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The Mechanism of Cl^- Transport at the Plasma Membrane of *Chara corallina* I. Cotransport with H^+

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Summary. Cl^- transport into cells of *Chara corallina* was studied in relation to that of other ions which have been proposed as cosubstrates for the Cl^- transport system. Although there appears to be a partial mutual dependence between K^+ and Cl^- for transport in intact cells, this is not apparent in cells which have been perfused internally. Moreover, in intact cells, the fluxes of K^+ and Cl^- show a large degree of independence in their responses to Cl^- starvation. Cl^- transport is electrogenic in a direction indicating the transport of excess positive charge into the cell. In the absence of any other likely counter ion, it is suggested that Cl^- is cotransported with H^+. Response of Cl^- influx to internal and external pH in perfused cells is consistent with this suggestion. There appears, in addition, to be a role for ATP in transport as judged by fourfold stimulation of Cl^- influx in perfused cells when 1 mM ATP is incorporated in the perfusion medium.

There exist three hypotheses (not necessarily mutually exclusive) to explain entry of Cl^- into giant internodal cells of fresh-water Characean algae. These are (i) Cl^-/Cl^- exchange diffusion (Hope, Simpson & Walker, 1966; Findlay et al., 1969); (ii) Cl^-/H^+ cotransport (or its operational equivalent, Cl^-/OH^- exchange), proposed originally by Spear, Barr and Barr (1969) and Smith (1970). A more recent modification of this proposal (Smith & Walker, 1976) suggests that if energized by H^+ or OH^-, the stoichiometry for Cl^- influx would be $2H^+:1Cl^-$; (iii) cotransport of Cl^- with alkali cations (Findlay et al., 1969; MacRobbie, 1974) with ATP as the putative supplier of energy (Smith & West, 1969; Smith & Raven, 1974).

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In a previous paper (Sanders, 1980*a*) simple 1:1 exchange diffusion of Cl^- across the plasma membrane was considered to be unlikely because under some circumstances (i.e., in the dark) the magnitude of transstimulated Cl^- efflux (that portion stimulated by external Cl^-) is greater than the absolute value of influx. Moreover, Cl^- influx is not transstimulated by internal Cl^-, as would be predicted for an exchange diffusion system. The existing evidence for $Cl^-/2H^+$ cotransport has been reviewed in the light of data demonstrating homeostatic control of Cl^- influx (Sanders, 1980*b*). The conclusion is that none of the experimental evidence so far invoked in favor of the hypothesis of $Cl^-/2H^+$ cotransport is sufficiently rigorous to exclude an interpretation based on control phenomena, as opposed to direct effects of imposed treatments on the transport system. An analogous argument can be used to dispute the validity of evidence which lead to the proposal of a Cl^-/alkali cation system. Thus, although Cl^- influx is inhibited when K^+ is withdrawn from solution, as is K^+ influx when external Cl^- is withdrawn (Findlay et al., 1969), this mutual dependence for entry was observed after long periods of pretreatment in solutions free of the appropriate ion. It is possible that during this time regulatory controls acted to reduce influx of the counter ion through a separate transport system.

It is apparent that there is no strong evidence either for or against the existence of either of the proposed Cl^- cotransport systems (with H^+ or alkali cations). The aim of the present work is to identify the ions (if any) which are cosubstrates with Cl^- for transport into the cell.

In the experimental conditions most frequently used, Cl^- is concentrated by a factor of 10 in the cytoplasm (Coster, 1966) against a plasma membrane potential difference of -200 mV (Richards & Hope, 1974). Thus an energy input of about 25 kJ mol^{-1} (260 mV) is required. An attempt has also been made

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in the present work, therefore, to identify the energy source for Cl^- influx.

Materials and Methods

Biological Material

Internodal cells of the alga *Chara corallina* were used. These were cultured under standard conditions (Sanders, 1980*a*) and cut from neighboring internodal cells the day before use. They were then stored overnight under continuous illumination.

Solutions

The composition of artificial pond water bathing medium (APW) was usually 1 mM NaCl, 0.2 mM K_2SO_4, 1 mM $CaSO_4$, 2 mM MES-NaOH, pH 5.4 to 5.5. In experiments involving Na^+-free solution, the composition of APW was 1 mM NaCl, 0.2 mM K_2SO_4, 2 mM MES-$Ca(OH)_2$, 0.5 mM $CaSO_4$ (total $[Ca^{++}]=1$ mM); this facilitated removal of Na^+ without leading to problems involving buffer adjustment. Subtractions of cations from these solutions were made by keeping $[Cl^-]=1$ mM with SO_4^{2-} as the variable anion.

Changes in the pH of APW were obtained through the use of Good buffers. These were added from a freshly made up solution of 100 mM buffer, titrated approximately to its pK with NaOH. Buffers were used at a final concentration of 2 mM and in some cases (especially at higher pH) this resulted in a final APW pH below the pK of the buffer. At the membrane surface, uptake of H^+ will discharge the protonated form of the buffer, and, taking into account the unstirred layer, a diffusion gradient will be set up from the membrane surface to the bulk solution for the deprotonated buffer. When deprotonated buffer concentration in bulk solution is low, i.e., pH is significantly less than pK of the buffer, this will lead to a proportional increase in pH in the vicinity of the membrane. However, for the worst case in the present experiments (highest rates of H^+ uptake and biggest discrepency between pH and pK), this pH gradient between bulk solution and membrane will be only 0.3 unit. Bulk solution pH has, therefore, been used as reference, as the pH range of interest (about 4 units) was large in comparison with the error. APW was made up immediately before use in experiments involving high pH to prevent significant formation of HCO_3^-.

Perfusion medium (PM) composition is given by Sanders (1980*b*). In making additions to and subtractions from this medium, the following were always maintained constant as described: (a) osmotic pressure = 850 kPa (350 mOsM) by variation of sorbitol concentration; (b) K^+ activity = 43.5 mM by variation of K_2SO_4; (c) Free $[Mg^{++}]=4$ mM by variation of $MgSO_4$. The solution was free of Ca^{++}. The buffering capacity of PM (provided by 5 mM TES and 50 mM EGTA) was measured as 19 to 30 mM/(pH unit) for pH 7.00 to 7.75, respectively. Cl^-, which is known to exert considerable influence on Cl^- influx (Sanders, 1980*b*), was usually not present, though exceptions are noted.

Ion Fluxes in Intact Cells

The methods for measurement of unidirectional Cl^- influx into intact cells have been detailed previously (Sanders, 1980*a*). Briefly, batches of about 10 internodal cells were immersed in $^{36}Cl^-$-APW, usually for 0.3 ksec, at 22 °C and under white light. Cells were washed in APW for 0.2 ksec to exchange all $^{36}Cl^-$ present in the cell wall, then measured and radioactivity determined in an end-window Geiger counter. For K^+ and Na^+, $^{42}K^+$-APW and $^{22}Na^+$-APW were used. In these cases the wash period was extended to 1.2 ksec to ensure that all cation present in the negatively-charged cell wall was fully exchanged; a doubling of the wash period to 2.4 ksec resulted in no further reduction of apparent influx of cation. Similarly, in double-labeling experiments, in which influx of both $^{42}K^+$ and $^{36}Cl^-$ were followed simultaneously, extension of the wash period for Cl^- from 0.2 ksec to 1.2 ksec did not lead to further loss of isotope from the cells. Double-labeled cells were recounted after all $^{42}K^+$ had decayed.

