10–15 s) wash was given to avoid contamination of the influx solution with the anion in question. This step would have been sufficient to remove all anion from the cell wall (8).

**Intracellular Perfusion and Ion Fluxes on Perfused Cells.** Intracellular perfusion and ion fluxes were performed as described previously (24). Intracellular perfusion removes the tonoplast and most of the streaming cytoplasm: this gives direct access of the perfusion medium to the inside of the plasma membrane.

**Measurement of Cell Turgor.** Measurement of cell turgor was by incipient plasmolysis. Using sorbitol as an osmoticum, cells were placed in solutions of increasing osmotic pressure until dimpling of the chloroplast layer was observed. This was taken as the point of incipient plasmolysis at which $\pi_i = \pi_o$. With $\pi_i$ known, cell turgor ($\Delta P$) was calculated for any $\pi_o$ as

$$\Delta P = \pi_i - \pi_o$$

This method was checked by direct measurement of the osmotic pressure of diluted vacuolar sap with an osmometer. Incipient plasmolysis consistently overestimated $\pi_i$ by 10%. This probably arises from adhesion of the plasma membrane to the cell wall at zero turgor, giving the appearance that the cell has not completely lost turgor. Incipient plasmolysis was the preferred method for the present experiments as it enabled sequential measurements to be made on the same cell. The results were corrected for the overestimation of $\Delta P$ by this method.

## RESULTS

**Selectivity of the $Cl^-$ Transport System for $Cl^-$.**

*External Site.* To study the effects of anions on the external site for $Cl^-$ influx, it is important to ensure that secondary effects of the treatment do not result. These secondary effects may take the form of controls via metabolism on $Cl^-$ transport or, more directly, interaction of the applied anion with the internal "inhibitor" site on the transport system. (This latter possibility is investigated below.) To overcome the possibility of secondary effects, the anion to be tested was applied for as short a period as possible. Thus, no pretreatment of the anion was given, and the period of influx of $^{36}Cl^-$ was restricted to 300 s (the shortest time in which measurable radioactivity enters the cells).

Table I shows the effect of 1 mM $Br^-$ on $Cl^-$ influx. Reference to lines 1 and 2 shows that $Br^-$ reduces $Cl^-$ influx by a factor of about 2. With $Br^-$ influx measured concurrently in the same cells, it is shown that this reduction of $Cl^-$ influx is compensated by that of $Br^-$. Thus, total halide influx is the same whether $Br^-$ is present or not. This is the expected result if $Br^-$ and $Cl^-$ enter on the same transport system, as the $K_m$ for $Cl^-$ transport is 40 $\mu$M (26) transport should be saturated and constant whether halide is present at 1 or 2 mM. Further evidence for entry of $Br^-$ on the $Cl^-$ transport system is that in the double-labeling experiment of Table I (line 2) there existed a strong positive correlation between $Br^-$ influx and $Cl^-$ influx in individual cells (data not shown). When $Cl^-$ influx is enhanced by prior starvation of $Cl^-$ (Table I, lines 3 and 4) again the total halide influx is the same in the presence and absence of $Br^-$. $Br^-$ influx also appears to be stimulated by $Cl^-$ starvation, although in this experiment not by as large a factor as $Cl^-$ influx.

In contrast to $Br^-$, no other anion reduced $Cl^-$ influx (Table II). This suggests that none of these ions is a substrate for the $Cl^-$ transport system. Alternatively, if any is a substrate, it must have a very high $K_m$ for transport and must be noneffective as a competitor at physiological concentrations. The stimulation of transport by some ions, although not statistically significant in this or in any other replicate experiment, was observed repeatedly. This applied especially to $NO_3^-$ and $H_2PO_4^-$. The origins of this stimulation are further considered under "Discussion." Generally, however, the effects of non-halide anions on $Cl^-$ influx are clearly small compared with those of $Br^-$.

