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Plasmalemma Chloride Transport in *Chara corallina*[1]

INHIBITION BY 4,4'-DIISOTHIOCYANO-2,2'-DISULFONIC ACID STILBENE

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ABSTRACT

Chloride transport, presumably via a Cl^--$2H^+$ co-transport system, was investigated in *Chara corallina*. At pH 6.5, the control influx (3.1 picomoles per centimeter² per second) was stimulated 4-fold by an 18-hour Cl^- starvation. The stimulated influx was inhibited to 4.7 picomoles per centimeter² per second after a 60-minute pre-exposure to 0.5 millimolar 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS). This compares with a nonsignificant inhibition of the control under similar conditions. At 2 millimolar DIDS, both stimulated and control influx were inhibited to values of 1.1 and 2.2 picomoles per centimeter² per second, respectively; in all cases, DIDS inhibition was reversible. Over the pH range 4.8 to 8.5, the control and DIDS-inhibited influx showed only slight pH sensitivity; in contrast, the stimulated flux was strongly pH dependent (pH 6.5 optimum). Inasmuch as changes in pH alter membrane potential, *N*-ethylmaleimide was used to depolarize the membrane; this had no effect on Cl^- influx. A transient depolarization of the membrane (about 20 millivolts) was observed on restoration of Cl^- to starved cells. The membrane also depolarized transiently when starved cells were exposed to 0.5 millimolar DIDS, but the depolarization associated with Cl^- restoration was inhibited by a 40-minute pretreatment with DIDS. Exposure of control cells to DIDS caused only a small hyperpolarization (about 7 millivolts). DIDS may have blocked Cl^- influx by inhibiting the putative plasmalemma H^+-translocating ATPase. Histochemical studies on intact cells revealed no observable effect of DIDS on plasmalemma ATPase activity. However, DIDS application after fixation resulted in complete inhibition of ATPase activity.

The differential sensitivity of the stimulated and control flux to inhibition by DIDS may reflect an alteration of transport upon stimulation, but could also result from differences in pretreatment. The stimulated cells were pretreated with DIDS in the absence of Cl^-, in contrast to the presence of Cl^- during pretreatment of controls. The differential effect could result from competition between Cl^- and DIDS for a common binding site. Our histochemical ATPase results indicate that Cl^- transport and membrane ATPase are separate systems, and the latter is only inhibited by DIDS from the inside of the cell.

In *Chara corallina*, as in other plants, chloride is actively accumulated against a large electrochemical potential gradient. The electric potential across the *Chara* plasmalemma usually falls within the range −190 to −250 mv (cytoplasm negative), depending on media conditions (10, 11). In external solutions of 1 mM Cl^-, it has been estimated that cytoplasmic Cl^- is 7 to 10 mM (17). Although fewer measurements are available for the potential across the tonoplast, the value is probably somewhere near +20 mv, the vacuole being positive with respect to the cytoplasm (9). In *C. corallina*, vacuolar Cl^- is about 106 mM (8). Since the vacuole constitutes approximately 95% of the cell volume, substantial amounts of Cl^- must be transported across both the plasmalemma and tonoplast in order to provide the osmotic activity necessary for cell expansion (7).

Transport across both these membranes may involve the indirect coupling of Cl^- movement to an ATP-dependent electrogenic H^+ current. Considerable evidence exists in support of the hypothesis that an electrogenic H^+ efflux system operates across the *Chara* plasmalemma (for a review, see Ref. 23). The concept that Cl^- transport may be coupled to the resulting proton gradient was first postulated by Spear *et al.* (24) and subsequently developed by Smith (21) and Smith and Walker (22). It has been proposed that the transport stoichiometry for Cl^- uptake is $2H^+/Cl^-$ transported across the plasmalemma (22). Only recently has transport into the vacuole been examined in terms of the possible mechanism(s) involved. Using isolated plant membrane fractions, Bennett and Spanswick (1) have obtained results consistent with the operation of an electrogenic H^+ ATPase located in the tonoplast. This ATPase appears to be Cl^--dependent and may be the system responsible for driving Cl^- transport into the vacuole.

In his recent studies on Cl^- transport into *C. corallina*, Sanders (16–18) found that rates of transport across the plasmalemma were not controlled by the actual thermodynamic driving force, but were under kinetic control. The transport model, proposed by Sanders and Hansen (19) to explain their experimental data, suggests that a depletion of the cytoplasmic Cl^- level acts in a kinetic sense to greatly enhance the flux across the plasmalemma. Despite these details, little is known about the spatial and/or mechanistic relationship(s) between the Cl^--$2H^+$ co-transport process and the H^+-translocating ATPase of the plasmalemma.

Anion transport processes in animal cell membranes (*e.g.* into red blood cells [3]) can be inhibited irreversibly by the membrane-impermeable aminoreactive agent, DIDS[3] (2, 3, 20). Recently, Lin (13) showed that DIDS was an effective inhibitor of Cl^- and SO^{2-}_4 transport into isolated corn root protoplasts. Lin also reported that DIDS inhibited the ATPase activity of isolated corn root plasma membranes; this result is consistent with the work of Churchill and Sze (4).

In the present paper, we report on the inhibitory effect of DIDS on Cl^- transport into *Chara corallina*. We found that DIDS was

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[3] Abbreviations: DIDS, 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene; APW, artificial pond water; NEM, *N*-ethylmaleamide; CPW, *Chara* pond water.

very effective in inhibiting the stimulated component of Cl^- influx, *i.e.* the enhanced component that was obtained by an 18-h pretreatment of cells in Cl^--free medium. However, unlike the animal anion transport systems, the DIDS inhibition could be reversed, indicating that an irreversible covalent bond had not been formed. Plasma membrane ATPase activity (as evidenced by histochemical studies) was not affected by DIDS, except when this aminoreagent was included in the reaction medium following tissue fixation for electron microscopy. Our results are consistent with DIDS acting as a competitor for the Cl^- binding site, presumably on the membrane carrier.