In experiments involving flux measurements during the period immediately after removal of a given ion, cells were bathed for 5 to 10 sec prior to the influx experiment in a solution identical in composition to the radioisotope solution. This ensured removal of those ions present in the surface film of water which could not be otherwise removed without blotting the cells to dryness. When applied to Cl^-, this treatment would also have removed all Cl^- from the wall (Dainty & Hope, 1959). The treatment would not have removed Na^+ and K^+ from the wall (Dainty & Hope, 1959), though as one of the aims of the experiments was to investigate the immediate effect of ion withdrawal, the problem of complete removal was not solvable. However, it was assumed that by keeping $[Ca^{++}]=1$ mM, most cation binding sites in the wall would be occupied by the divalent ion.

Measurement of Membrane Potential

A cell was placed in a narrow glass-sided chamber, capacity 1.5 ml, and held at one end with a piece of greased perspex. Solution flow into the chamber was through a hole in the base which was linked via nylon tubing to a gravity feed reservior. During the course of measurements, solution was flowed constantly past the cell at a rate of 0.15 ml s^{-1}. The small volume of the chamber and the relatively fast flow rate ensured 90% replacement of one medium by another within 50 sec (*see* Fig. 1).

A 3 M KCl-filled electrode of tip diameter about 1 μm was inserted into the vacuole of the cell. The reference electrode was filled in the tip (diameter 10 to 20 μm) with 3 M KCl in 2% agar, and in the shank with 3 M KCl. It was positioned downstream from the cell to prevent possible contamination of the cell with Cl^- during Cl^- starvation experiments. Both electrodes were connected via bridges of 3 M KCl in 2% agar to calomel half-cells. The electrical potential difference across tonoplast and plasma membrane in series (hereafter referred to as "membrane potential") was measured on a high impedance Keithley 603 electrometer amplifier and recorded on a Tekman TE 200 potentiometric recorder. Illumination was provided by a fiber optics light source and the cell viewed with a binocular microscope. All experiments were performed at ambient temperature (20 to 22 °C).

Intracellular Perfusion

Full details of the method of intracellular perfusion and for ion flux measurements on perfused cells are given by Sanders (1980*b*). The technique involves the loss of tonoplast and most of the streaming cytoplasm from the cell and thereby enables direct access via diffusion between the perfusion medium and inside of the plasma membrane. After perfusion, the cell was tied at each end to enable turgor regeneration; this, however, restricted measurement of ion influx to a single set of conditions on each cell.

One possible reason for caution in the extrapolation of results obtained with perfused cells to intact cells is that, under the conditions of perfusion used in all experiments reported in this paper, the membrane potential was −95 to −110 mV and insensitive to internal and external pH (Sanders, 1978). This contrasts with

intact cells (Richards & Hope, 1974) and other perfusion conditions (Tazawa & Shimmen, 1980) where sensitivity of membrane potential to pH is observed. The causes of this discrepancy have not been investigated, but a likely reason is that the perfusion medium used in the present work contained EGTA at a concentration of 50 mM. This would have complexed essentially all internal Ca^{++}. Other workers (e.g., Tazawa, Kikuyama & Shimmen, 1976) have used lower concentrations of chelators, which may have left some free Ca^{++} inside the cell. However, as pointed out previously (Sanders, 1980*b*), the absolute value of Cl^- influx in perfused cells compares favorably with that observed in intact cells under similar conditions. The discrepancy of response of membrane potential to pH may, therefore, represent a specific effect of perfusion on the electrogenic H^+ pump, rather than a general alteration of membrane properties. For example, it is possible that the H^+ pump requires trace amounts of Ca^{++} for its operation.

Results are expressed in the form mean ± SEM (number of replicates or cells from which mean was obtained).

Results

Is Cl^- Cotransported with K^+ or Na^+?

The proposal by Findlay et al. (1969) that Cl^- is cotransported with K^+ (by a so-called "salt pump") was made on the experimental basis that, after withdrawal from external solution of either Cl^- or K^+ for a period in excess of 7 ksec, the flux of the counter ion was observed to have fallen. This long pretreatment was used to ensure the complete removal of the given ion from the cell wall, but was probably unnecessary. Figure 1 shows that even for K^+, which is retained in the negatively-charged wall for longer periods than is Cl^-, a response of membrane potential to withdrawal of K^+ from solution is observed within a few seconds and is complete after 100 sec. This demonstrates that removal of K^+ from the vicinity of the membrane occurs readily when bulk external K^+ is withdrawn. Therefore, if Cl^- is cotransported with K^+ or Na^+, an immediate effect on the flux should occur when the counter ion is withdrawn.

Figure 2 shows that for K^+ influx the initial effects of Cl^- withdrawal are small or nonexistent. Thus, in each of three experiments, during the first 0.3 ksec of Cl^- withdrawal, a decline in K^+ influx of 10 to 20% was observed, though this was smaller than the SEM for each batch of cells. It is clear that significant inhibitory effects of Cl^- withdrawal on K^+ influx are not seen until after 2 to 3 ksec.

The converse situation was also investigated – the effect of removal of alkali cations on influx of Cl^-. The data of Table 1 correspond to the first three points and the last point of Fig. 2. A similar behavior is found for Cl^- influx in response to K^+ removal as for K^+ influx in Fig. 2. Thus, during the first 0.3 ks of K^+ withdrawal (experiments 1 and 2), a decrease (of about 50%) in Cl^- influx is found. A K^+-dependent decline in Cl^- influx is also found in cells which have had Cl^- influx enhanced by prior Cl^- starvation (experiment 4). With 0.3 ksec pretreatment in K^+-free solution, however, the flux is restored to the control level (experiment 5). In addition, if influx is measured over a longer period of 1.8 ksec in K^+-free solution (experiment 6), no significant inhibition of Cl^- influx is apparent, presumably because that occurring in the first 0.3 ksec is obscured by the large natural intercellular variability of Cl^- influx. As with K^+ influx, after long (greater than 7 ksec) pretreatment in counter-ion-free solution, Cl^- influx falls again (experiments 7 and 8). The magnitude of this "late" fall is similar to that observed by Findlay et al. (1969) and on which they based their postulate of a salt pump.

In contrast to K^+, whose removal is equivalent to $(Na^+ + K^+)$ removal (experiments 1, 2, 4, and 7), the effects of Na^+ removal are not consistent (experiments 1, 3, 4, and 7). Na^+ does not, from this, appear to play a major role in the mechanism of Cl^- influx.

The above experiments could indicate the involvement of a KCl or "salt" pump, based on the following interpretation. Directly after counterion removal (0.3 ksec is the first portion of the time scale accessible) there is a reduction in KCl transport as the KCl transport system is inoperative. Subsequently, however, a control system shifts K^+ (or Cl^-) transport to another transport system, thus explaining the recoveries in Fig. 2 and Table 1. The following experiments were designed to test this hypothesis.

If the initial inhibition is due simply to lack of cosubstrate for a KCl pump, a similar inhibition should also be apparent for Cl^- transport into perfused cells. Table 2 shows that, for cells in which $[Cl^-]_i = 0$, there is a barely significant stimulation of influx immediately after cation withdrawal. Although there are not enough data to assess whether the stimulation is genuine or the result of sampling error, in no case was there apparent an inhibition of Cl^- influx approaching the 50% for intact cells when $(Na^+ + K^+)$ are removed. Preliminary experiments were also conducted with $[Cl^-]_i = 1$ mM and with a short pretreatment in $(Na^+ + K^+)$-free APW, and, so far as the accuracy of the data allows, no inhibition of influx was seen here either (Table 2).