*Internal Site.* A preliminary attempt was made to assess the degree to which other anions can fulfill the role of $Cl^-$ as an internal transinhibitor of $Cl^-$ influx. To preload cells with the anion to be tested, cells were starved of $Cl^-$ in the presence of the anion, and $Cl^-$ influx was measured in the absence of the anion at the end of the starvation period. Evidence has been obtained previously (24) that during starvation, the cytoplasm is depleted of $Cl^-$ (lost primarily to the vacuole, but also to the external medium) and that this in turn stimulates $Cl^-$ influx. If any of the anions tested is able to abolish the starvation-stimulated flux, then this result could be taken as evidence that it acts in a similar way to internal $Cl^-$. The validity of the experimental method, however, relies on two assumptions: first, that the anion under test actually enters the cell, and second, that the long pretreatment in the presence of the anion does not lead to the activation of other controls on $Cl^-$ influx.

The starvation-stimulated influx ($\phi^s$) was defined as the difference between influx after $Cl^-$ starvation in $Cl^-$-free APW containing the anion under study and influx after treatment in $Cl^-$-free APW + 1 mM NaCl. The reference point for $\phi^s$ was taken as the influx after pretreatment in solutions containing $SO_4^{2-}$ as the only anion. Within the limitation of the experimental method outlined above, it is possible to state with reference to Table III that $Br^-$ again appears to be the only significantly effective inhibitor, reducing starvation-stimulated influx by a factor of 4. The only other possible candidate as a transinhibitor is malate which causes weak, nonsignificant inhibition of starvation-stimulated influx. $NO_3^-$ appears to stimulate influx though not significantly so. This was apparent in two replicates of the experiment in Table III. The role of $NO_3^-$ is further considered under "Discussion."

Clearly, $Br^-$ is the only anion tested which acts at either internal or external sites for $Cl^-$. This similarity in ion specificity is consistent with the suggestion (26) that these sites differ only topographically and do not constitute chemically separate entities. If the latter were true (for example, the internal inhibitor site were "allosteric") then different affinities of the two sites for other anions might be expected.

**Does *Chara* Control Turgor?**

*Is $Cl^-$ Transport Turgor-sensitive?* Cell turgor was adjusted experimentally by addition of various concentrations of sorbitol to the external medium. $Cl^-$ influx was measured at intervals of about 150 kPa over the whole range of $\Delta P$ from 10 kPa to full turgor (610 kPa in these experiments). At no value of $\Delta P$ did influx deviate from the control level at full turgor (data not shown). Similarly, variation of the pretreatment time from 0 to 15 h before measurement of influx at any given turgor gave no indication of an effect of turgor on $Cl^-$ influx once water equilibrium is achieved (data not shown). The same conclusion was reached for influx of $^{86}Rb^+$ in *Chara* and of $^{36}Cl^-$ and $^{42}K^+$ in *Nitella flexilis*, a related Characean.

*Is Carbon Fixation Turgor-sensitive?* Even though no turgor-dependent ion fluxes could be found, it is possible that *Chara* does control turgor, by increased synthesis of an organic osmoticum,

Table I. *Effect of $Br^-$ on Influx of $Cl^-$*

Influx of radiotracer took place over 300 s in the light. Each batch consisted of 10 cells. In lines 2 and 4, influx of $Br^-$ and $Cl^-$ was measured simultaneously on the same cells. These samples were counted twice (before and after decay of $^{82}Br^-$) to enable calculation of the separate $Br^-$ and $Cl^-$ fluxes. The data are the mean ± SE of the mean.

| Pretreatment Solution (15 h) | Influx Solution | $Cl^-$ Influx | $Br^-$ Influx | Total Halide Influx |
|---|---|---|---|---|
| | | | *nmol $m^{-2}$ $s^{-1}$* | |
| BPW[a] | BPW | 15.9 ± 5.6 | | 15.9 ± 5.6 |
| BPW | BPW + 1 mM $Br^-$[b] | 8.7 ± 4.1 | 5.9 ± 2.4 | 14.6 ± 6.4 |
| $Cl^-$-free BPW[c] | BPW | 31.0 ± 6.2 | | 31.0 ± 6.2 |
| $Cl^-$-free BPW | BPW + 1 mM $Br^-$ | 24.0 ± 5.8 | 7.6 ± 1.3 | 31.6 ± 6.4 |

[a] BPW was of the same composition as APW, but contained in addition 0.5 mM $Na_2SO_4$.

