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Inhibition of Anion Transport in Corn Root Protoplasts[1]

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ABSTRACT

The effects of several amino-reactive disulfonic stilbene derivatives and *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate on Cl^-, SO_4^{2-}, and inorganic phosphate (Pi) uptake in protoplasts isolated from corn root tissue were studied. 4-Acetamido-4′-isothiocyano-2,2′-stilbenedisulfonic acid, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid, 4,4′-diamino-2,2′-stilbenedisulfonic acid, and NAP-taurine inhibited Cl^- and SO_4^{2-} but not Pi and K^+ uptake in corn root protoplasts; whereas mersalyl inhibited Pi but not Cl^- or SO_4^{2-} uptake. The rate of uptake of all anions decreased with increasing external pH. In addition, these reagents markedly inhibited plasmalemma ATPase activity isolated from corn root tissue. Excised root segments were less sensitive to Cl^- and SO_4^{2-} transport inhibitors.

A growing interest has emerged on the nature of anion transport in several biological systems. For example, carrier proteins for phosphate, chloride, and sulfate transport have been recently identified and isolated from bacteria (1, 20), fungi (6), and human red blood cells (see reviews 10, 24). In the red blood cell studies (2–4, 8, 11, 17), several nonpermanent, amino-reactive reagents (*e.g.*, SITS,[2] DIDS, DADS, and NAP-taurine), which covalently bind to the plasmalemma, have been used to identify and characterize the band 3 membrane protein as the carrier for Cl^- and SO_4^{2-} transport.

Virtually no information is available on the nature of the carrier proteins responsible for ion transport in plant cells, particularly root cells which are the major sites of entry for ions into the plants. The lack of such information is due, in part, to the lack of suitable technique for isolating functional protoplasts from roots which are, by their nature, more amenable to techniques that have been used to characterize membrane transport. Recently, we developed techniques for the large scale and rapid isolation of protoplasts from corn roots (13) which are capable of transporting several ions (K^+, $H_2PO_4^-$, and H^+). In this study, several chemical modifiers which have been used to study anion transport into red blood cells were employed to study anion transport into corn root protoplasts with the intent of providing further insight into anion transport in plant cells.

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[2] Abbreviations: SITS, 4-acetamido-4′-isothiocyano-2,2′-stilbenedisulfonic acid; DIDS: 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; DADS, 4,4′-diamino-2,2′-stilbenedisulfonic acid; NAP-taurine, *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate; FCCP, (*p*-trifluoromethoxy) carbonyl cyanide phenylhydrazone; DIFP, diisopropylfluorophosphate.

MATERIALS AND METHODS

A modification of our previously reported method (13) was used to isolate protoplasts from young corn roots. As outlined in Figure 1, 0.1% pectolyase Y-23 (16) substituted for pectinase and hemicellulase in the digestion enzyme mixture. Also, the tissue incubation time in the digestion mixture was shortened to 1.5 to 2 h and the precentrifugation steps were omitted. The yield of 10^6 protoplasts/g root tissue and viability of the protoplasts were similar to those previously described (13). Transport rates for Pi and K^+ were 2-fold higher and linear for longer periods of time (10–20 min longer) than in the previously described technique (Ref. 13; Table I and Fig. 2). To avoid possible adverse effects on the surface proteins of the protoplast by proteases from the cell wall digestion enzyme mixture (15), 0.05% BSA and 10 mM DIFP, a specific inhibitor for fungal serine proteases (15), were added to the digestion enzyme mixture.

The previously described rapid separation technique (13) was used to measure ion influx into protoplasts. About 0.5 million protoplasts were used in each measurement. Carrier-free $^{36}Cl^-$, $^{35}SO_4^{2-}$, $H_2{}^{32}PO_4^-$, and $^{86}Rb^+$ (New England Nuclear) in 1 mM KCl, K_2SO_4, KH_2PO_4, and KCl were added in 1 mM Hepes (pH 6.0), 0.2 mM $CaCl_2$, and 0.65 M mannitol to measure Cl^-, SO_4^{2-}, Pi, and K^+ influx, respectively. NaOH or H_3PO_4 were used to adjust the pH of the absorption media in the pH studies. Except for the time course experiments, a 15-min radioactive exposure period was employed. Previously described methods were used to measure the ion influx into 4 h-washed excised root segments at 0.2 mM salt concentration (12) and plasmalemma ATPase activity (14). All experiments were duplicated.