In a further experiment with intact cells, K^+ and Cl^- fluxes were measured simultaneously at the termination of variable periods of Cl^- starvation. Fig. 3 shows that there is a complete independence of the starvation-stimulated portion of Cl^- influx from influx of K^+, and that, as in the case where influx of K^+ is measured without termination of Cl^- starvation in Cl^--free medium (Fig. 2), K^+ influx is inhibited only gradually by Cl^- starvation. Table 3 ex-

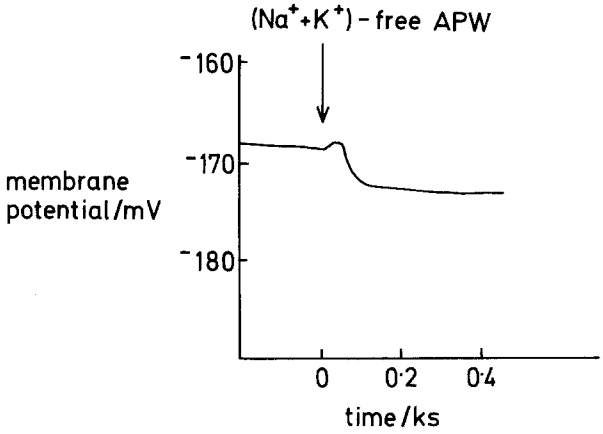

Fig. 1. Response of combined plasma membrane and tonoplast potential difference to $(Na^+ + K^+)$-free solution. Measurements made in light. At time = 0, the flowing solution was changed from APW to $(Na^+ + K^+)$-free APW

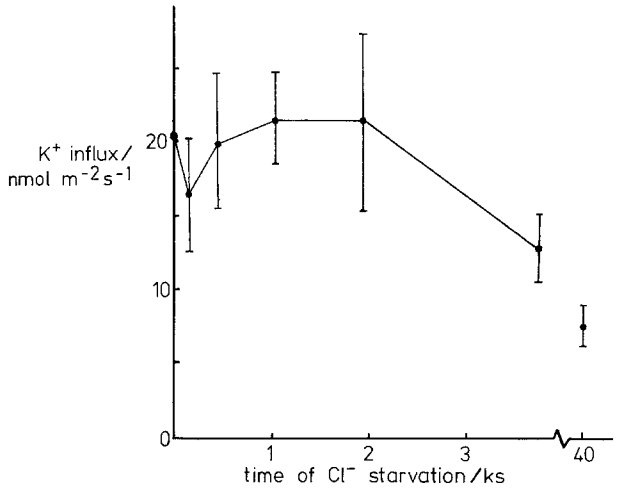

Fig. 2. Effect of Cl^- starvation on K^+ influx. Cells were pretreated in Cl^--free APW and influx of K^+ from Cl-free $^{42}K^+$-APW measured over 0.3 ksec. Data are plotted at time of pretreatment $+0.5 \times$ influx period. Vertical bars are SEM for batches of 10 cells

Table 1. Effects of monovalent cation withdrawal on Cl^- influx in intact cells[a]

Experiment number	Control Cl^- flux/ nmol m^{-2} s^{-1}	Time of pretreatment in cation-free APW/ksec	Cl^- influx as proportion of control		
			$-K^+$	$-Na^+$	$-(K^+ + Na^+)$
1	14.8±4.0	0	0.53±0.09	0.47±0.08	0.50±0.09
2	17.6±3.3	0			0.51±0.13
3	21.0±4.9	0		1.16±0.20	
4	40.2±2.9	0	0.79±0.09	1.13±0.12	0.67±0.13
5	12.5±4.3	0.3	1.11±0.36		
6	5.5±1.7	0: influx measured over 1.8 ksec	0.84±0.19		
7	14.8±4.0	7.2	0.42+0.06	0.88±0.26	0.52±0.05
8	12.5±4.3	60	0.30±0.06		

[a] Each value is mean ± SEM for batch of 9 to 11 cells. In all experiments, influx was measured in $^{36}Cl-APW$ free of the relevant cation for 0.3 ksec (except experiment 6: 1.8 ksec). Experiments 1 to 3 and 5 to 7: cells pretreated in APW overnight, followed by pretreatment for the specified period in cation-free solution. Experiment 4: cells pretreated in Cl^--free APW overnight. Experiment 8: cells pretreated in K^+-free APW overnight.

Table 2. Effect of alkali cation-free solutions on Cl^- influx in perfused cells[a]

$[Cl^-]$ in PM/mM	External solution		Cl^- influx/ proportion control	No. cells
	Pretreatment (0.3 ksec)	Influx (0.3 ksec)		
0	APW	$^{36}Cl-APW$	1.00	5
	APW	$(K^+ + Na^+)$-free $^{36}Cl-APW$	1.19±0.16	3
	$(K^+ + Na^+)$-free APW	$(K^+ + Na^+)$-free $^{36}Cl-APW$	1.27	1
1	APW	$^{36}Cl-APW$	1.00	2
	APW	$(K^+ + Na^+)$-free $^{36}Cl-APW$	0.88	1
	$(K^+ + Na^+)$-free APW	$(K^+ + Na^+)$-free $^{36}Cl-APW$	1.14, 0.97	2

[a] pH of perfusion medium (PM) was 7.45. For $[Cl]_i = 0$, the results in lines 1 and 2 are for 3 separate experiments on cells from different cultures. Control fluxes: 34.6, 23.0, 40.2 nmol m^{-2} s^{-1}. The proportional effect of $(K^+ + Na^+)$-free treatment was then averaged. For $[Cl^-]_i = 1$ mM, control influx (comparable with the first of the experiments listed above) was 17.0 nmol m^{-2} s^{-1}.

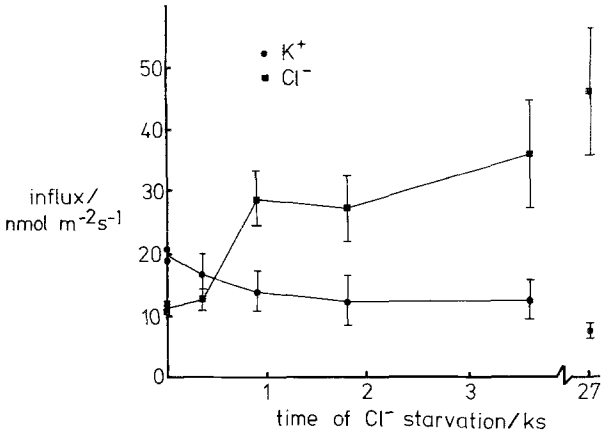

Fig. 3. Effect of termination of Cl^- starvation on Cl^- and K^+ influx. Cells were pretreated in Cl^--free APW for period shown. Influx time 0.3 ksec. Influx solution $^{42}K^+ - ^{36}Cl^- - APW$. Vertical lines are SEM for batches of 10 cells

