Plant Physiol. (1988) 86, 759–763
0032-0889/88/86/0759/05/$01.00/0

Nitrate Absorption by Corn Roots[1]

INHIBITION BY PHENYLGLYOXAL

Received for publication July 9, 1987 and in revised form November 3, 1987

KANWARPAL S. DHUGGA[2], J. GILES WAINES, AND ROBERT T. LEONARD*
Department of Botany and Plant Sciences, University of California, Riverside, California 92521-0124

ABSTRACT

Nitrate transport in excised corn (*Zea mays* L.) roots was inhibited by phenylglyoxal, but not by 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid (DIDS) or fluorescein isothiocyanate (FITC). Inhibition of nitrate uptake by a 1-hour treatment with 1 millimolar phenylglyoxal was reversed after 3 hours, which was similar to the time needed for induction of nitrate uptake. If induction of nitrate uptake occurs by *de novo* synthesis of a nitrate carrier, then the resumption of nitrate uptake in the inhibitor-treated roots may occur because of turnover of phenylglyoxal-inactivated nitrate carrier proteins. All three chemicals inhibited chloride uptake to varying degrees, with FITC being the strongest inhibitor. While inhibition due to DIDS was reversible within 30 minutes, both FITC and phenylglyoxal showed continued inhibition of chloride uptake for up to 3 hours after removal from the uptake solution. Assuming that the anion transporter polypeptide(s) carries a positive charge density at or near the transport site, the results indicate that the nitrate carrier does not carry any lysyl residues that are accessible to DIDS or FITC, whereas the chloride carrier does. Both chloride and nitrate carriers, however, seem to possess arginyl residues that are accessible to phenylglyoxal.

With the increased cost of farm inputs such as nitrogen fertilizers, it is important to breed crop cultivars that absorb and use soil nitrogen more efficiently. The absorption of nitrate by plants is mainly determined by the root surface area and/or the capacity of nitrate uptake by root cells which, in turn, depends in part upon the number and/or efficiency of the nitrate carriers per unit area of the plasma membrane (1). Although the involvement of carriers in the transport of ions across the plant plasma membrane has long been recognized, it has been difficult to biochemically identify and characterize such carriers. A biochemical identification of a nitrate carrier, for example, is essential for effective manipulation of nitrate uptake by plants at the molecular level. This requires the development of certain probes that can be used to label and subsequently identify the polypeptide(s) involved in nitrate transport.

Group-specific chemical reagents have been successfully used to identify the functionally essential amino acid residues in a number of enzymes (15, 27). Because of the high pKa (12.5) of their side chains, arginyl residues are positively charged at physiological pH. Based on this observation, it was suggested that enzymes which have anionic substrates will most likely have arginyl residue(s) at or near their active sites (27), and this hypothesis has received considerable support (4, 24, 26, 31). The other amino acid residues that can provide a positive charge at physiological pH are lysine (pKa 10.5) and histidine (pKa 6.0).

Phenylglyoxal, a diketone that binds the guanidinium group of arginine (30), strongly inhibited chloride transport in mammalian red blood cells (3). Anion transport in red blood cells was also inhibited by the disulfonic acid stilbene and eosin derivatives, both of which bind the essential lysyl residue of the band 3 protein, which composes the anion transporter (17, 18, 22, 25). Likewise, DIDS,[3] a disulfonic acid stilbene derivative, inhibited chloride uptake in corn roots and *Chara* (16, 19, 20). ^{14}C-Phenylglyoxal, tritiated DIDS, and FITC were successfully used to specifically label and identify the anion transporter in red blood cells (3, 9, 29). Inhibition of chloride uptake by DIDS in corn roots and *Chara* was reversible, however, such that radiolabeled DIDS could not be used to label the chloride transporter protein of plant cells (16, 19).