MATERIALS AND METHODS

Culture Material. *Chara corallina* Klein ex Willd., em R.D.W. (= *Chara australis* R. Br.) was cultured in the laboratory (10). Internodal cells (2–3 cm for electrophysiology; or 4–8 cm long, containing 1–3 cells, for flux studies) were cut the day before the experiments. After measuring their surface areas, cells were preincubated (overnight) in either APW-6.5 or Cl^--free APW-6.5 under continuous light (10 w m^{-2}, approximately 18 h). Cells used to investigate the effect of NEM on $^{36}Cl^-$ influx were pretreated in CPW-6.5 and given an 8-h dark period, followed by 3-h illumination (10 w m^{-2}) before being used in an experiment.

Experimental Solutions. The basic solution used for the DIDS experiments, APW-6.5, was prepared using high purity, glass-distilled H_2O and contained (mM): 1.0 NaCl, 0.2 K_2SO_4, 1.0 $CaSO_4$, 2.0 Mes, adjusted to pH 6.5 with freshly dissolved NaOH. Chloride-free APW-6.5 was as above but with 0.5 mM Na_2SO_4 substituting for the 1.0 mM NaCl. The DIDS (Calbiochem) was added to the experimental solutions just before use and the solution pH was adjusted to the desired value. In some experiments, solution pH values other than 6.5 were used. In those cases, the pH and buffers used were: pH 4.8, Mes; pH 5.7, Mes; pH 7.5, Hepes; and pH 8.5, 3-{[tris(hydroxymethyl)methyl]amino}propanesulfonic acid (Taps); all at 2.0 mM.

In experiments with NEM, a slightly different basic solution was used, CPW, which contained (mM): 1.0 NaCl, 0.2 KCl, 0.2 $CaSO_4$, and either 5 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) at pH 6.5 or 5 mM 3-{[tris(hydroxymethyl)methyl]amino}propanesulfonic acid at pH 8.0. A 100 mM stock solution of NEM was prepared and stored at 4°C for up to 3 d. The concentration (0.1 mM) of NEM in the experimental solution was assayed spectrophotometrically (at *A* 302 nm; extinction coefficient = 620 M^{-1} cm^{-1}).

Electrophysiological Measurements. The equipment, electrodes, and methods used in these experiments have been previously described (10). However, most DIDS experiments were performed in nonflowing solutions to reduce the amount of reagent utilized per experiment.

Measurements of $^{36}Cl^-$ Influx. Radioactive ^{36}Cl was obtained from ICN (Irvine, CA) as a sterile aqueous solution of NaCl. Experimental solutions were prepared such that they contained 1.0 mM total Cl^- for the DIDS experiments and 1.2 mM Cl^- for the NEM experiments; all solutions had a specific activity of approximately 80 μCi/mmol chloride. Following the prescribed pretreatment, cells were transferred from Petri dishes into sealed tubes that were placed in a thermostated water bath (25°C) in the light (20 w m^{-2}) for a 1- to 2-h equilibration period. Solutions used for this pretreatment will be specified in the text. In experiments in which we examined the effects of pH, the pretreatments were done at the same pH values used during uptake. A $^{36}Cl^-$ uptake period of 10 min was employed because the stimulation in Cl^- influx, caused by cell exposure to Cl^--free conditions, decreases rapidly (17). A 1-min wash, followed by a second 5-min wash, was used to remove $^{36}Cl^-$ present in the cell wall. Individual cells were then blotted and placed into scintillation vials where they were treated (15 min) with 0.1 ml of 30% H_2O_2 and 0.1 ml of 7% $HClO_4$ to digest the tissue and prevent Chl quenching during scintillation counting. Tissue was suspended in 10 ml of a toluene-based scintillation fluid (containing 300 ml Triton X-100, 40 ml ethanol, 5 g 2,5-diphenyloxazole, and 0.1 g POPOP plus toluene to make 1 L final volume) and radioactivity was measured in a Beckman (LS 9800) liquid scintillation system. Influx into each cell was calculated using its measured surface area. Typically, each treatment consisted of 12 cells; in some cases, treatments were pooled. Results are reported as average ± SE (*n* = total number of cells measured).

ATPase Localization. Shoots were cut from the *Chara* cultures and incubated in APW-6.5 or Cl^--free APW-6.5 for 18 h (continuous light, 10 w m^{-2}). Some Cl^--starved shoots were incubated in 0.5 mM DIDS (80 min) and fixed directly, or the 0.5 mM DIDS treatment was followed by a 1- or 15-min wash with Cl^--free APW-6.5 prior to tissue fixation. Primary fixation was in 1.25% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 2 h at 4°C. This was followed by a 2-h rinse in 50 mM sodium cacodylate buffer (three changes) and a 15-min rinse in 50 mM Tris-maleate buffer (pH 7.2). The shoots were then incubated for 2 h (in dark; room temperature) in reaction medium containing 2 mM $Pb(NO_3)_2$, 2 mM ATP, and 2 mM $Mg(NO_3)_2$ in 50 mM Tris-maleate buffer (pH 7.2). They were then washed with Tris-maleate buffer for 15 min followed by a 30-min wash with sodium cacodylate buffer. Postfixation was for 1 h at 23°C in 1% OsO_4 (25 mM cacodylate buffer, pH 7.2). Shoots were then rinsed with distilled H_2O, dehydrated with an acetone series, and embedded in Spurr's resin (25). Thin sections were examined, unstained, using a JEOL 100S or Zeiss 10 transmission electron microscope.

In one experiment, DIDS was added both to the Tris-maleate wash (15-min) and the following ATPase reaction medium. Controls for the specificity of the ATPase reaction in this tissue have been previously described (7).