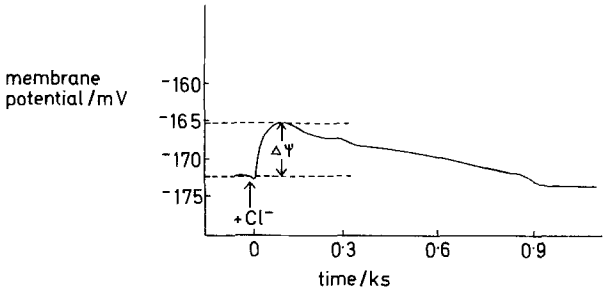

Fig. 4. Effect of termination of Cl^- starvation on membrane potential. Cell was starved of Cl^- overnight in Cl^--free APW. At time = 0, flowing solution changed to APW. $\Delta\psi$ = initial depolarization

Table 3. Effect of Cl^- starvation on influxes of Cl^-, K^+, Na^+[a]

Experiment number	Control flux/nmol m^{-2} s^{-1}			Flux at termination of Cl^- starvation/ proportion control		
	Cl^-	K^+	Na^+	Cl^-	K^+	Na^+
1	24.1 ± 5.9	53.6 ± 6.5		2.28 ± 0.29	0.45 ± 0.10	
2	12.0 ± 2.9	20.9 ± 5.2		3.85 ± 0.85	0.36 ± 0.06	
3	10.7 ± 2.4	19.1 ± 2.4		4.32 ± 0.95	0.38 ± 0.06	
4	13.8 ± 2.9	43.3 ± 6.9		3.62 ± 1.07	0.52 ± 0.06	
Mean 1–4				3.52 ± 0.44 (4)	0.43 ± 0.04 (4)	
5	10.1 ± 2.7		6.0 ± 0.8	1.62 ± 0.29		0.93 ± 0.07

[a] Each value is mean ± SEM for batch of 9 to 11 cells. Cl^- starvation in Cl^--free APW was for periods in excess of 25 ksec. Fluxes of K^+ and Cl^- were measured simultaneously in $^{42}K^+ - ^{36}Cl^- - APW$. Fluxes of Na^+ and Cl^- were measured separately. Influx period was 0.3 ksec.

pands this point, showing that concurrent with an enhancement of Cl^- influx by prior Cl^- starvation, K^+ influx is inhibited by a factor of about 2, even though Cl^- is present in bathing medium during measurement of K^+ influx. Na^+ influx remains unstimulated during the enhanced entry of Cl^-. The implication of these results for the existence of a salt pump is discussed below.

Membrane Potential Changes Accompanying Onset and Cessation of Cl^- Starvation

The results presented in Fig. 3 and Table 3 suggest that it is unlikely that Cl^- starvation results in increased activity of a "salt pump" when Cl^- is reprovided; the increased flux of Cl^- into the cell under these conditions must be balanced electrically either by an increase in the influx of H^+ or efflux of anions or by a decrease in cation efflux.

The possibility that the Cl^- transport system is electrogenic can be investigated by measurement of membrane potential changes which occur as a result of removal or resupply of external Cl^- to the cell.

Previous attempts to study the properties of the characean Cl^- transport system in this way have yielded variable results. Thus, when external Cl^- was removed, a depolarization was found in *Chara* by Findlay et al. (1969); small hyperpolarizations in *Chara* and *Nitella* by Lefevre and Gillet (1970), Pickard (1973) and Spanswick (1974); and no effect in *Chara* by Smith and Walker (1976). However, the latter workers have not ruled out the possibility that there exists an electrogenic Cl^- pump, as they suggest that any change in Cl^- current, might, on the basis

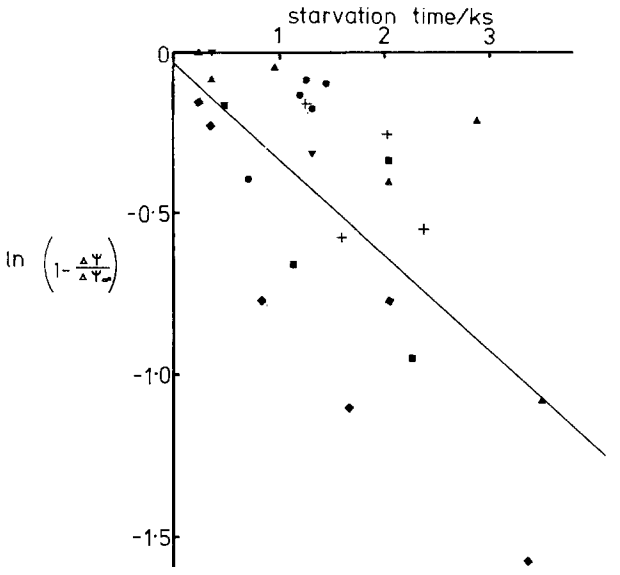

Fig. 5. Effect of Cl^- starvation time on initial depolarization ($\Delta\psi$). Each point is value from a single cell. Individual cells are identified with separate symbols. $\Delta\psi_\infty$ = initial depolarization after overnight starvation = 9.8 ± 1.3 (5) mV. Data are transformed assuming a (1-exp) rise to $\Delta\psi_\infty$. Slope of fitted line = -0.30 ksec^{-1} (95%confidence limits ± 0.15 ksec^{-1}). Time constant for rise is therefore 3.4 ksec. Additional data (marked +) were obtained from 2 of the above five cells: for these cases, Cl^- starvation and its termination were carried out in ($Na^+ + K^+$)-free solutions

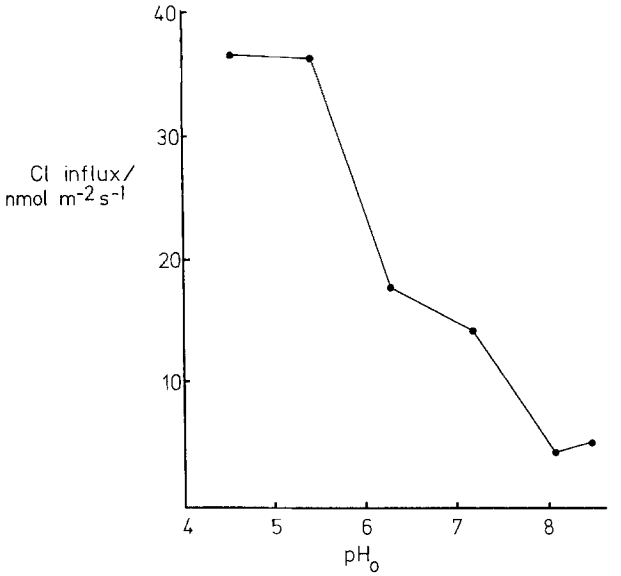

Fig. 7. Effect of external pH (pH_o) on Cl^- influx in perfused cells. Each point is mean of 2 values. Perfusion medium: pH 7.45, $[Cl^-] = 0$. External medium: $^{36}Cl - APW + 250$ mM sorbitol, buffered at appropriate pH with following buffers (each at 2 mM, adjusted with NaOH): pH 4.55, 5.40, MES; pH 6.30 MOPS; pH 7.20, HEPES; pH 8.10, CHES; pH 8.50, CAPS