[b] $^{82}Br^-$ was added at the expense of $K_2SO_4$ as the $K^+$ salt to a final concentration of 0.4 mM, and diluted with 0.6 mM NaBr. $Na^+$ was maintained constant by removal of $Na_2SO_4$.

[c] $Cl^-$-free BPW was obtained by omission of NaCl from BPW.

Table II. *Effect of Anions on Influx of $Cl^-$*

Cells were pretreated in APW at the appropriate pH for 15 h. Influx was in the light in the presence of the anion indicated, for 300 s. Fluxes are for batches of 9 or 10 cells. Variability between controls is probably a reflection of seasonal fluctuation of fluxes. The data are the mean ±SE of the mean.

| Date | Solution pH | Anion Added to APW[a] | $Cl^-$ Influx |
|---|---|---|---|
| | | | *nmol $m^{-2}$ $s^{-1}$* |
| March 25, 1978 | 5.5 | $SO_4^{2-}$ | 6.59 ± 1.45 |
| | | $NO_3^-$ | 8.95 ± 3.14 |
| | | $H_2PO_4^-$ | 10.20 ± 3.53 |
| April 9, 1978 | 5.5 | $SO_4^{2-}$ | 4.26 ± 1.50 |
| | | Malate$^-$ | 4.88 ± 1.63 |
| June 1, 1977 | 5.5 | $SO_4^{2-}$ | 18.77 ± 3.88 |
| | | $CO_2(/HCO_3^-)$ | 26.37 ± 5.10 |
| June 1, 1977 | 8.5[b] | $SO_4^{2-}$ | 7.94 ± 1.37 |
| | | $HCO_3^-$ | 9.49 ± 1.55 |

[a] Anions were added to the influx solution as the $Na^+$ salt and to a final concentration of 1 mM.

[b] Buffering in this solution was with 2 mM Ches-NaOH.

Table III. *Effect of Selected Anions on Development of Starvation-stimulated $Cl^-$ Flux*

Cells were pretreated for 12-15 h in $Cl^-$-free APW in the presence of the anion indicated. $Cl^-$ influx was then measured over 300 s in the light and in the absence of the anion present during pretreatment. Data are for batches of 9-11 cells. Influx in APW + 0.5 mM $Na_2SO_4$ was chosen as the reference point for starvation-stimulated influx (see 24). Variability between controls is probably a reflection of seasonal fluctuation of ion fluxes. The data are the mean ±SE of the mean.

| Date | Anion Added to $Cl^-$-free APW[a] | $Cl^-$ Influx |
|---|---|---|
| | | *nmol $m^{-2}$ $s^{-1}$* |
| April 9, 1978 | $Cl^-$ | 4.3 ± 1.5 |
| | $SO_4^{2-}$ | 13.1 ± 3.6 |
| | $NO_3^-$ | 17.8 ± 6.3 |
| March 25, 1978 | $Cl^-$ | 2.8 ± 0.5 |
| | $SO_4^{2-}$ | 13.5 ± 4.0 |
| | $H_2PO_4^-$ | 14.7 ± 3.7 |
| | $CO_2(/HCO_3^-)$ | 15.1 ± 4.1 |
| June 13, 1978 | $Cl^-$ | 6.6 ± 1.5 |
| | $SO_4^{2-}$ | 33.7 ± 5.9 |
| | Malate$^-$ | 27.5 ± 2.7 |
| | $Br^-$ | 13.5 ± 1.5 |

[a] Anions were added to the pretreatment solution as $Na^+$ salts to a final concentration of 1 mM.