Pectolyase Y-23 was purchased from Seishin Pharmaceutical Co., Japan, Cellylysin and DIFP from Calbiochem, SITS, DIDS, and NAP-taurine from Pierce Chemical Co., DADS from Eastman Kodak Co., and mersalyl from Sigma. All other chemicals were ACS reagent grades.

RESULTS AND DISCUSSION

The inhibition of Cl^- and SO_4^{2-} uptake in red blood cells by membrane impermeable amino-reactive disulfonic stilbene derivatives (SITS, DIDS, and DADS) was proposed to result from the covalent binding of these chemicals to the outer peptide chain of the band 3 protein, the major anion transport carrier (10, 24). Figure 2 shows SITS and DIDS also rapidly and completely inhibited the Cl^- and SO_4^{2-} accumulation in corn root protoplasts, whereas, in excised root segments, higher concentrations of these chemicals and a 10 to 15 min lag were required to produce limited inhibition. This suggests that an amino-reactive protein may also be involved in Cl^- and SO_4^{2-} in plant cells. The concentration dependency of the SITS and DIDS inhibition of chloride and sulfate transport into protoplasts and root segments is shown in Figure 3. The maximum inhibition of Cl^- uptake by SITS and DIDS in protoplasts was 80%, whereas in root segments higher than 95% inhibition was observed at higher SITS or DIDS con-

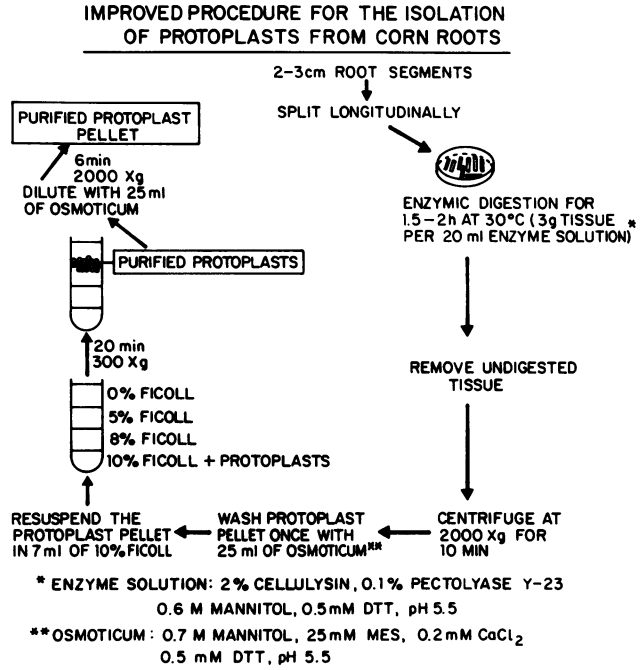

FIG. 1. An improved procedure for the isolation of protoplasts from corn roots. 0.05% BSA and 10 mM DIFP were added in enzyme solution and osmoticum was included throughout the Ficoll step gradient.

Table I. *Effects of Chemical Modifiers on Anion Transport in Corn Root Protoplasts and Segments*

Radioactive exposure periods for protoplasts and segments were 15 and 30 min, respectively.