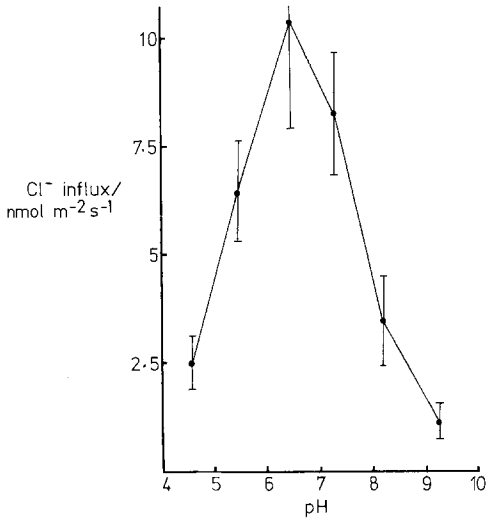

Fig. 6. Effect of pH on Cl^- influx in intact cells. Cells were pretreated overnight in APW with MES substituted by the appropriate buffer. Influx time 0.3 ksec. Influx solution $^{36}Cl^- - APW$ at same pH as pretreatment. Vertical bars are SEM for batches of 9 to 11 cells. Buffers (at final concentration of 2 mM adjusted with NaOH) as follows: pH 4.58, 5.47, MES; pH 6.40, MOPS; pH 7.28, HEPES; pH 8.23, CHES; pH 9.25, CAPS

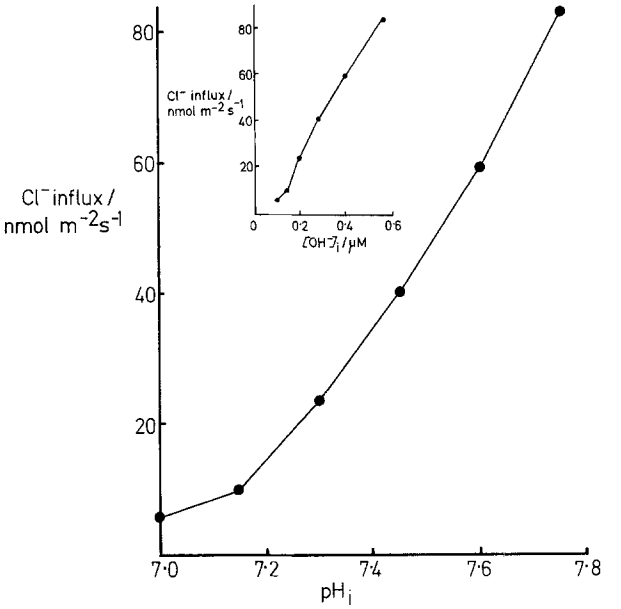

Fig. 8. Effect of internal pH (pH_i) on Cl^- influx in perfused cells. Each point is mean of 2 values. Perfusion medium: $[Cl^-] = 0$, titrated to appropriate pH with KOH. Total K^+ concentration to 155 mM with K_2SO_4 (K^+ activity in all solutions 43.5 mM). External medium: $^{36}Cl^- - APW + 250$ mM sorbitol (pH 5.4). Inset: replot of data with respect to internal $[OH^-]$

of isotopic flux measurements, be too small to be detected, given the relatively high membrane conductance. Thus the maximum expected change in membrane potential when external Cl^- is removed is calculated as 4 mV, given an electrogenic Cl^- flux of 15 nmol m^{-2} s^{-1} and a membrane conductance of 350 mS m^{-2}. Experiments such as that given in Fig. 3 demonstrate that it is possible, simply by prior starvation of the cell, to raise the rate of Cl^- transport by a factor of 3 to 4. It therefore follows that if the flux is electrogenic, its presence should be more easily detectable electrically following a starvation period, provided that one or more equivalent of charge is carried per ion.

Figure 4 shows the effect on membrane potential of resupply of Cl^- to a cell which had been starved of Cl^- overnight. An immediate depolarization is noted, followed by a slower recovery. In five cells this depolarization was 9.8 ± 1.3 mV. Similar results were obtained in solutions containing only benzenesulphonate and MES as anions: a change in the equilibrium potential for SO_4^{2-} when Cl^- is resupplied at the expense of SO_4^{2-} is, therefore, ruled out as the cause of the depolarization. When Cl^- was removed from cells which had been re-equilibrated with Cl^- for at least 7 ksec, a small hyperpolarization was sometimes apparent. The mean value of the hyperpolarization was about 2 mV for seven cases in which it occurred. In a further five cases there was no measurable potential change when Cl^- was removed.

Cells were starved of Cl^- overnight and a value obtained for the magnitude of the initial depolarization when Cl^- is resupplied ($\Delta\psi_\infty$). Values were also obtained after shorter periods of starvation, and in Fig. 5 these are linearized assuming a (1-exp) rise with time to $\Delta\psi_\infty$. Although the confidence limits of the fitted relationship are wide, it is clear that there is an increase in the magnitude of depolarization ($\Delta\psi$) with starvation time. Moreover, the time constant for the rise of $\Delta\psi$ (3.5 ksec) agrees closely with that for the rise in Cl^- influx after Cl^- starvation (4.0 ksec) (Sanders, 1980*b*). It is, therefore, likely that the depolarization is due directly to enhanced Cl^- influx, which, as a result of Cl^- starvation, rises by a factor of 3 to 4 (Sanders 1980*b*). Activation of a transport system in which Cl^- is the sole substrate would be expected to hyperpolarize the membrane. However a depolarization is observed, so it is suggested that the entry of more than one positively charged ion (or exit of more than one negative ion) must accompany each Cl^- transported.

Which ion carries this inward movement of positive charge? Also shown in Fig. 5 are values of $\Delta\psi$ after Cl^- starvation and resupply both in $(K^+ + Na^+)$-free APW. Although possibly slightly smaller than the values obtained in normal APW [as is the Cl^- flux itself sometimes after prolonged $(K^+ + Na^+)$-starvation: Sanders, 1978], the depolarization occurs essentially independently of the presence in solution of K^+ and Na^+. This is consistent with the flux data of Table 3 and Fig. 3 in suggesting that Cl^- entry after starvation is not coupled to entry of K^+ or Na^+. No known anion efflux, including that of Cl^-, is large enough to equal or exceed Cl^- influx under these conditions. Thus, although Cl^- efflux is transstimulated by external Cl^-, the absolute magnitude of this flux in the light is so small (10 to 20% that of the starvation-stimulated influx: Sanders, 1980*a*) that the depolarization cannot have its origins in the opening of a Cl^- conductance by external Cl^-. Similarly, cessation of efflux of K^+ and Na^+ would not provide sufficient charge (*see* values given by Raven, 1976). The remaining candidate for coentry with Cl^- is H^+ (or countertransport with OH^-; these two formulations are used interchangeably in the present context as they are operationally indistinguishable).

The Effects of Internal and External pH on Cl^- Influx in Intact and Perfused Cells

The proposal by Smith (1970) of a Cl^-/OH^- exchange system has led to several previous attempts to examine the pH-dependence of Cl^- influx. The modified proposal of $Cl^-/2OH^-$, as considered by Smith and Walker (1976), predicts that a pH-dependence will be caused by variation of the function $(2\Delta\bar{\mu}_H - \Delta\bar{\mu}_{Cl})$, the free energy of the transport reaction for $Cl^-/2OH^-$ exchange. Below an external pH of 7, the function has a value around -30 kJ mol^{-1}, though as external pH is raised to 9, $(2\Delta\bar{\mu}_H - \Delta\bar{\mu}_{Cl})$ falls to 0. The evidence for Cl^- cotransport with H^+ in the present work also would lead to an expectation of a degree of pH-dependence of Cl^- influx, though this could result either from a variation of the value of $\Delta\bar{\mu}_H$ if the transport system exists at or near reversible equilibrium or simply from a dependence of the activity of the system on H^+ as substrate (kinetic control). In both situations it would therefore be expected that maximum Cl^- influx occurs at low external pH (pH_o). Figure 6 shows that for intact cells this is not so; below an optimum pH of 6.3, influx is strongly inhibited by pH_o. This observation alone does not, of course, invalidate the hypothesis of Cl^-/H^+ cotransport.