rather than by increased ion influx. Table IV shows the result of a preliminary experiment designed to test this possibility. First, at high pH, $HCO_3^-$ influx into *Chara* was measured (lines 1-3). Under these conditions, plasma membrane transport is rate-limiting to C fixation (16). At two widely different turgor pressures, there is no effect of turgor pressure on $HCO_3^-$ influx. The experiment was repeated at lower external pH (lines 4-6). Here, entry of $^{14}C$, which occurs mainly as $H_2CO_3$, is probably not rate-limiting to C fixation (19). There is 1.7-fold stimulation of $^{14}C$ fixation under conditions where turgor is reduced from 545 to 60 kPa after overnight pretreatment at low turgor, and almost as much stimulation if only 1-h pretreatment is given. It seems possible that this stimulation represents enhanced production of organic osmoticum to compensate for the lowered turgor. In order to investigate the possibility that $\pi_i$ rises with $\pi_o$ to maintain turgor constant, a long-term experiment was performed.

*Effects of $\pi_o$ on $\pi_i$.* When *Chara* internodal cells are cut from their neighbors and stored at room temperature in APW, $\pi_i$ increases (by about 30%) over the ensuing days to a maximum level of 750 kPa. If *Chara* controls turgor, this process should be stimulated at high $\pi_o$, the resulting higher $\pi_i$ would tend to keep the pressure difference (turgor) constant. Figure 1 shows the results of an experiment in which cells were stored for several days after cutting in APW or in APW with sorbitol added to decrease turgor. Clearly, there is no difference in the final value of $\pi_i$ in any of the conditions; nor is the rate at which this maximum value is attained stimulated by high $\pi_o$. Thus, it seems unlikely that turgor is controlled in *Chara* either by enhancement of ion influx or by any other mechanism.

**Regulation of $Cl^-$ Influx by Temperature.** The results of Raven and Smith (21) showed that in short and medium term experiments (up to 24 h) $Cl^-$ influx at 5 C is significantly lower than at 15 or 25 C. However, after longer times (99 h) at 5 C, influx may actually exceed that at higher temperatures. A similar time-dependent rise in $Cl^-$ influx at low temperature was noted in cells used for the present experiments (data not shown). This suggests that at low temperature, regulatory mechanisms may act to restore $Cl^-$ influx to levels equal to, or greater than, at higher temperatures. The characteristics of this regulatory mechanism are further investigated here.

If cells are pretreated at 4 C for several hours and then returned

Table IV. *Effect of Turgor Pressure on $HCO_3^-$ Influx/$CO_2$ Fixation*

Influx was measured in the light over 30 min. After influx and measurement of cell dimensions, cells were placed in a scintillation vial with 0.1 N HCl and dried overnight to drive off unfixed $CO_2$. Means are for batches of 9 or 10 cells. All solutions contained 1 mM $NaHCO_3$ in addition to APW. Cell turgor was lowered to 60 kPa by addition of 200 mM sorbitol. Buffer at pH 6 was 5 mM Mes-NaOH. Buffer at pH 9 was 5 mM Ches-NaOH.

| Cell Turgor | | Influx Solution (pH) | $^{14}C$ Fixation |
|---|---|---|---|
| Overnight (15–18 h) | 1 h before and during influx | | |
| *kPa* | | | *nmol $m^{-2}$ $s^{-1}$* |
| 545 | 545 | $HCO_3^-$ APW (9) | 24.8 ± 7.5 |
| 545 | 60 | $HCO_3^-$ APW (9) | 27.4 ± 9.0 |
| 60 | 60 | $HCO_3^-$ APW (9) | 25.6 ± 5.8 |
| 545 | 545 | $CO_2$-APW (6) | 228 ± 23 |
| 545 | 60 | $CO_2$-APW (6) | 342 ± 18 |
| 60 | 60 | $CO_2$-APW (6) | 396 ± 31 |