It is highly probable that the nitrate carrier of the plant cell plasma membrane also carries arginyl and/or lysyl residue(s) at or near the transport site. To our knowledge, no inhibitors, reversible or irreversible, for nitrate transport have so far been reported in plants. The availability of an irreversible inhibitor of nitrate transport could facilitate the identification of the plasma membrane-associated polypeptide(s) involved in nitrate transport. This study reports on the inhibition of nitrate transport by phenylglyoxal as it relates to the induction of nitrate uptake in excised corn roots. It further reports on the inhibition of chloride uptake by DIDS, FITC, and phenylglyoxal and demonstrates the involvement of different mechanisms in nitrate and chloride transport in corn roots.

MATERIALS AND METHODS

Plant Materials. Seeds of corn (*Zea mays* L.) hybrid WF 9 × MO 17 were grown in glass baking trays on a single layer of blotter paper saturated with 0.1 mM $CaCl_2$ solution. The trays were covered with plastic wrap, which was perforated to allow gas exchange, and kept at 28°C in a growth chamber in the dark. Primary roots from 3.5-d-old seedlings were excised, cut into segments about 4 cm long, and placed in aerated, ice-cold water.

Nitrate Uptake Assay. For studying the induction of nitrate uptake, 3 g of the fresh root segments were weighed into 50 ml capacity glass test tubes kept on ice. Uptake was started by adding 30 ml of uptake solution (0.5 mM KNO_3, 0.25 mM $Ca[NO_3]_2$, 2 mM Mes, pH 6.0). Assay tubes were kept in a water bath maintained at 30°C and were aerated throughout the uptake period. Uptake was studied by monitoring the depletion of nitrate from the solution. Aliquots of 0.2 ml were taken at hourly intervals and nitrate was determined colorimetrically by the method

[1] Supported in part by a Chancellors Patent Fund Award to K. S. D., and funds from the California Agricultural Experiment Station and the University of California, Riverside Botanic Gardens to J. G. W.

[2] Present address: Department of Biological Sciences, Stanford University, Stanford, CA 94305.

[3] Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid; FITC, fluorescein isothiocyanate.

of Cataldo *et al.* (7). Data are shown for representative experiments consisting of duplicate nitrate assays for duplicate root samples.

Nitrate Pretreatment. It was determined that nitrate uptake in excised root segments from corn seedlings grown as described above, reached its maximum rate after about 3 h. Therefore, the root segments were routinely pretreated for 3 h in a solution containing 5 mM nitrate (2.5 mM KNO_3 and 1.25 mM $Ca[NO_3]_2$) in 5 mM Mes (pH 6.0) unless specified otherwise.

Inhibition of Nitrate Uptake. Inhibitors were added to the uptake assays to a final concentration ranging from 0.25 to 1.00 mM. The root segments were incubated with the inhibitor for 1 h before measurement of nitrate absorption. In one experiment (Fig. 4), the nitrate-pretreated roots were incubated in 1 mM inhibitor solution in 2 mM Mes (pH 6.0) for 1 h, and then uptake was studied either in the presence or absence of the inhibitor over a period of 6 h.

Inhibition of Chloride Uptake. For measurement of chloride uptake, root segments were pretreated for 3 h in an aerated solution consisting of 0.1 mM $CaSO_4$ in 2 mM Mes (pH 6.0). The pretreated root segments were then incubated in a solution containing 0.5 or 1.0 mM inhibitor, 0.1 mM $CaSO_4$, 2 mM Mes (pH 6.0) for 1 h, at the end of which time they were transferred to uptake solution containing 0.5 or 1 mM inhibitor. The uptake solution consisted of 1 mM KCl, 0.1 mM $CaSO_4$, 2 mM Mes (pH 6.0), and $^{36}Cl^-$ at a specific activity of 25 to 30 μCi per mmol chloride. After incubation for 10 min, the root segments were washed twice with ice-cold 0.1 mM $CaSO_4$ solution for 5 min each and weighed into scintillation vials for determination of radioactivity in a liquid scintillation counter. Data are shown for representative experiments consisting of duplicate root samples.