	Cl^-		SO_4^{2-}		Pi	K^+
	Proto-plasts	Seg-ments	Proto-plasts	Seg-ments	Proto-plasts	Proto-plasts
	nmol/ 10^6 prot·h	*μmol/ g·h*	*nmol/ 10^6 prot·h*	*μmol/ g·h*	*nmol/ 10^6 prot·h*	*nmol/10^6 prot·h*
Control	9.96	1.74	1.54	0.28	5.16	27.38
SITS, 0.1 mM	4.84	1.84	0.59	0.26	5.53	23.27
DIDS, 0.1 mM	2.11	1.75	1.08	0.23	5.16	27.92
NAP-taurine, 0.1 mM	3.79	1.81	1.01	0.23	5.92	24.37
DADS, 0.1 mM	4.85	1.93	0.99	0.24	9.89	24.92
Mersalyl, 25 μM	9.86		1.28		1.29	
FCCP, 10 μM	5.21	0.07	0.78	0.05	1.34	1.37

centrations. Similar results were found for SO_4^{2-} uptake in protoplasts *versus* segments. The requirement of higher reagent concentrations for the inhibition of Cl^- and SO_4^{2-} transport in root segments is probably due to either decreased rate of penetration of the reagent to the membrane or reaction of the reagent with cell wall constituents.

Accumulation of Cl^- and SO_4^{2-} ions leveled off after 30 min of incubation (Fig. 2). When 1 mM glucose was added in the uptake medium, ion accumulation was extended by 10 to 15 min before leveling off but without any change in the initial rate of accumulation. The presence of 1 mM glucose in the medium did not alter the inhibitory effect of the anion transport inhibitors tested. The observation that glucose extends the accumulation of ions by 10 to 15 min into protoplasts suggests that O_2 is not limiting under the transport assay condition.

In bacterial and red blood cells (2–5, 10, 23), SITS, DIDS, and DADS were found to be specific inhibitors for Cl^- and SO_4^{2-}

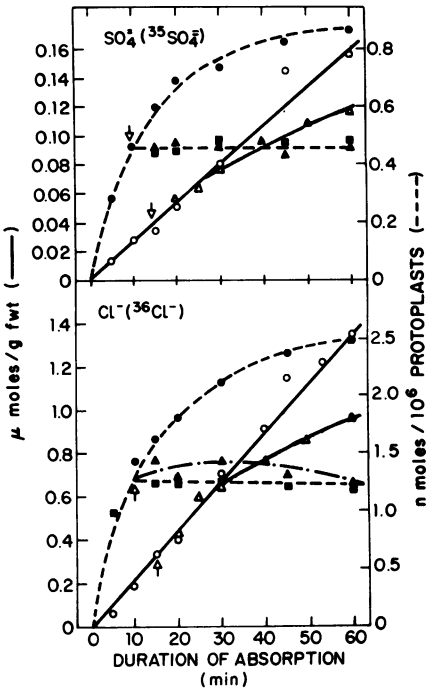

FIG. 2. The effect of SITS (△ and ▲) and DIDS (□ and ■) on Cl^- (lower panel) and SO_4^{2-} (upper panel) uptake in isolated corn root protoplasts (▲, ■, ●) and excised root segments (△, □, ○). SITS or DIDS was added at the time indicated by arrows to protoplasts (0.1 mM) or segments (0.5 mM). ○ and ● are the controls.

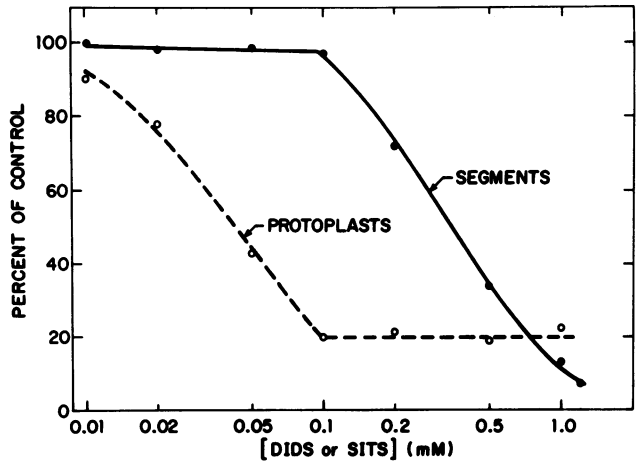

FIG. 3. Function of SITS or DIDS concentrations on Cl^- uptake in isolated corn root protoplasts and excise root segments (SITS and DIDS gave identical results). Control rate for protoplasts were 10.15 nmol/10^6 protoplast·h and 1.82 μmol/g·h for segments.