For our NEM studies, cells were cut and pretreated as described under "Culture Material." In some experiments, *Chara* cells were exposed, for 15 min, to 1 mM NEM (CPW-6.5) prior to primary fixation. The tissue was then rinsed with CPW-6.5, placed into fixative solution, and further processed as outlined above. The effect of including 1 mM NEM in both the Tris-maleate buffer rinse and the ATPase incubation medium was also examined.

RESULTS

Electrophysiological Measurements. Representative membrane potential changes are illustrated in Figure 1; the potentials represent values measured between the vacuole and the bathing medium. Because the potential across the tonoplast is small, we will refer to our measured potentials as representing the value across the plasmalemma. Reintroducing Cl^- to cells that had been pretreated for 18 h in Cl^--free APW-6.5 elicited a large transient depolarization in the potential across the plasmalemma (Fig. 1A). This response is similar to that reported by Sanders (18); however, the magnitude of our depolarization was larger, being 20 ± 4 (*n* = 5) mv (*cf.* 9.8 ± 1.3 mv [18]). Treatment of control (*i.e.* not Cl^--starved) cells with 0.5 mM DIDS resulted in a small, 4- to 10-mv hyperpolarization of the potential (Fig. 1B). When DIDS was applied to cells that had been treated in Cl^--free APW-6.5 for approximately 18 h, the membrane potential depolarized by 10 to 20 mv, but then quickly hyperpolarized to a value 21 ± 6 (*n* = 9) mv more negative than the initial potential (Fig. 1C). After 40 to 60 min in DIDS solution, we introduced Cl^- in the presence of DIDS. As shown in Figure 1D, this introduction of Cl^- had little effect on the potential.

In the experiments outlined above, the treatments had, at most, only a small influence on measured values of membrane resistance.

DIDS Inhibition of $^{36}Cl^-$ Influx. The results presented in Figure 1 suggest that DIDS may have an inhibitory effect on Cl^- transport

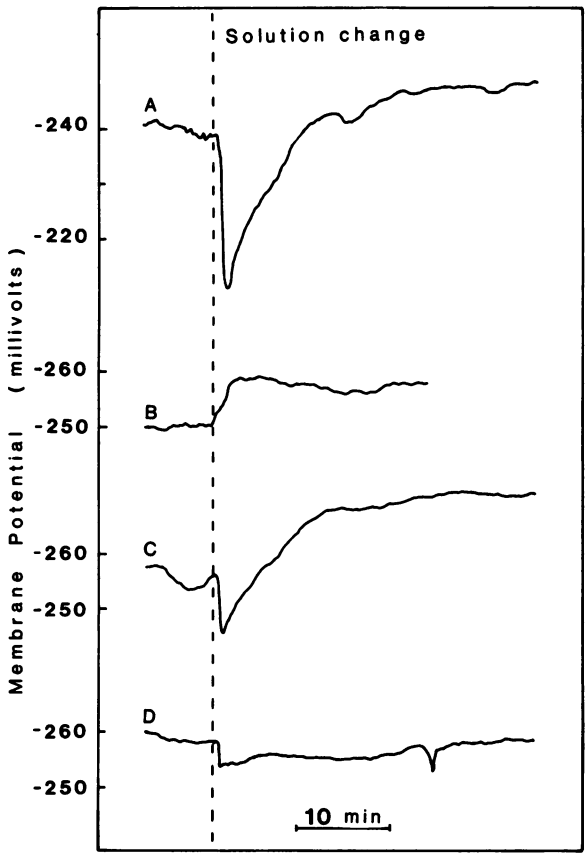


FIG. 1. Membrane potential measurements in *Chara*. A, Cell pretreated for 18 h in Cl^--free APW-6.5; then, at the dashed line, the experimental medium was changed to APW-6.5 containing 1.0 mM Cl^-. B, The cell was pretreated for 18 h in APW-6.5; this cell was then exposed to APW-6.5 + 0.5 mM DIDS. C, After an 18-h treatment in Cl^--free APW-6.5, cells were exposed to Cl^--free APW-6.5 + 0.5 mM DIDS. D, following 40 to 60 min in the solution in C, the medium was changed to APW-6.5 + 0.5 mM DIDS.

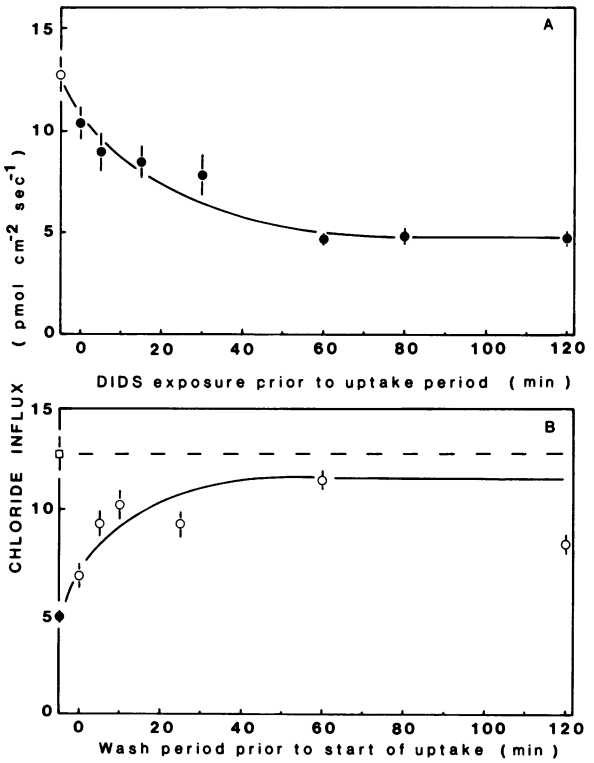


FIG. 2. Measurement of $^{36}Cl^-$ influx following an 18-h treatment in Cl^--free media. A, Chloride uptake was measured in APW-6.5 + 0.5 mM DIDS following increasing periods of pre-exposure to 0.5 mM DIDS under Cl^--free conditions (●), with a control (○) in the absence of DIDS. B, After a 60-min exposure to 0.5 mM DIDS (●), $^{36}Cl^-$ influx was measured following increasing periods of exposure to DIDS-free solution (Cl^--free APW-6.5) (○), and compared with a control (□) that was not exposed to DIDS.