For the time-course experiment (Fig. 6), the pretreated root segments were incubated in a solution containing 1 mM inhibitor, 2 mM Mes, 0.1 mM $CaSO_4$ (pH 6.0) for 1 h, at the end of which time they were washed twice with water for 5 min each. The inhibitor-treated roots were kept in the aerated solution of the same composition as above minus the inhibitor. Chloride uptake was measured as a function of time as described above.

RESULTS AND DISCUSSION

Inhibition of Nitrate Uptake. Both DIDS and FITC carry amino reactive isothiocyanate group(s) that can react with the epsilon amino group of lysyl residues and the N-terminal α-amino group of polypeptide chains (12, 25). DIDS or FITS had little inhibitory effect on nitrate uptake in corn roots (Figs. 1 and 2). The slight inhibition by DIDS may simply result from general anionic competition at the transport site because DIDS has two well exposed negative charges on the sulfonate groups. In comparison to DIDS, FITC is relatively less negatively charged at physiological pH (32). It has one relatively hidden, negatively charged carboxyl group and a well exposed, but partially deprotonated hydroxyl group (pK = 6.5). A slight apparent stimulation in nitrate uptake, shown by 1 mM FITC, probably resulted from the simultaneous depletion of both FITC and nitrate from the uptake solution over the period of uptake. The absorption spectra of FITC and nitrate, in the solution used for the colorimetric nitrate assay, were found to slightly overlap each other (data not shown). This would result in inflated estimates of apparent nitrate loss from the uptake solution because at each time interval, both loss of nitrate and FITC were being assayed.

DIDS has long been known as a potent inhibitor of anion transport in mammalian red blood cells (6). The following observations demonstrated the specificity of DIDS for the anion transporter (band 3 protein) in red blood cells. One mole of DIDS per mole of the anion transporter completely inhibited sulfate flux into red blood cells (29). In the same study, almost 90% of the total radiolabeled DIDS bound to the red blood cells

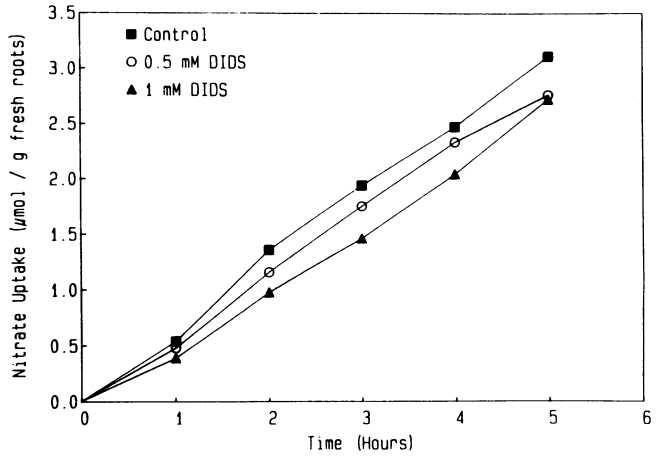

FIG. 1. Effect of DIDS on nitrate in corn roots. Uptake was studied on nitrate-pretreated roots in the presence of the indicated concentrations of DIDS. The rates of uptake for the control, 0.5, and 1.0 mM DIDS were 0.63, 0.57, and 0.57 μmol/(g roots/h), respectively.

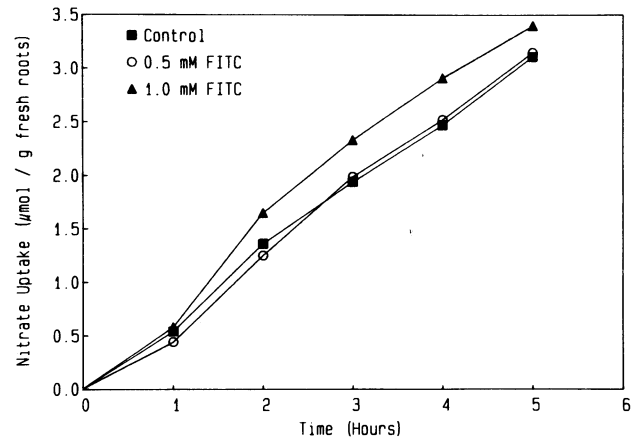

FIG. 2. Effect of FITC on nitrate uptake in corn roots. Uptake was studied on nitrate-pretreated roots in the presence of the indicated concentrations of FITC. The rates of uptake for the control, 0.5, and 1.0 mM DIDS were 0.63, 0.67, and 0.69 μmol/(g roots/h), respectively.