transport. Importantly, a similar specificity of inhibition by these chemicals was observed in protoplasts isolated from corn roots (Table I) in that they markedly inhibited Cl^- and SO_4^{2-} uptake, while Pi and K^+ uptake was essentially unaffected. The degree of inhibition of Cl^- uptake was similar to that caused by the uncoupler FCCP. A similar but smaller inhibition of SO_4^{2-} uptake was found in the same experiment. The fact that Pi and K^+ were not affected by these chemicals suggests that the protoplasts suffered no general damage during the uptake period. The failure of the uncoupler to inhibit anion uptake strongly in protoplasts may be due to a higher passive influx of ions into the protoplasts.

The specificity of SITS and DIDS inhibition for Cl^- uptake is further demonstrated in Figure 4. Very little inhibition of Pi

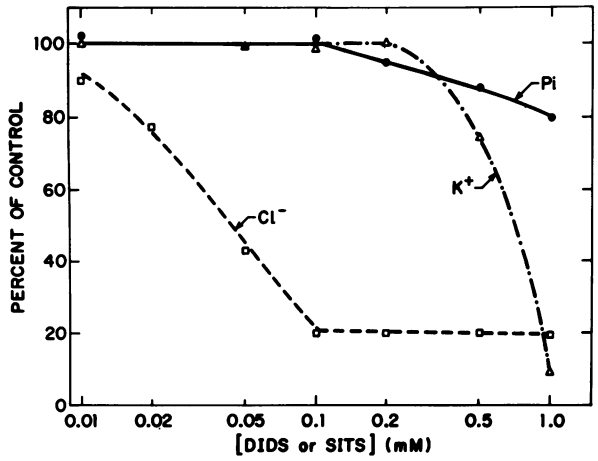

FIG. 4. Function of SITS or DIDS concentrations on Cl^-, K^+, and Pi uptake in isolated corn root protoplasts (SITS and DIDS gave identical results). Control rates were 10.15, 28.02, and 4.98 nmol/10^6 protoplast·h for Cl^-, K^+, and Pi, respectively.

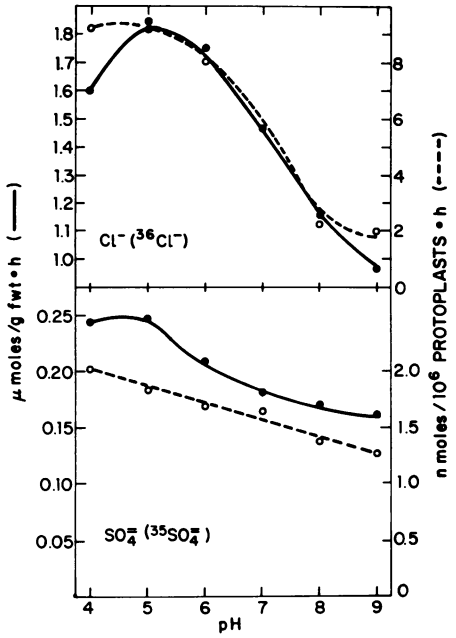

FIG. 5. The effect of pH on Cl^- (upper panel) and SO_4^{2-} (lower panel) uptake in isolated corn root protoplasts (---) and excised root segments (——).

uptake can be found in the concentration range tested, suggesting different transport systems for Pi and Cl^- or SO_4^{2-}. The inhibition of K^+ influx by high concentrations of DIDS or SITS (>0.2 mM) may be an indirect effect on K^+ transport since at these concentrations Cl^- transport was inhibited to a maximal level, which would tend to increase the cytoplasmic pH (20, 21) and possibly affect K^+ transport.

Excised root segments were insensitive to the specific anion transport inhibitors at low concentrations (Table I, Fig. 3), indicating that removal of cell wall is desirable in studying the effects of these compounds. The water-soluble sulfhydryl-binding mercurial, mersalyl, inhibited Pi uptake, by protoplasts more effectively than in root segments (12), presumably through its inhibition of the Pi/OH^- antiporter, but Cl^- and SO_4^{2-} transport was essentially unaffected. These results support the conclusion that the Cl^- and SO_4^{2-} transport system(s) is distinct from that for Pi. At present, the reason for the strong stimulatory effect of DADS on

Table II. *Effect of Chemical Modifiers on Plasmalemma ATPase Activity in Corn Root Tissues*

Plasmalemma fraction (20–25 μg protein) was used in the assay. Reaction time was 20 min.