One possible explanation for these results is that pH_o acts in other ways on the transport system than those simply concerning variation of substrate H^+;

these nonspecific effects would be analogous to those which pH ultimately has on the activity of any enzyme, whether H^+ is involved in the catalyzed reaction or not. However, several secondary effects of pH_o occur in intact cells which it is possible to eliminate in perfused cells. For example, membrane potential is highly sensitive to pH_o in intact cells (Richards & Hope, 1974), and yet in perfused cells this dependence does not occur (*see* Materials and Methods). In addition, the cytoplasmic pH (pH_c) of intact cells is a function of pH_o (Smith & Walker, 1976), while in perfused cells, the overall buffering capacity is much greater and internal pH is probably constant during short-term changes in pH_o. Although the dependency of pH_c on pH_o in intact cells is small (Smith & Walker, 1976), it is nevertheless possible that small changes of pH_c could have significant effects on transmembrane fluxes.

The effect of pH_o on Cl^- influx into perfused cells was therefore examined. Figure 7 shows that the trend for an optimum pH_o for Cl^- influx at pH 6.3 has now disappeared. A simpler relationship emerges, in which Cl^- influx is approximately inversely correlated with pH_o over the range pH_o 5 to 8. If this represents the direct response of the transport system to pH_o (rather than being mediated by a secondary factor), then one possible control over Cl^- influx in intact cells is, as described above, pH_c. For this reason the effect of varying internal pH in perfused cells was also investigated. The results are shown in Fig. 8. Comparison with Fig. 7 shows that influx is more sensitive to internal pH than to pH_o. However, as with the effects of pH_o, the response is again in a direction consistent with that of a pump transporting H^+ into (or OH^- out of) the cell. In the inset, the data have been replotted with respect to internal $[OH^-]$.

The Dependence of Cl^- Influx on ATP

The data presented so far have suggested that Cl^- coenters the cell with H^+. Moreover, with a H^+/Cl^- stoichiometry of $>1:1$, the free energy gradient up which Cl^- must be moved to cross the plasma membrane is significantly lowered. If the stoichiometry is in fact 2:1, then Smith and Walker (1976) suggest there is sufficient energy stored in the Cl^- and H^+ gradients alone to drive net Cl^- influx in all conditions of external pH over which Cl^- influx was investigated: no other energy input to the system would be required. For the present experiments with perfused cells, a similar situation obtains. Thus, for all conditions used, the free energy stored in the combined ion gradients of Cl^- and H^+ creates an inward driving force on a $2H^+/Cl^-$ transport system. This raises the interesting problem of whether the transport system shows a more direct coupling to metabolism.

Table 4 shows that Cl^- influx into perfused cells is stimulated almost fourfold by the inclusion of 1 mM ATP into the perfusion solution. (For this reason ATP was routinely incorporated into the perfusion medium used in all other experiments.) Raising the concentration of ATP to 10 mM inhibits influx compared with the effects of 1 mM ATP, although there is nevertheless a twofold stimulation over ATP-free controls. In the single experiment in which it was used, ADP gave rise to a slight, but nonsignificant, stimulation. It is possible that any residual adenylate kinase not washed out by perfusion could have contributed to this effect by synthesizing ATP.

Discussion

The Involvement of a KCl Pump

In intact cells, the inhibition of the fluxes of K^+ and Cl^- during the first 0.3 ksec of counterion removal could be taken to indicate the contribution of a KCl pump. However, such a pump would not seem to account for the major portion of the fluxes, especially after Cl^- starvation, where Cl^- influx exceeds that of K^+ by a factor of 2 to 6 (Table 3).

In addition, the lack of dependence of Cl^- influx on external K^+ in perfused cells is strong evidence against the existence of a KCl pump. The question then arises: why is there a partial mutual link between the fluxes of K^+ and Cl^- in intact cells?

One possible explanation for the apparent link between K^+ and Cl^- in intact cells invokes the mediation of internal (cytoplasmic) pH. Thus, if K^+ entry were in exchange for H^+ efflux (for which there is no direct proof), then sudden removal of K^+ could lead to a decrease in pH_c. Fig. 8 shows that this would in turn inhibit Cl^- influx. Similarly, if K^+ influx is inhibited at high pH, cessation of Cl–H cotransport would reduce this flux. However, the magnitude of the internal pH change would be expected to be far greater in the case of intact cells because of the restricted volume of the compartment (cytoplasm) directly inside the plasma membrane. The size of this compartment is increased by a factor of about 40 by perfusion and is, therefore, better buffered than cytoplasm, in spite of the similarity in buffering capacity per unit volume of perfusion medium and plant cell cytoplasm (*see* Raven & Smith, 1976). It is then possible that recovery observed for fluxes of both K^+ and Cl^- after 0.3 ksec in solution free of the counterion (Fig. 2 and Table 1, experiments 5 and 6)

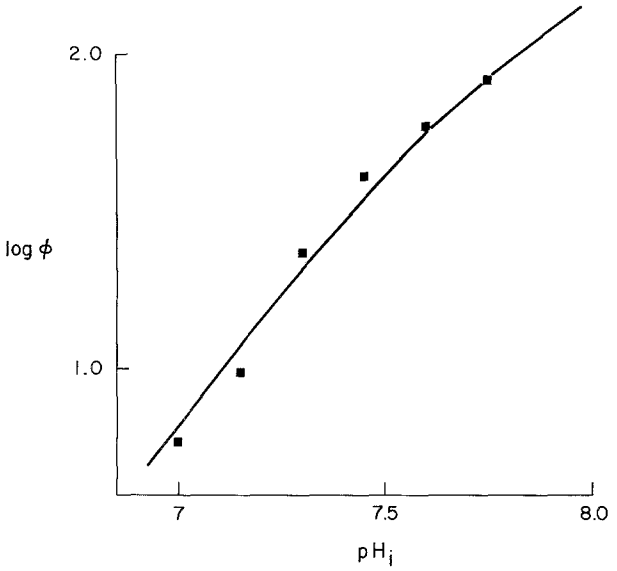

Fig. 9. Dependence of Cl^- flux (Φ) on pH_i. Replot of data of Fig. 8. The line has been fitted assuming the relation

$$\log \Phi = \log \Phi' - 2 \log (1 + H^+/K_h)$$

in which Φ' is the maximum flux (425 nmol $m^{-2} s^{-1}$) and K_h the dissociation constants for H^+ of 2 sites. $K_h = 1.41 \times 10^{-8}$; i.e., the pK for the two sites is 7.85

occurs as a result of subsequent regulatory adjustment of pH_c (Hansen, 1978).