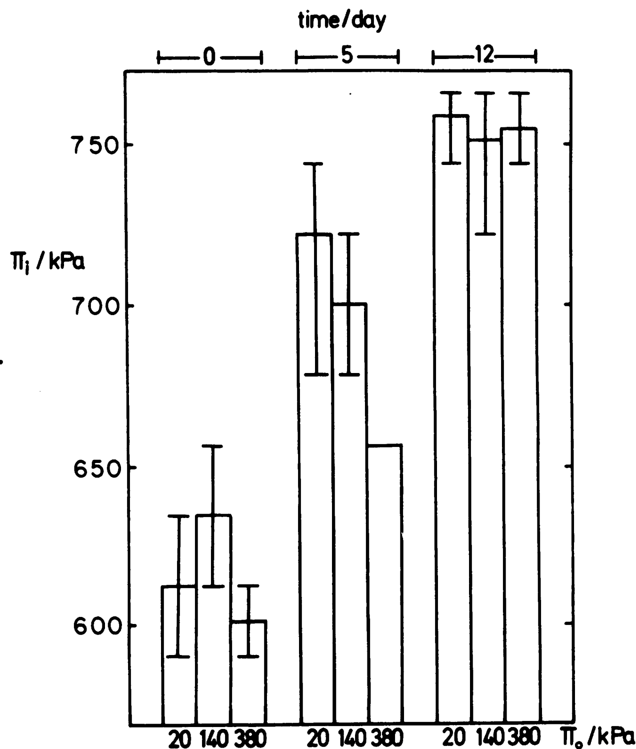


FIG. 1. The effect of $\pi_o$ on $\pi_i$ in isolated internodal cells. Internodal cells were cut from their neighbors on day 0, and $\pi_i$ measured. Cells were stored in one of the following three bathing solutions: APW ($\pi_o$ = 20 kPa) APW + 50 mM sorbitol ($\pi_o$ = 140 kPa) or APW + 150 mM sorbitol ($\pi_o$ = 380 kPa). Solutions were changed every 2 or 3 days, and storage was under continuous illumination at 21 C. Error bars give the range about the mean value of $\pi_i$ for 2 to 4 cells.

to 20 C, a dramatic (6–10-fold) stimulation of $Cl^-$ influx is observed in comparison with cells maintained at 20 C throughout. The time course for the decay of this stimulation is shown in Figure 2. The stimulation clearly persists for up to 8 h after return to 20 C, although it has decayed by 13 h.

One question which arises concerning the nature of the temperature-activated control system is its location, membrane or cytoplasmic. For example, it could be envisaged that some metabolic intermediate (cytoplasmic) acts on the $Cl^-$ transport system to raise influx. Alternatively, changes in membrane properties could

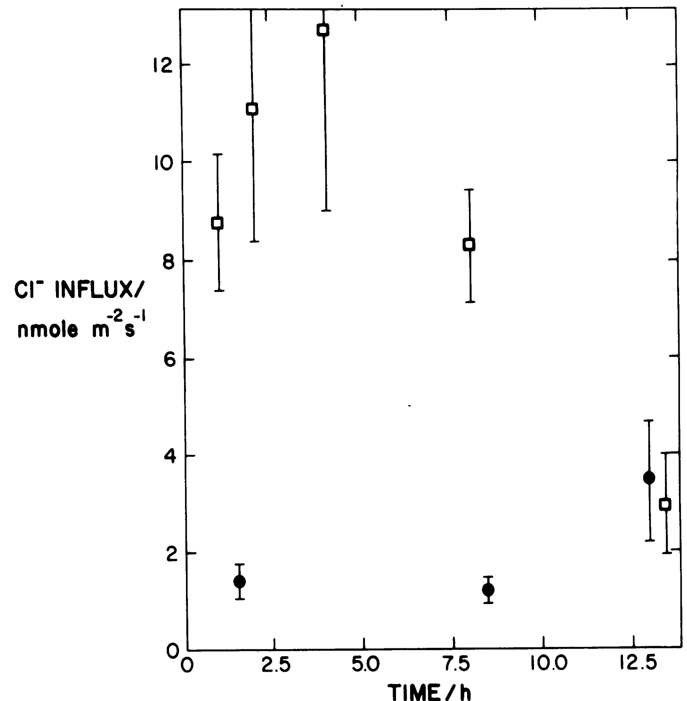


FIG. 2. Decay of low temperature-induced enhancement of $Cl^-$ influx. Internodal cells were pretreated for 21 h at 20 C (●) or at 4 C (■) and influx measured over 600 s at various times thereafter. Pretreatment and influx were in the light. Each point is mean ±SE for batch of 9 or 10 cells.

occur, such as an increased number of transport systems. An attempt to discriminate between the two possibilities was made by the use of intracellular perfusion. This procedure largely replaces the cytoplasm by a medium whose composition is under experimental control. Thus, if the control system is cytoplasmic, perfusion should abolish stimulation after low temperature pretreatment.