	ATPase Activity		Δ KCl
	+KCl	−KCl	
	μmol Pi/mg protein·h		*μmol Pi/mg protein·h*
Control	14.78	9.93	4.85
SITS, 0.1 mM	9.61	6.53	3.08
DIDS, 0.1 mM	6.80	5.96	0.84
NAP-taurine, 0.1 mM	9.31	8.54	0.77
DADS, 0.1 mM	11.38	9.04	2.34
Mersalyl, 25 μM	9.31	6.55	2.76
FCCP, 10 μM	19.51	11.32	8.19

Pi uptake in the protoplast suspension is unknown.

Previous studies with corn root segments (12) and protoplasts (13) suggested that Pi was taken up through a Pi/OH^- antiporter. It was shown that Pi uptake decreased with increasing external pH. Figure 5 shows a similar pH dependency for Cl^- and SO_4^{2-} influx. The data may be consistent with an OH^- exchanging or H^+ co-transporting system being involved in the Cl^- and SO_4^{2-} transport. In other tissues, H^+ and OH^- or HCO_3^- have been suggested as counter-ions for Cl^- uptake (9, 21, 22).

An ATPase mediated electrogenic Cl^- transport mechanism has been proposed in both animal (22) and plant (9, 18, 19, 21) tissues. All chemical modifiers which inhibited anion uptake also inhibited the KCl-stimulated plasmalemma ATPase activity at pH 6.5 (Table II) which is regarded as that ATPase component associated with active transport (7). FCCP increased ATPase activity probably through an uncoupling effect.

Microsomal ($Na^+ + K^+$)-ATPase activity in both turtle bladders and eel electric organs has been reported to be strongly inhibited by SITS (5). Since K^+ uptake, which is generally thought to be directly coupled to the plasmalemma ATPase activity in higher plant tissues (12, 19), was not inhibited by the anion transport inhibitors (Table I), the inhibition of plasmalemma ATPase by these chemicals was unexpected. There are three possible reasons for this apparent disparity. First, the current adapted technique (7) for the isolation and determination of the plasmalemma ATPase from plant tissue may not be able to separate different ATPases (if there are any) which might be responsible for different ion transport in the plasmalemma. Consequently, the ATPase affected by anion transport inhibitors (Table II) might be directly involved in the Cl^- and SO_4^{2-} uptake and may not be the same as that for K^+ uptake. Secondly, the isolation of the plasmalemma may have caused a rearrangement of the membrane and exposed the ATPase to the external solution, thereby resulting in an *in vitro* inhibition of ATPase activity by these chemicals. A similar result was found in a membrane-impermeable Pi/OH^- antiporter inhibitor, mersalyl, which did not affect the K^+ uptake but strongly inhibited plasmalemma ATPase activity (Table II). Lastly, the inhibition of ATPase activity observed here may be a secondary effect of these inhibitors which is separable from the Cl^- and SO_4^{2-} transport system. Further studies with the photoaffinity and radioisotope-labeled anion transport inhibitors should provide some insight into the transport mechanism, especially the involvement of the membrane bound ATPase on anion uptake.

CONCLUSIONS

The present study shows that isolated protoplasts are quite sensitive to several anion transport inhibitors which have been used with red blood cells, bacteria, and fungi, and suggests the

presence of comparable transport proteins in the plant cell plasmalemma. Transport of Cl^- or SO_4^{2-} is indicated to be similar to anion carriers, but the phosphate carrier is distinct. All the inhibitors affect the K^+-stimulated ATPase of isolated plasmalemma, but the data do not permit the conclusion that anion transport is directly coupled to ATP hydrolysis. The anion transport inhibitors are only effective on root segments at higher concentrations, possibly due to binding and inactivation of the inhibitors in the cell wall.

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