in *Chara*. To investigate this further, we examined the effect of 0.5 mM DIDS treatment (60 min) on $^{36}Cl^-$ uptake. Influx into control cells (APW-6.5, no Cl^--free treatment) was 3.1 ± 0.3 (n = 22) pmol cm^{-2} s^{-1}, whereas in the presence of DIDS, the value was 3.0 ± 0.3 (n = 12) pmol cm^{-2} s^{-1}. However, Cl^--starved cells showed a much greater level of DIDS sensitivity. A time course for DIDS inhibition of Cl^- influx into Cl^--starved cells is presented in Figure 2A. As a comparison, our stimulated influx, in the absence of DIDS, was 12.7 ± 0.9 (n = 32) pmol cm^{-2} s^{-1}, a value 2 to 3 times larger than that reported by Sanders (18).

The inhibition of Cl^- uptake by DIDS could be reversed by its removal from the experimental medium (Fig. 2B); this result is at variance with the irreversible effect observed on animal anion transport systems.

pH Effects on Cl^- Influx. Figure 3 shows the pH dependence of Cl^- influx in control cells and cells that were starved for approximately 18 h in Cl^--free media. The inhibitory effect of 0.5 mM DIDS on Cl^--starved cells is also shown. This DIDS treatment inhibited the stimulated Cl^- influx to values close to those obtained for control cells (not Cl^--starved). Since 0.5 mM DIDS had little effect on control influx (see earlier section), the DIDS concentration was increased to 2.0 mM to determine whether the stimulated influx could be inhibited to a value below that of the control. At pH 6.5 and in the presence of 2 mM DIDS, the stimulated Cl^- influx was inhibited to 1.1 ± 0.1 (n = 12) pmol cm^{-2} s^{-1}. For a comparison, $^{36}Cl^-$ influx into 2 mM DIDS-treated control cells (*i.e.* not Cl^--starved) was 2.2 ± 0.2 (n = 12) pmol cm^{-2} s^{-1}.

The pH dependence of 2.0 mM DIDS inhibition on the stimulated influx is also illustrated in Figure 3. The degree of inhibition becomes more apparent when the inhibited flux is given as a percentage of the noninhibited flux; these values are 3% at pH 4.8, 13% at pH 5.7, 9% at pH 6.5, 26% at pH 7.5, and 35% at pH 8.5.

DIDS Inhibition at Different External Cl^- Concentrations. The effectiveness with which 2.0 mM DIDS inhibited Cl^- influx (measured always in 1.0 mM total Cl^-) was determined after a 60-min DIDS pretreatment in the presence of various external Cl^- concentrations (0, 0.5, 0.1, 0.2, and 1.0 mM). The results of two experiments are shown in Figure 4, along with the relevant controls in which cells were given a 60-min pretreatment at the specified Cl^- concentrations, but in the absence of DIDS. The control treatments illustrate the stimulatory effect of pretreatment of reduced exogenous Cl^- levels on $^{36}Cl^-$ influx and serve to demonstrate the complexity of the observed inhibition.

Cation Effects on DIDS Inhibition. Inhibition of the stimulated Cl^- influx by DIDS became more effective at higher H^+ concentrations (Fig. 3). Experiments were performed in the presence of higher Na^+ and Ca^{2+} levels to ascertain whether this increased inhibition was due to a screening of the fixed negative charges in the cell wall. When 5 mM Na_2SO_4 or 5 mM $CaSO_4$ was added to the pretreatment and uptake solutions, we observed no significant influence on either the stimulated Cl^- influx or its inhibition by DIDS (Table I).

Plasma Membrane ATPase Activity. In a recent study, Franceschi and Lucas (7) suggested that the charasome (for structure and development, see Refs. 5, 6, and 14) may be a special plasma membrane modification adapted to function as a site for Cl^--$2H^+$

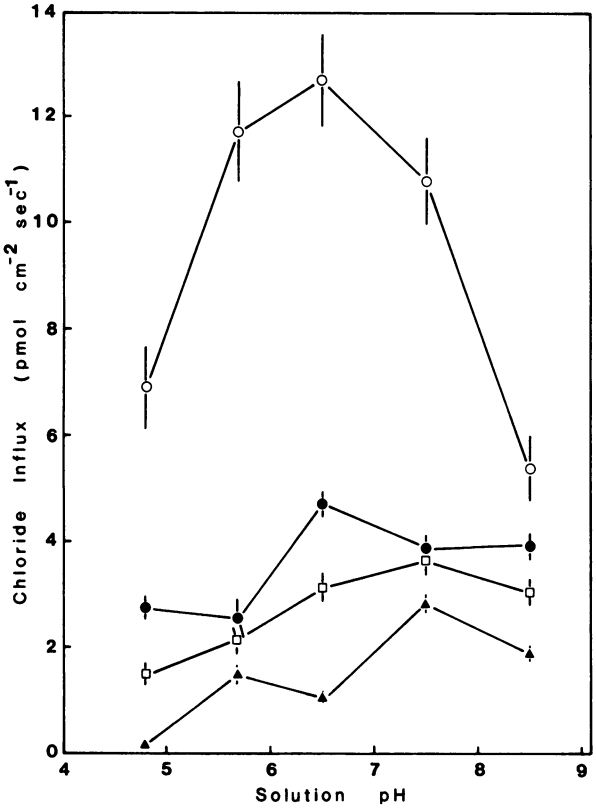


FIG. 3. Chloride influx measured as a function of external pH. All $^{36}Cl^-$ fluxes were measured after overnight pretreatment in the following solutions: APW-6.5 (□); Cl^--free APW-6.5 (○); Cl^--free APW-6.5 for 18 h plus 60 min in either Cl^--free APW-6.5 + 0.5 mM DIDS (●); or Cl^--free APW-6.5 + 2.0 mM DIDS (▲).