was recovered in the anion transporter. The DIDS binding site was later identified as a single lysyl residue located in a transmembrane segment of the anion transporter (25). A lysyl residue different from the one modified by DIDS was, however, modified by phenylisothiocyanate, another inhibitor of anion transport (14). Eosin derivatives, eosin isothiocyanate, and eosin-5-maleimide, strongly inhibited anion transport in red blood cells and occupied a common site with DIDS (22). FITC, a fluorescein derivative which is similar to eosin minus the bromide groups, fluorescently labeled the anion transporter with a high specificity (9).

Based on the above discussion, it appears that in contrast to the anion transporter of red blood cells, the transport site of the nitrate carrier in corn roots does not recognize DIDS or FITC. Although these results (Figs. 1 and 2) do not rule out the involvement of lysyl residues in nitrate transport, they do demonstrate that the nitrate carrier does not contain lysyl residues that are accessible to DIDS or FITC.

Phenylglyoxal, a diketone, covalently modifies the guanidinium group of arginyl residues in proteins with a stoichiometry of two phenylglyoxal molecules per guanidinium group (2, 3, 30), although only a 1:1 ratio was observed in other studies (4, 23, 24). In either case, however, the loss of enzyme activity is

directly related to phenylglyoxal binding to essential arginyl residue(s) on the enzyme.

Phenylglyoxal strongly inhibited nitrate uptake by corn roots (Fig. 3). Inhibition was proportional to phenylglyoxal concentration, and at equimolar concentration with nitrate (1 mM), phenylglyoxal almost completely inhibited uptake. These results are consistent with the earlier reports on yeast hexokinase and rat liver S-adenosylhomocysteinase, where the modification of arginyl residue was found to be proportional to phenylglyoxal concentration (23, 31).

To see whether phenylglyoxal irreversibly inhibited nitrate uptake, the nitrate-pretreated root segments were incubated in a 1 mM solution of phenylglyoxal for 1 h and then uptake was studied both in the presence and absence of phenylglyoxal (Fig. 4). Phenylglyoxal pretreatment strongly inhibited nitrate uptake for the first 3 h (uptake rate 0.24 μmol/g roots/h) as compared to the control (0.77 μmol/g roots/h). During the next 3 h, though, roots resumed normal uptake, and the rate (0.67 μmol/g roots/h) was similar to that of the control. When, however, 0.5 mM phenylglyoxal was also included in the uptake medium, the control rate of nitrate uptake never resumed (uptake rate 0.24 μmol/g roots/h).

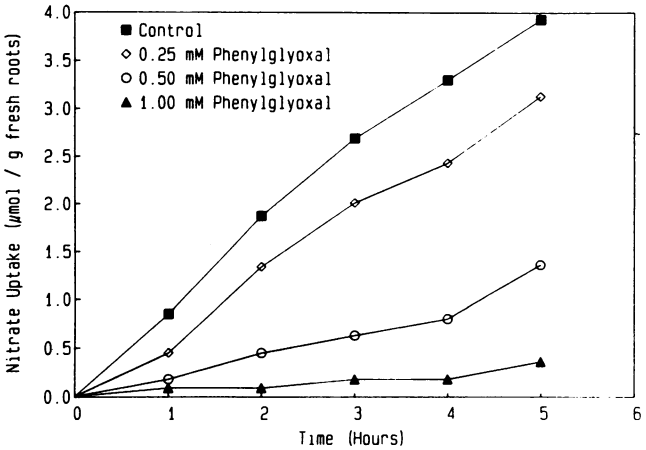

FIG. 3. Effect of phenylglyoxal on nitrate uptake in corn roots. Uptake was studied on nitrate-pretreated roots in the presence of the indicated concentrations of phenylglyoxal. The rates of uptake for the control, 0.25, 0.5, and 1.0 mM phenylglyoxal were 0.75, 0.65, 0.27, and 0.06 μmol/(g roots/h), respectively.