Clearly the acceptability of this explanation relies heavily on assumptions about the nature of K^+ influx—its ionic coupling (if any) and internal control. Previous workers (Kitasato, 1968; Walker & Hope, 1969) have concluded, in contradiction to the above proposal, that K^+ influx and efflux are not ionically coupled (passively electrogenic) as the fluxes respond steeply to membrane potential in the direction expected for transport of positive charge. However, these results could also be interpreted in terms of K—H countertransport as follows. It is known that the ion with the highest plasma membrane conductance is H^+ (Kitasato, 1968). Thus, when clamping at negative membrane potentials, pH_c will tend to fall. If K^+ influx is stimulated by low pH_c, as suggested above, this would give rise to the observed effects of voltage clamp on K^+ influx, which would be indirect. Recent work by Bielby and Walker (1980) has shown a large discrepancy between the effect of long and short term voltage clamp on Cl^- current in *Chara*, and the explanation of pH_c change induced by change of membrane potential could apply here too.

The decline in K^+ and Cl^- influx after long (greater than 3 ksec) periods in solutions free of the counter-ion seems to be caused by another feedback mechanism. This could be analogous to that in *Neurospora*, which in times of starvation of any one particular ion or nutrient, shuts down transport in general except for the ion or nutrient which is deficient in the outer medium (Slayman, 1980). Clearly not appropriate as an explanation for this long term fall in fluxes, however, is the proposal of Findlay et al. (1969), that a salt pump is responsible.

Table 4. Effect of ATP on Cl^- influx in perfused cells[a]

Exp.	Relative Cl^- influx			
	Control	+ATP (1 mM)	+ATP (10 mM)	+ADP (10 mM)
A	1.00±0.18 (3)	3.76±0.81 (3)	2.38±0.35 (3)	–
B	1.00±0.14 (5)	–	2.31±0.20 (10)	1.35±0.50 (5)

[a] Perfusion medium composition: ATP or ADP added as K_2Na_2 salt. $[Na^+]$ adjusted to 20 mM with Na_2SO_4 where necessary. Free $[Mg^{++}]$ was adjusted to 4 mM using binding constants to EGTA given by Portzehl, Caldwell, and Rüegg (1964) and to ATP given by Wood, Davies, and Lochmüller (1966). pH was 7.45. External solution: ^{36}Cl—APW+250 mM sorbitol. Control for experiment A: 12.0 nmol $m^{-2} s^{-1}$; for experiment B: 10.3 nmol $m^{-2} s^{-1}$.

Evidence for Coupled H^+/Cl^- Entry and the Stoichiometry of the Transport System

The proposal for Cl—H cotransport was justified in the Results section on the basis that (a) the magnitude of the depolarization at the end of Cl^- starvation shares a common rise time with that of the induction of the isotopically labeled flux and is, therefore, a Cl^- current; (b) the Cl^- current is essentially independent of external alkali cations, and the isotopically measured flux larger than that of any measured ion efflux. Measurements of current flow produced when Cl^- is provided to starved *Chara* under voltage clamp conditions have enabled a similar conclusion to be drawn by Bielby and Walker (1980).

Two points of interest arise concerning the nature of the depolarization of membrane potential when Cl^- is resupplied to starved cells. Firstly, there is always observed a repolarization of the membrane with a time constant of 0.3 to 0.6 ksec (Fig. 4). In contrast, the time constant for decay of the starvation-stimulated Cl^- flux in the presence of external Cl^- is 1.7 ksec (Sanders, 1980*b*) so the repolarization cannot have its origins in the decreasing activity of the transport system. A similar complete or partial repolarization of the membrane is noted for all other fungal and plant H^+ cotransport systems so far investigated, including glucose transport in *Neurospora* (Slayman & Slayman, 1974; Slayman, Slayman & Hansen, 1977), in *Chorella* (Komor & Tanner, 1976)

and in parenchyma cells of *Impatiens* (Jones, Novacky & Dropkin, 1975), for glycine and NO_3^- transport in *Lemna* (Novacky et al., 1978), and for various amino acids and carbohydrates in oat coleoptile (Etherton & Nuovo, 1974). A possible explanation for this is that the electrogenic H^+ pump increases activity due to increased entry of H^+ in order to restore cytoplasmic pH. That the feedback system which is assumed to regulate pH_c in characean cells has a similar time constant (Hansen, 1978) to that found for repolarization is in support of this suggestion.

A second consideration arises from the near-zero intercept for the rise of peak depolarization with Cl^- starvation time (Fig. 5). As the maximum starvation-stimulated Cl^- influx is greater than control Cl^- influx by a factor of only 3.3 (Sanders, 1980*b*), it would be expected that $\Delta\psi_\infty$ (depolarization after overnight starvation) should exceed $\Delta\psi_o$ (depolarization when Cl^- is resupplied immediately after it is withdrawn) by this factor also. This would give an intercept on the ordinate of Fig. 5 at -0.36 (i.e., 3.0 mV), which, in spite of the large scatter of the data, would clearly not be justified. The explanation for this discrepancy may be that membrane resistance increases with time of Cl^- starvation; this phenomenon is well characterized in *Neurospora* (Slayman, 1980), where starvation of a wide range of organic metabolites and inorganic ions can lead to greater effects of substrate reprovision on membrane potential than would be present were the initiation of electrogenic cotransport occurring across normal membrane resistance. Thus, it is predicted that the magnitude of depolarization when Cl^- is reprovided to starved cells reflects not only a change in H^+-coupled Cl^- flux, but also a progressive change in membrane resistance.

Considerable attention has been focused by other workers (*see*, for example, Hope & Walker, 1975) on Smith's (1970) hypothesis for Cl^-/OH^- exchange. However, in an earlier paper (Sanders, 1980*b*) I have suggested that all data used in support of the hypothesis can more satisfactorily be explained in terms of control processes. Nevertheless, the net result of the present investigations is a confirmation of the revised form of the original proposal (Smith & Walker, 1976) in that Cl^- is cotransported with more than one H^+. The effects of internal and external pH on Cl^- influx corroborate, but do not prove, the hypothesis that Cl^- and H^+ are cosubstrates for the same transport system.

An attempt to estimate the stoichiometry of $H^+:Cl^-$ for the transport system can be made from measurements of depolarization and the known Cl^- flux at the termination of Cl^- starvation. The mean depolarization after overnight starvation is 9.8 ± 1.3 mV, so assuming a membrane conductance of 800 mS m^{-2} (Findlay & Hope, 1964), the current flowing through the cotransport system is 7.8 ± 1.0 mA m^{-2}. Thus the charge-carrying activity of the transport system is 81.2 ± 10.8 neq $m^{-2}s^{-1}$ under these conditions. However, as outlined above, Cl^- starvation may result in a lower conductance than that measured with Cl^- present. Taken in conjunction with the initial starvation-stimulated Cl^- influx of 58.7 nmol m^{-2} s^{-1} (Sanders, 1980*b*), this could suggest net inward transport of one positive charge per Cl^- at termination of starvation, i.e., $2H^+:1Cl^-$. It should be pointed out, however, that there exists considerable variation in conductance estimates of *Chara* plasma membrane (Hope & Walker, 1975). For example, that of Smith and Walker (1976) of 350 mS m^{-2} would give $H^+:Cl^- = 1.4$ to 1.8. In addition, the assumption that membrane conductance is invariant with voltage may not be justified.

A more thorough investigation of the problem of $H^+:Cl^-$ stoichiometry has been conducted recently by Bielby and Walker (1980), who also suggest $H^+:Cl^- = 2:1$.