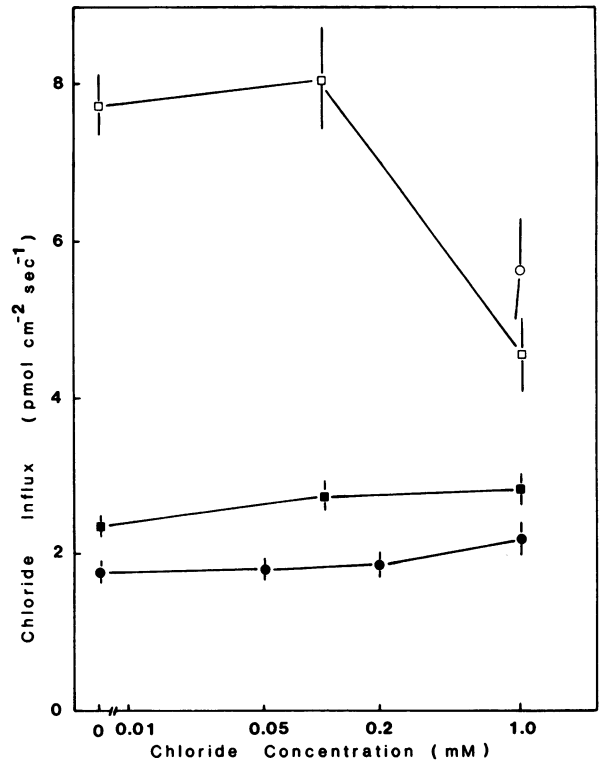


FIG. 4. Chloride influx measured following a 60-min pretreatment in solutions of different Cl^- concentration. Controls (○, □) and, following 60-min exposure to 0.5 mM DIDS, simultaneous with Cl^- pretreatment (●, ■).

co-transport. We used a histochemical approach to investigate the influence of DIDS on the ATPase activity along the *Chara* plasma membrane. ATPase activity reaction product was abundant in the charasomes of Cl^--starved cells (Fig. 5). No detectable difference in the amount of reaction product within the charasomes could be discerned between Cl^--starved cells and cells that were not starved of Cl^-. The reaction product often occurred as very dense patches within the cytoplasmic tubules of the charasomes and at the inferface of the charasome and the gel layer of the cytoplasm. The same distribution pattern was found in Cl^--starved shoots treated with DIDS (Fig. 6) or with DIDS followed by a 1- or 15-min wash (Fig. 7). The mitochondria of cells exposed to the above conditions were generally devoid of reaction product (Figs. 5–7), while the chloroplasts always contained slight amounts of reaction product (Figs. 5–8). Charasomes in cells exposed to DIDS after fixation (included in both reaction medium and prior buffer rinse) were almost completely devoid of reaction product (Fig. 8). However, we observed that the mitochondria in these cells now contained reaction product. Under these conditions, the chloroplasts also contained reaction product at a level similar to that seen after the other treatments previously described. Ultrastructural preservation of charasomes, mitochondria, and chloroplasts of cells treated with DIDS after fixation was the same as for all other experimental treatments examined.

Influence of NEM on $^{36}Cl^-$ Influx and Charasome ATPase Activity. The results presented in Figure 3 indicate that $^{36}Cl^-$ influx into our control cells was not markedly sensitive to external pH. Inasmuch as the membrane potential of *Chara* is extremely pH-sensitive (11, 23), this implied that the transport process responsible for Cl^- influx must be relatively insensitive to the potential across the plasmalemma. This aspect was investigated by using the permeant sulfhydryl reagent NEM, which has been shown to open the K^+ channels in the plasmalemma of *Chara* (12). Since Lichtner *et al.* (12) did their experiments at pH 8.0, we exposed cells to NEM at both pH 8.0 and 6.5. Cells that were given 1- or 2-min pretreatment in 0.1 mM NEM had $^{36}Cl^-$ influx values similar to those of the controls (Table II).

Table I. *Cation Effect on DIDS Inhibition of $^{36}Cl^-$ Uptake into Chara corallina*