Cells were pretreated for 16 to 20 h at 3.6 or 19 C and returned to 19 C for 2 h before intracellular perfusion and measurement of $Cl^-$ influx at this temperature. In three experiments, $Cl^-$ influx was stimulated by a factor of 2.4 ± 0.6 by low temperature pretreatment. Reference to Figure 2 shows that this stimulation is not as great as in intact cells after a similar recovery period at the higher temperature. The reasons for this discrepancy are discussed below.

## DISCUSSION

**Specificity of $Cl^-$ Transport System.** Of the anions tested, only $Br^-$ appeared to have affinity for either internal or external sites on the transport system. The general suitability of $Br^-$ as an analog for $Cl^-$ in characean cells has been appreciated since 1927, when it was shown (11) that accumulation of $Br^-$ in the vacuole occurs at the expense of $Cl^-$. Preliminary attempts were made (Sanders, unpublished experiments) to examine whether $Cl^-$ acts as a competitive inhibitor of $Br^-$ transport as expected if the two ions compete for the same transport system. Large changes in the apparent $K_m$ for $Br^-$ transport were detected in the presence of $Cl^-$, although smaller changes were also present in $V_{max}$. Thus, it is not yet possible to state unequivocally that the two ions compete for the same system, although most of the evidence favors this interpretation, especially the constancy of the total halide flux in the presence and absence of $Br^-$. An estimate for the inhibition constant ($K_i$) for $Br^-$ on $Cl^-$ transport can be obtained with the information (Table I) that 1 mM $Br^-$ inhibits $Cl^-$ influx to 0.55 of control, and that the $K_m$ of $Cl^-$ transport is 40 μM (26). Assuming

that $Br^-$ affects only $K_m$ and not $V_{max}$ of $Cl^-$ transport, the apparent $K_m$ rises to 904 $\mu$M in the presence of $Br^-$, which gives $K_i$ = 46 $\mu$M. This is in good agreement with the $K_m$ for $Br^-$ transport in *Chara* of 27 to 36 $\mu$M (26) and may be taken as further evidence that the two ions enter on the same system. In barley roots, high affinity $Cl^-$ uptake is competitively inhibited by $Br^-$ with a $K_i$ of 36 $\mu$M, but is otherwise specific for $Cl^-$ (9), as in the present case.

The absence of an ability of any of the other anions tested to compete with $Cl^-$ for entry or to inhibit transport from inside the cell (assuming that the anions do, in fact, enter) is significant in the light of work on other plant species. Thus, internal $NO_3^-$ concentration is thought to have a role in controlling $Cl^-$ influx in barley and carrot roots (5) and citrus leaf slices (29). Inhibitory effects of $HCO_3^-$ on $Cl^-$ influx also occur in carrot and barley, and may be related to the ability of these tissues to use $Cl^-$ or malate (synthesized from $HCO_3^-$) as alternative vacuolar osmotica (6) (although in malate-loaded tissue, there is apparently no tendency for decrease in $Cl^-$ influx). Although *Chara* can probably also utilize organic anions as a vacuolar osmoticum when $Cl^-$ is unavailable (Sanders, manuscript in preparation), there appear to be no such effects of $HCO_3^-$ on $Cl^-$ influx here. The failure of external $CO_2$ (and hence, presumably cytoplasmic $HCO_3^-$) to inhibit $Cl^-$ influx in *Chara* is also noteworthy in view of the finding that in *Nitella*, 1 mM $CO_2$ (but not external $HCO_3^-$) will inhibit $Cl^-$ influx (30). The reason for the discrepancy between the results on *Nitella* and those here on *Chara* is unclear.