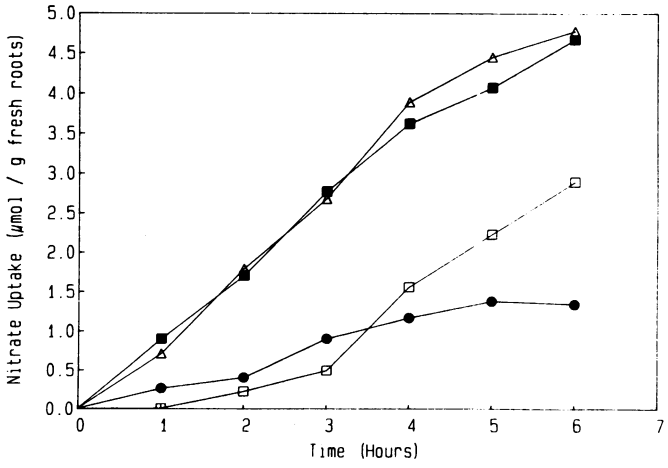

FIG. 4. Effect of DIDS and phenylglyoxal on nitrate uptake in corn roots. Nitrate-pretreated root segments were incubated with 1 mM inhibitor for 1 h prior to measurement of nitrate uptake in the presence or absence of the inhibitor. Control (■), uptake for phenylglyoxal-treated roots in the absence of phenylglyoxal (□), uptake for phenylglyoxal-treated roots in the presence of 0.5 mM phenylglyoxal (●), and uptake for DIDS-treated roots in the absence of DIDS (△).

There is strong support for the ubiquity of arginyl residues at or near the ligand binding site of enzymes that have anionic substrates or cofactors (14, 24, 26, 27, 31). The essential arginyl residue in creatine kinase reacted with phenylglyoxal 15 times faster than with free arginine (5). As a result, only the essential residue(s) were modified, although there are several other arginyl residues in the protein (2, 5, 30). Similar results were obtained with human red cells where phenylglyoxal strongly inhibited chloride transport and yet only one arginyl residue was modified per anion transporter (3). Thus, in keeping with the available evidence, the results on nitrate uptake in corn roots suggest the involvement of phenylglyoxal-accessible arginyl residue(s) in nitrate transport through the plasma membrane.

When the excised roots from nitrate-starved corn seedlings were transferred to nitrate solution, after an initial lag phase, an apparent induction of nitrate uptake was observed (13, 21). This induction was postulated to occur by *de novo* synthesis of a nitrate carrier in the corn root plasma membrane. The present study shows that after a lag phase of 3 h, a maximum rate of nitrate uptake was observed (Fig. 5). The length of time taken by the roots to recover from inhibition by phenylglyoxal was similar to the length of the lag phase for apparent induction of nitrate uptake (Figs. 4 and 5). As originally postulated, if the induction of nitrate uptake occurs due to the synthesis of a nitrate carrier, then this study would suggest that it takes about 3 h for the synthesis and functioning of such a carrier to reach steady state. This would seem quite probable in light of the rapid rates of synthesis and degradation observed for different proteins (28). Furthermore, enzymes that catalyze either the first or the rate-limiting step of a biochemical pathway, have been shown to have short half-lives (10, 11). A rapid rate of turnover allows the level of such an enzyme to fluctuate rapidly in response to environmental changes, thus providing the organism with an evolutionary advantage in terms of plasticity in response, which in turn, would widen the range of adaptation of the organism. Assuming that nitrate is a major source of nitrogen for plants and that virtually all of it is taken up through the roots, the nitrate carrier would be the first enzyme involved in nitrogen metabolism in plants. The recovery time of 3 h needed to overcome the inhibition by phenylglyoxal may be explained in terms of the turnover of the covalently modified, nonfunctional carriers and insertion of the newly synthesized, functional ones into the plasma mem-