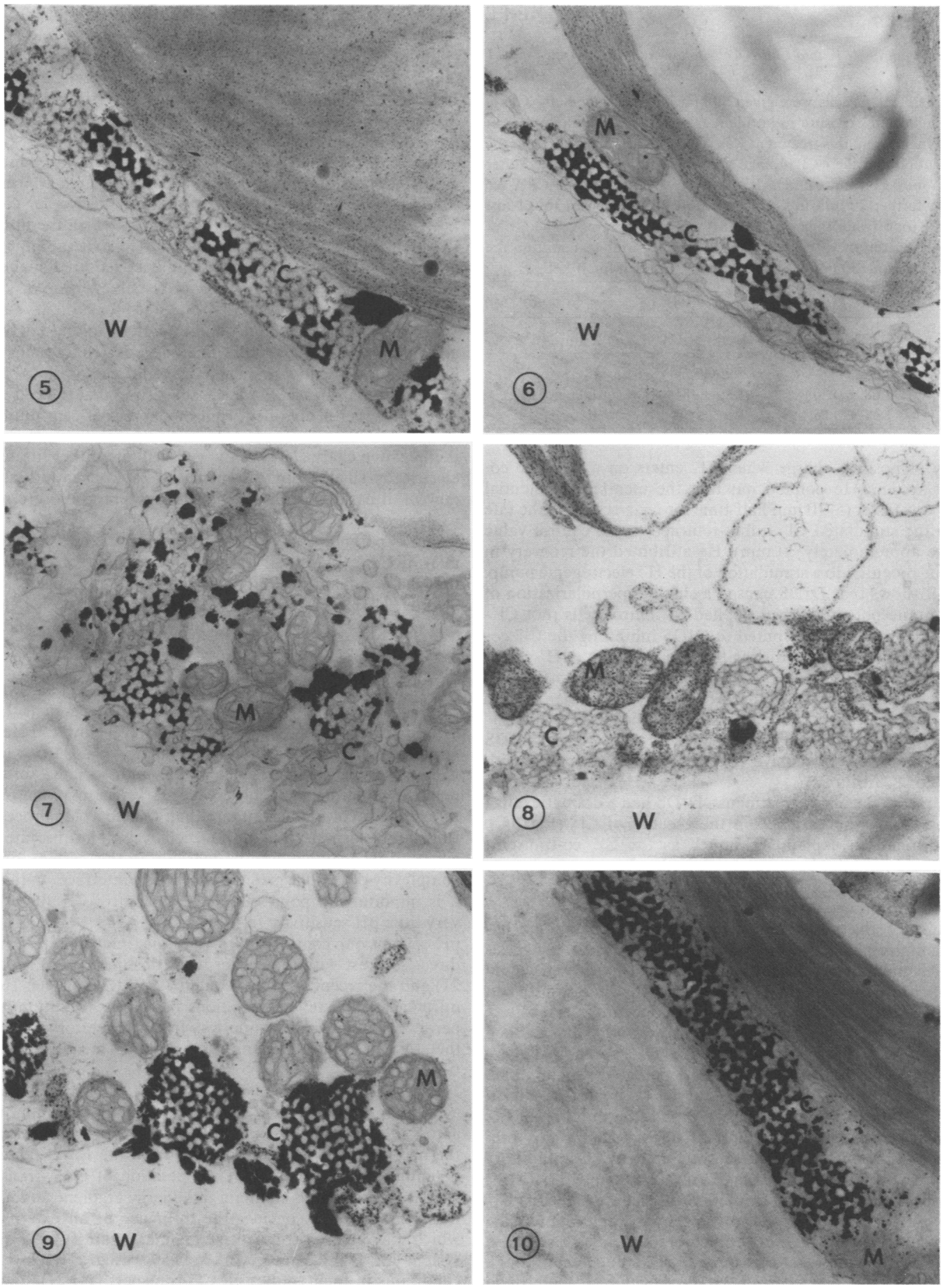
After an 18-h treatment in Cl^--free APW-6.5, in continuous light, the cells received a 1-h pretreatment in Cl^--free APW-6.5 containing the additions as indicated. Chloride influx was measured in APW-6.5 (10-min uptake period) containing the same cation concentration as the pretreatment. The results are the mean for 12 cells ± SE.

Solution Additions	Cl^- Influx
	pmol cm^{-2} s^{-1}
No addition	11.5 ± 1.1
0.5 mM DIDS	4.4 ± 0.5
5.0 mM Na_2SO_4	13.9 ± 1.1
5.0 mM Na_2SO_4 + 0.5 mM DIDS	4.8 ± 0.4
5.0 mM $CaSO_4$	11.2 ± 1.1
5.0 mM $CaSO_4$ ± 0.5 mM DIDS	4.4 ± 0.3

In our histochemical study, we found that NEM did not inhibit the charasome ATPase activity when used either prior to tissue fixation (Fig. 9) or in the ATPase incubation medium (Fig. 10). However, NEM did appear to inhibit most of the ATPase activity that was associated with the chloroplast membranes (compare Figs. 5, 6, and 10).

DISCUSSION

Electrophysiology. Figure 1A shows the transient depolarization that results when Cl^- is restored to the cell following a period of Cl^- starvation. Sanders (16) attributed this depolarization to the



FIGS. 5–10. ATPase localization along the cortical region of branch internodal cells of *Chara corallina*. No poststaining was employed. Charasome (C), mitochondrion (M), cell wall (W).

FIG. 5. ATPase reaction product observed in cells pretreated in Cl^--free APW-6.5 for approximately 18 h. × 24,000.

FIG. 6. Reaction product pattern observed after pretreatment as in Figure 5, except that cells were also given an 80-min exposure to 0.5 mM DIDS Cl^--free APW-6.5, prior to chemical fixation. × 24,000.

FIG. 7. Pretreatment and DIDS exposure as in Figure 6. Tissue was washed in Cl^--free APW-6.5 for 15 min before chemical fixation. Section shown is tangential to the wall. × 24,000.

FIG. 8. Tissue treated as above, except that 0.5 mM DIDS was applied after fixation and during incubation in the ATPase reaction medium. × 24,000.

FIG. 9. Section from shoot incubated in 1 mM NEM for 15 min prior to chemical fixation. × 23,200.

FIG. 10. Reaction product pattern observed when 1 mM NEM was included in the ATPase incubation medium. × 28,700.

Table II. *Influx of $^{36}Cl^-$ into Chara Cells following Pretreatment with 0.1 mM NEM*

In pretreatment A, cells were given 60 min in control medium (CPW-6.5). The indicated exposure to an identical solution containing 0.1 mM NEM was then given; this treatment was stopped by returning cells to the control solution and the $^{36}Cl^-$ uptake period (30 min) was initiated after a further 5 min. For pretreatment B, cells were given 30 min in CPW-6.5 and then a further 20 min in CPW-8.0 before being exposed to 0.1 mM NEM (pH 8.0); a 1-min rinse in CPW-8.0 was used to remove the NEM. Cells were then returned to CPW-6.5 for 10 min prior to initiating the $^{36}Cl^-$ uptake period (30 min). The results are the mean for 10 cells ± SE.