The slight stimulatory effects of $NO_3^-$ on $Cl^-$ influx could be related to the presumed result of $NO_3^-$ reduction in raising $pH_c$ (20), which would in turn stimulate $Cl^-$ influx. However, this would not explain the stimulatory effects of $H_2PO_4^-$. A more likely explanation is that the effect is related to the observation in *Neurospora* that starvation of any one of a variety of nutrients shuts down electrical leaks in the membrane (28). Thus, it might be expected that termination of starvation of $NO_3^-$ or $H_2PO_4^-$ in *Chara* may allow the reopening of such leaks, which would include $Cl^-/2H^+$ co-transport. Clearly, the only direct tests which can eliminate such secondary control systems must come from studies on intracellularly perfused cells.

A major conclusion of the present work is that with intact cells, even with pretreatment and influx times reduced to a minimum, interpretation of results can be difficult if the control systems react quickly. Possibly, therefore, the $Cl^-$ transport systems of other plant species are just as specific as that of *Chara* for halides. Physiological control of $Cl^-$ transport by other anions in other plant species may result from indirect effects of these ions on $Cl^-$ transport; this is of homeostatic rather than mechanistic significance.

**Absence of Turgor Control in *Chara*.** The present results confirm the findings of previous workers on *Nitella* (13, 34) that fresh water characean cells do not control turgor. A recent investigation of $HCO_3^-$ influx in *Chara* has also failed to uncover any low turgor-induced stimulation for this ion (1). The reason for stimulation of the apparent C fixation rate is unknown, although it seems clear that it does not reflect a large increase in the net synthesis of vacuolar osmoticum. As has been pointed out previously (12), fixation of $^{14}C$ is not a good indicator of net synthesis and may represent simply a faster turnover of organic compounds. This criticism can also be applied to recent results obtained with *Valonia* (14) in which it was shown that low turgor stimulates $^{14}C$ incorporation into sucrose. It is not possible to draw conclusions about net synthesis from this type of experiment where turnover rates are not known.

The absence of control of turgor does not seem to be common to all species in the *Chara* genus. In Figure 3, ion concentration data from Collander (4) are replotted. These data were obtained from cells of the brackish water alga *Chara tomentosa* (formerly *C. ceratophylla* (37)) which were collected from localities of varying degrees of salinity. To calculate $\pi_i$ and $\pi_o$, the assumption has been made that $Cl^-$ was the counter ion for the four cations measured within the cell and in the external medium, although in reality, $Cl^-$ accounted for 75 to 85% of the cation equivalents. *C. tomentosa* does appear to control turgor, although not with 100% efficiency as is found for some marine algae. The original data indicate $Na^+$ and $Cl^-$ to be the ions primarily involved in turgor control. The brackish water charophyte *Lamprothamnium* also maintains turgor constant over a wide range of $\pi_o$ using mainly $K^+$ and $Cl^-$ as regulatory osmotica (3).

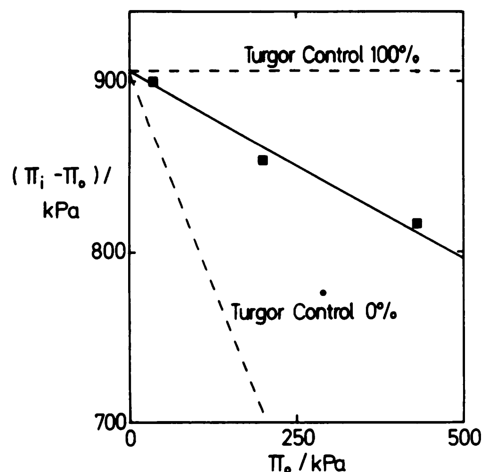


FIG. 3. Cell turgor ($\pi_i$-$\pi_o$) as a function of external osmotic pressure in *C. tomentosa*. Data are taken from the ion concentrations measured by Collander (4) and corresponding osmotic pressures calculated as described in the text. The data can be fitted assuming turgor control = 78% (solid line). Turgor control is defined as $[1 - (\pi_i - [\pi_i]^0)/\pi_o]$ 100% where $\pi_i$ is internal osmotic pressure at any given external osmotic pressure ($\pi_o$) and $[\pi_i]^0$ the internal osmotic pressure at zero external osmotic pressure.