Kinetic Effects of pH_i on Cl^- Transport: Possible Support for $2H^+:1Cl^-$

In Fig. 9 are replotted the data of Fig. 8 on a log scale *vs.* pH_i. From such a plot, it should be possible to determine the pK of the ionizable group(s) which must be deprotonated for transport to occur, provided the following conditions are met (*see* Cleland, 1970): (i) Transport rate is measured under conditions where all substrates are saturating. The data are for $[Cl^-]_o = 1$ mM, which is about 25 times the K_m for the transport system for Cl^- in these cells (D. Sanders, *in preparation*) and at optimum external pH (Fig. 7). (ii) The deprotonation step being looked at is solely rate-limiting for transport. There is no information on this point, so the estimate of pK must be taken as a minimum.

The data cannot be fitted assuming the deprotonation of a single site; the maximum slope this could generate would be +1, whereas the experimental points exceed this value. However, there is a good fit if it is assumed that two dissociable sites control transport rate. (In Fig. 9 it is assumed that the two sites have (equal) pK's of 7.85, though data have not been collected over a wide enough range of pH to ascertain whether different pK's either side of this value would provide a better fit.)

It is possible that the deprotonation steps represent unbinding of transported H^+ inside the cell (or loading of substrate OH^-). This interpretation would support the suggestion above that more than $1H^+$

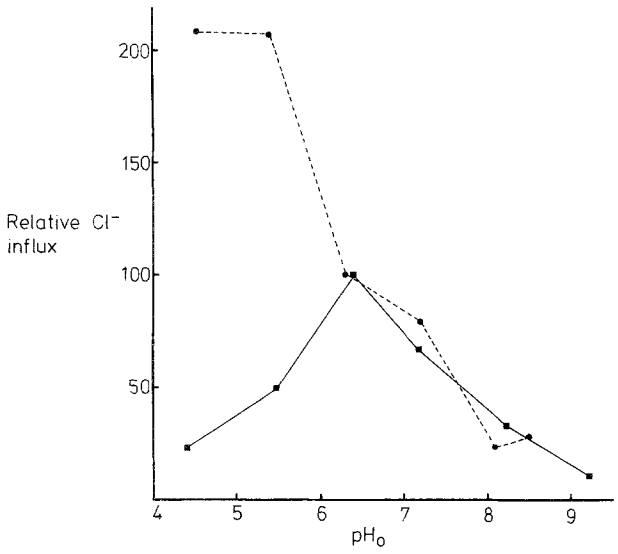

Fig. 10. Comparative effects of external pH on Cl^- influx in intact (■) and perfused cells (●)

is transported per Cl^-, though the possibility of a stoichiometry greater than $2H^+:1Cl^-$ is not excluded.

Energy Sources for Cl^- Influx

Smith and Walker (1976) have suggested that if Cl^- is cotransported with $2H^+$, there exists across the plasma membrane a combined electrochemical potential gradient for both ions sufficient to drive net Cl^- influx under all conditions of external and internal pH for which net influx is observed. This is also true for all the perfusion conditions used in the present experiments. At first sight, therefore, the stimulation of Cl^- influx in perfused cells by ATP (Table 4) is surprising, and recent work by Reid and Walker (1980) has shown only marginally significant effects of ATP on Cl^- influx in perfused *Chara*. The reasons for the discrepancy between their results and those reported in the present work are not known. Two possible indirect effects of ATP on Cl^- influx can be discounted. First, although other workers have obtained a hyperpolarization of the membrane when ATP is included in the perfusion medium (Shimmen & Tazawa, 1977), a sustained effect of ATP on membrane potential was not observed using the present perfusion system (Sanders, 1978). In addition, even if ATP stimulates the proton pump under the present conditions without causing significant electrogenesis, it is unlikely that the pH of the strongly buffered perfusion medium would change significantly during the flux measurement period. Although the external medium was more weakly buffered, reference to Fig. 7 shows that lowering of external pH by one unit to 4.5 results in no further stimulation of Cl^- influx.

The conclusion, therefore, is that ATP has a more direct effect on Cl^- transport. In this respect one possible explanation is that ATP acts catalytically to induce some conformational change in the transport system without net hydrolysis. However, under some circumstances, additional energy input to Cl^- transport, besides that stored as $\Delta\bar{\mu}_H$, may be required. This might apply especially to the conditions experienced by *Chara* in its natural habitat, where both Cl^- and H^+ are at low concentration externally (Stroede, 1933; Hutchinson, 1975). To test the possibility of an additional energy source such as ATP, the critical experiments have not yet been performed in which an attempt is made to create a net influx of Cl^- in the absence of an ionic driving force provided by Cl^- and H^+ combined.

In the present work with perfused cells, the use of perfusion media free of Cl^- does not permit evaluation of the function $(2\Delta\bar{\mu}_H - \Delta\bar{\mu}_{Cl})$. The influence of the ion gradients of H^+ and Cl^- on Cl^- influx is examined in a subsequent paper (D. Sanders, *in preparation*).

Physiological Implications of the Effects of pH on Cl^- Transport in Intact and Perfused Cells

Similar effects of pH_o on Cl^- transport to those reported here have been obtained with *Chara* by Jayasuria (1975) and Smith and Walker (1976), and on *Nitella* by Spanswick and Miller (1977). The dependence of pH_c on pH_o demonstrated by a method of weak acid distribution (Smith & Walker, 1976), considered in relation to the high sensitivity of Cl^- influx to small changes in pH_c in perfused cells (Fig. 8), enables an explanation to be developed for the form of the relationship between Cl^- influx and pH_o in intact cells. The absence of stimulation of Cl^- influx at low pH_o in intact cells (in contrast to the situation in perfused cells, Fig. 7) can be explained if pH_c is also low at low pH_o. Thus pH_c will be rate-limiting to transport at low pH_o and only in the middle of the external pH range does optimal transport occur.

A semi-quantitative evaluation of this proposal is obtained by direct comparison of the effects of pH_o on Cl^- influx in intact and perfused cells (Figs. 6 and 7). These data have been normalized to pH 6.3/6.4 in Fig. 10. It is seen that above this pH there is good agreement between the two conditions, suggesting that pH_o is indeed rate-limiting. Below pH 6.3, Cl^- influx in intact cells is inhibited to 30% and 12% at pH_o 5.5 and pH_o 4.6 respectively, compared with the rates expected from perfused cells.

The extent to which this inhibition results from a dependence of pH_c on pH_o in intact cells can be estimated using the relationship between pH_c and pH_o given by Smith and Walker (1976). Thus, at $pH_o = 6.4$, $pH_c = 7.69$; at $pH_o = 5.5$, $pH_c = 7.49$; and at $pH_o = 4.6$, $pH_c = 7.29$. Cl^- influx at these internal pH's, as percentages of that at internal pH 7.69 (from Fig. 8), are 62% at pH 7.49 and 31% at pH 7.29. It is therefore predicted that if pH_c is the sole cause of inhibition of Cl^- influx at low pH_o, the inhibition will be to 62% at pH_o 5.5 (*cf.* the observed value of 30%) and to 31% at pH_o 4.6 (*cf.* 12%: Fig. 10). Whether this discrepency arises from the effects of pH_o on an additional factor which also acts on Cl^- influx is considered in a forthcoming paper (D. Sanders, *in preparation*).

The high sensitivity of Cl^- influx to internal pH may also explain some other observations in the literature. The inhibition of Cl^- influx in *Chara* by uncouplers of photosynthesis and respiration (Smith & Raven, 1974) should be considered primarily in terms of changes in pH_c (rather than ATP supply) resulting from the ability of these compounds to conduct H^+ flow through the plasma membrane (Felle & Bentrup, 1977).

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