Pretreatment	$^{36}Cl^-$ Influx		
	Control (0 NEM)	1-min NEM	2-min NEM
	pmol cm^{-2} s^{-1}		
A	1.68 ± 0.20	1.89 ± 0.14	1.47 ± 0.11
B	1.38 ± 0.23		1.40 ± 0.12

net entry of positive charge when Cl^- enters on a Cl^--$2H^+$ co-transport system. He pointed out that the membrane potential recovery was faster (5–10 min half-time for recovery) than the rate at which the stimulated Cl^- influx returned to the control value (half-time approximately 30 min). He attributed the recovery in membrane potential to a stimulation of the H^+ electrogenic pump. Figure 1B shows that DIDS caused a small hyperpolarization of the membrane potential when applied to control cells (not Cl^--starved). This could be attributed to DIDS inhibiting the Cl^- co-transport system, thereby reducing the return current of H^+ across the membrane and, thus, allowing the electrogenic pump to hyperpolarize the membrane slightly. However, this explanation is unlikely as DIDS, at 0.5 mM, has an insignificant effect on Cl^- influx, even after a 60-min pretreatment. It may be that DIDS affects, to some extent, passive leakage currents associated with either H^+ or K^+ influx.

In contrast to the above treatments, DIDS had a dramatic effect on the potential when applied in the absence of Cl^- (Fig. 1C). This is difficult to explain, inasmuch as the Cl^- co-transport system should not have been operating in the absence of substrate and as DIDS had no apparent effect on the ATPase activity when applied to the outer surface of the plasmalemma. Although it seems unlikely, the similarity between the depolarizing and hyperpolarizing responses observed in Figure 1, A and C, suggests that DIDS may have been entering the cell. However, it would seem more likely that under external Cl^--free conditions, sufficient Cl^- may leak into the inner cell wall space to provide substrate for the transport system, and it was this activity that was then inhibited. When Cl^- was restored to a DIDS-inhibited, Cl^--starved cell, the effect on the membrane potential was minimal, compared to restoring Cl^- to a Cl^--starved cell (compare Fig. 1, A and D). This effect is consistent with a DIDS inhibition of Cl^- influx and, hence, the large transient depolarization was prevented. This interpretation is also supported by the inhibitory effect of DIDS on the stimulated Cl^- influx system (Fig. 2A). It would appear that for DIDS inhibition to occur, exogenous Cl^- must either be absent or present in very low concentrations. The small effect of these treatments on membrane resistance can also be rationalized on the basis that the major conductance through the membrane is associated with the electrogenic H^+ pump and that the conductance of the Cl^--$2H^+$ co-transport is small in comparison.

Reversibility of DIDS Inhibition. In the present study, we found that the DIDS inhibition of Cl^- influx was reversible (*cf.* Fig. 2, A and B). This is in contrast to the case in red blood cells where DIDS irreversibly inhibits anion transport (3). In red blood cells, it is thought that DIDS binds reversibly to the anion transport site, which places DIDS in a position to react, covalently, with the ε-amino group of two lysine residues on the transport protein (2, 20). In other situations, the likelihood of this geometry for DIDS binding is low, and may account for the reversibility we observed. Since the inhibition is reversible, we cannot use this molecule to label the proteins involved with Cl^- transport.

Effect of the Plant Cell Wall. The slowness with which DIDS acts in *Chara* (60 min to saturate) and the high concentrations needed (0.5–2.0 mM), as compared to the micromolar concentrations used in red blood cells (3), probably reflect the influence of the plant cell wall. Lin (13) also reported that inhibition of Cl^- and SO_4^{2-} transport into root segments required higher DIDS concentrations than were necessary to achieve similar inhibition in root protoplasts.

Given that DIDS is quite a large divalent anion (mol wt of 456) and that the cell wall contains fixed negative charges, the ion may have some difficulty in penetrating the cell wall to the outer plasmalemma surface. Evidence suggesting that the cell wall may act as a barrier is obtained from the larger per cent inhibition by 2.0 mM DIDS at lower pH values. As the solution pH is lowered, the negative charge sites in the cell wall are either titrated or are electrically shielded by the increased H^+ concentration, thereby making it easier for the DIDS anion to enter and cross the cell wall.

Our experiments in which we increased the level of Na^+ (by 10 mM) or Ca^{2+} (by 5 mM) do not offer support for the hypothesis that the dominant wall effect is due to the influence of the fixed negative charges. Such a high concentration of Ca^{2+} would reduce the negative Donnan potential of the wall, but, under these conditions, we found no enhancement of DIDS inhibition on Cl^- influx (Table I).

DIDS could have been acting as a weak acid, where the concentration of the reactive species is pH-dependent. However, titration indicated that its pK value is well below 1.3. Hence, it is very unlikely that the increased sensitivity to DIDS, at lower pH values, is due to the presence of the undissociated form of this reactive molecule. The observed pH effect may, nevertheless, reflect the involvement of more than one complex process occurring at the plasmalemma of these cells. Also, the absolute effect of external pH may actually be masked, in this system, due to the complexity of the plasmalemma-cell wall interface. In this regard, it is important to point out that our control Cl^- fluxes showed very little pH sensitivity from pH 6.5 to 8.5 (see Fig. 3, □). In this pH region, our control fluxes were maximal; this response is in direct contrast with the earlier work of Smith (see Fig. 1 of Ref. 21) and the recent work of Sanders (see Fig. 6 of Ref. 16). These differences may reflect variations in culture conditions, and experiments are presently underway in our laboratory to investigate the effect of culture medium pH on charasome development and Cl^- transport.