Nakagawa *et al.* (17) have suggested that *Nitella* controls internal osmotic pressure (rather than turgor). From the considerable increase in $\pi_i$ with time after isolation of internodal cells (Fig. 2) it seems doubtful that $\pi_i$ is an efficiently controlled parameter in *Chara*.

Despite the apparent rise in $L_p$ at low turgor in *Chara* (40), the present results show that there is no homeostatic and long-lasting enhancement of solute fluxes under these conditions. In *Valonia*, where apparent $L_p$ and solute fluxes are both stimulated by low turgor, (10, 39), it is therefore possible by analogy with *Chara* that the apparent rise in $L_p$ is independent of the magnitude of the solute flux. This contrasts with the proposal that the homeostatic stimulation of solute fluxes is instrumental in causing the apparent $L_p$ to rise (33, 41). Convincing evidence has been presented (32) showing that the increase in $L_p$ in *Nitella* is not due to experimental artifact and that a large portion of the increase in $L_p$ is due to turgor reduction rather than decrease in $\pi_i$. Previously it had been found (15, 35) that a dependence of $L_p$ on $\pi_i$ could be interpreted in terms of the independence of the effects of $\pi_i$ and $\pi_o$ rather than the difference between them (turgor). In conclusion, both the cause of the increase in $L_p$ (low $\pi$ or low turgor) and the interpretation of the events at the membrane level remain unclear.

**Temperature-activated Controls on $Cl^-$ Influx.** It is anticipated that *Chara* possesses a temperature-compensation system which acts on $Cl^-$ influx to overcome the effects of the high $Q_{10}$ for $Cl^-$ transport. This control facilitates the recovery of influx from the initial inhibition by low temperature (21). It is present also at higher temperatures, where its activity decays only after several hours.

The experiments with perfused cells indicate that part of the mechanism for low temperature activation of $Cl^-$ influx resides at the plasma membrane. But plasma membrane control accounts

for only 2.4-fold stimulation after pretreatment at 4 C, whereas 6- to 10-fold stimulation is seen in intact cells. Cytoplasmic factors are thus also implicated in the stimulation. These cytoplasmic factors can be accounted for in terms of well established controls of $Cl^-$ transport, as follows. Raven and Smith (22) have shown that at 5 C, $pH_c$ is 0.1 unit higher than at 25 C. If this state were to persist after return to higher temperature, then it would account for 1.5-fold stimulation of $Cl^-$ influx (25). In addition, a rapid increase in $Cl^-$ efflux across the plasma membrane occurs in response to temperature upshift (Sanders, unpublished observations). If $[Cl^-]_c$ falls from 10 mM to 5 mM as a result, then $Cl^-$ influx would increase by a factor 1.7 given a $K_i$ for internal $Cl^-$ on $Cl^-$ transport at the plasma membrane of 1.94 mM at $pH_c$ 7.75 (26). Thus, the overall stimulation of $Cl^-$ influx in intact cells resulting from: (a) plasma membrane changes; (b) $pH_c$ increase; and (c) $[Cl^-]_c$ decrease would be, respectively $(2.4 \times 1.5 \times 1.7) =$ 6.1-fold, which is in the experimentally observed range.

What is the origin of the membrane change producing enhanced $Cl^-$ influx? The long time for its decay is consistent with the synthesis of an increased number of transport systems at low temperature. Recent work has documented a tendency for increased plasma membrane fluidity after cold pretreatment (36, 38). It seems this fluidity increase may be manufactured by changes in the degree of saturation of phospholipids (38), although other mechanisms have a role in some circumstances (36). In addition, a positive correlation is emerging between plasma membrane fluidity and transport activity in a variety of systems (18, 27). Thus it seems reasonable to propose that $Cl^-$ transport in *Chara* is similarly enhanced by increased plasma membrane fluidity resulting from low temperature pretreatment.

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