External Buffering and Cl^- Transport. The present model for active Cl^- transport into *Chara* proposes that this anion moves across the plasmalemma via a Cl^--$2H^+$ co-transport system, with the necessary energy being derived from the H^+ electrochemical potential gradient (16–19). The operation of a H^+-translocating ATPase is thought to maintain this energy gradient. In this regard, it is of interest to consider the influence of an external buffer which may act in a competitive manner, against the co-transport system for H^+. Various workers have used a range of buffer conditions in their studies on Cl^- transport (15, 21), and, except for the amine-type buffers, Cl^- influx does not appear to be sensitive to exogenous buffer. (However, in some experiments, buffers were used at pH values well below their pK, and so there would have been little chance for the conjugate base to compete for H^+ [15].) This insensitivity to exogenous buffer may be due, in part, to the anion-screening effect of the cell wall. Additionally, if the charasome plays a role in Cl^- uptake, its specialized periplasmic space may confine the protons such that they are not

readily accessible to the buffer.

DIDS Competition with Cl^-. The stimulated component of Cl^- influx that was elicited by Cl^- starvation could be almost completely eliminated by pretreatment with 0.5 mM DIDS. However, DIDS at the same concentration had no significant effect on the control Cl^- transport process. This suggested to us that there may be two Cl^- transport mechanisms—the normal transporter and another mechanism that carries the stimulated flux—with only the latter being sensitive to DIDS. Inasmuch as increasing the DIDS concentration to 2.0 mM caused a significant inhibition of normal Cl^- influx, this interpretation seems unlikely. Yet we must explain why 2.0 mM DIDS always produced a much greater inhibition of Cl^- transport into Cl^--starved *versus* control cells (not Cl^--starved). The basic difference between these two experiments is that, for DIDS inhibition of the stimulated Cl^- influx, DIDS was always applied for the last 60 min of the Cl^--free pretreatment. In control experiments, DIDS was applied for 60 min, but it was always in the presence of 1.0 mM Cl^-. On a speculative note, the differential effect of DIDS may indicate that Cl^- and DIDS are competing for the same binding site. If competition with Cl^- was actually preventing DIDS from effectively reaching the binding site, then a 60-min pretreatment with DIDS, under conditions of reduced exogenous Cl^-, should increase its ability to inhibit transport. These competition experiments were complicated by the stimulatory effect of even a short period of Cl^- starvation (16), so controls (no DIDS) were run for each pretreatment concentration of Cl^-. The pretreatment with DIDS at lower Cl^- concentration gave absolute flux values that were slightly lower, but with only a marginal level of significance (Fig. 4). However, the important point to note is that the relative inhibition by DIDS, compared to the control, is significantly larger at lower Cl^- pretreatment concentrations. Inasmuch as any effort to reduce competition between Cl^- and DIDS also stimulates Cl^- influx, the possibility still exists that there are two Cl^- transport mechanisms, with DIDS being more effective at inhibiting the mechanism associated with the stimulated Cl^- influx.

Membrane ATPase Activity. The apparent insensitivity of the *Chara* plasma membrane ATPase to DIDS, in both control and Cl^--starved situations, suggests that Cl^- inhibition by DIDS is not due to an indirect effect on the electrogenic H^+ pump. This interpretation is also supported by the observation that in control cells the membrane potential actually hyperpolarized in the presence of DIDS (Fig. 1B). Electrogenic H^+ pump inhibition would have resulted in a depolarization of the potential to the diffusion potential (11, 23).

The plasmalemma ATPase activity was inhibited, however, by including DIDS in the reaction medium (after tissue fixation). Thus, once DIDS can penetrate the tissue, it presumably finds reactive sites (lysine residues?) on the inner surface of the plasmalemma to which it binds, thereby inhibiting the ATPase system. This result and interpretation are in agreement with studies on isolated plasma membrane preparations in which ATPase sensitivity to DIDS was also observed (4, 13). In these membrane preparations, the microsomal vesicles are usually everted and, hence, the inner plasma membrane surface is accessible to the DIDS molecule. Bennett and Spanswick (1) also reported that their putative tonoplast vesicles derived from corn roots contained a H^+-translocating ATPase that was partially sensitive to DIDS. However, they interpreted this DIDS inhibition in terms of DIDS acting at a Cl^- transport site that was thought to be intimately associated with the H^+-translocating ATPase. *Chara* cell perfusion studies must now be performed to determine whether, in unfixed tissue, DIDS can inhibit the electrogenic H^+ ATPase of the plasmalemma; depolarization of the membrane potential in such cells would offer support for the above hypothesis.

Insensitivity of Cl^- Transport to Membrane Potential. A brief exposure of *Chara* cells to NEM results in an opening of K^+ channels within the plasmalemma (12). Thus, in our NEM-pretreated cells, the membrane potential would have been depolarized from −250 mv down to −150 mv (12). However, under these conditions, $^{36}Cl^-$ influx was not significantly different from values obtained with control cells (Table II). This result supports the hypothesis of Sanders and Hansen that Cl^- influx is under kinetic, rather than thermodynamic control (19). The limits of Cl^- transport insensitivity to membrane potential will be investigated in the near future.

The insensitivity of both Cl^- influx and plasmalemma-charasome ATPase activity to NEM also supports our recent hypothesis that the plasmalemma H^+-translocating ATPase remains operational in the presence of open K^+ channels (10).

Conclusions. DIDS is an effective inhibitor of the *Chara* plasma membrane Cl^- transport system, a system presently thought to operate as an electrogenic Cl^--$2H^+$ co-transport process. Transport inhibition appears to result from competition between DIDS and Cl^- for an external binding site, although a more complex form of interaction between DIDS and the transport system cannot be discounted. The ability of DIDS to form covalent bonds with the ϵ-amino group of lysine plays no role in its inhibition in *Chara*, suggesting that there are no lysine residues in the vicinity of the external binding site of the Cl^- transport system.

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