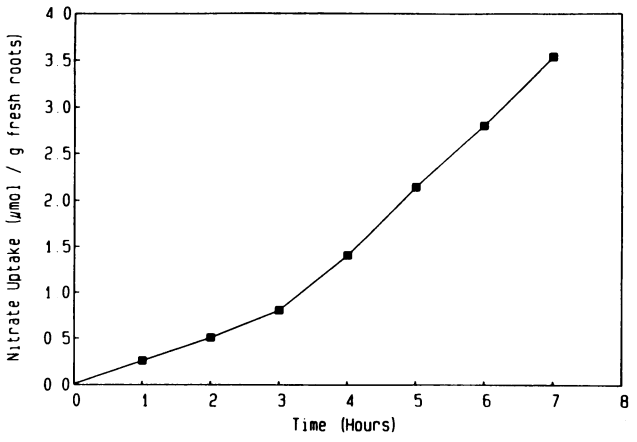

FIG. 5. Induction of nitrate uptake in nitrate-starved corn roots. Roots, fresh from the seedlings, were transformed to the uptake solution and uptake was determined over a 7-h period.

brane. More definitive experiments, however, are needed to confirm or reject the hypothesis that the reversal of inhibition occurs due to the turnover of nitrate carriers.

Inhibition of Chloride Uptake. DIDS, FITC, and phenylglyoxal inhibited chloride uptake (Table I). FITC was a stronger inhibitor than either DIDS or phenylglyoxal at equimolar inhibitor concentrations. The results for DIDS agree with the earlier reports where it was found to inhibit chloride uptake in corn roots (19, 20), corn root protoplasts (20), and *Chara* (16). It appears that the chloride carrier contains both lysyl residue(s) that are accessible to DIDS and FITC and arginyl residues that are accessible to phenylglyoxal. This is in contrast to the nitrate carrier where no DIDS- or FITC-accessible lysyl residues were detected indicating, in agreement with kinetic data (8), that distinctly different transporters are involved in chloride and nitrate transport in corn roots.

To see whether this set of inhibitors affected chloride uptake irreversibly, pretreated roots were treated with 1 mM of each of the inhibitors for 1 h before transfer to an inhibitor-free buffer solution and assay of chloride uptake in the absence of the inhibitors over a 6-h period (Fig. 6). While the results for DIDS agreed with the earlier reports, where it was found to be a reversible inhibitor (16, 19), FITC and phenylglyoxal both inhibited chloride uptake for about 2 to 3 h after the removal of the inhibitor. This lag period is similar to that observed for restoration of phenylglyoxal inhibited nitrate uptake (Fig. 4) and is consistent with the view that uptake resumes due to turnover of the modified chloride carriers. This implies that carriers for both chloride and nitrate have relatively short half-lives.

Table I. *Effect of Chemical Inhibitors on Chloride Uptake in Excised Corn Roots*

Pretreated root segments were transferred to a 0.1 mM $CaSO_4$ solution containing inhibitor concentrations as indicated. After an incubation of 1 h, root segments were transferred to the uptake solution containing $^{36}Cl^-$ at a specific activity of 25 μCi/mmol chloride and the inhibitor concentrations as indicated. Uptake was measured over a 10-min period.

Inhibitor	Concentration	Rate of Cl^- Uptake
	mM	*% of control*
Control		100[a]
DIDS	0.5	50
	1.0	44
FITC	0.5	28
	1.0	28
Phenylglyoxal	0.5	50
	1.0	50

[a] The control rate of chloride uptake was 0.18 μmol/g fresh roots per 10 min.

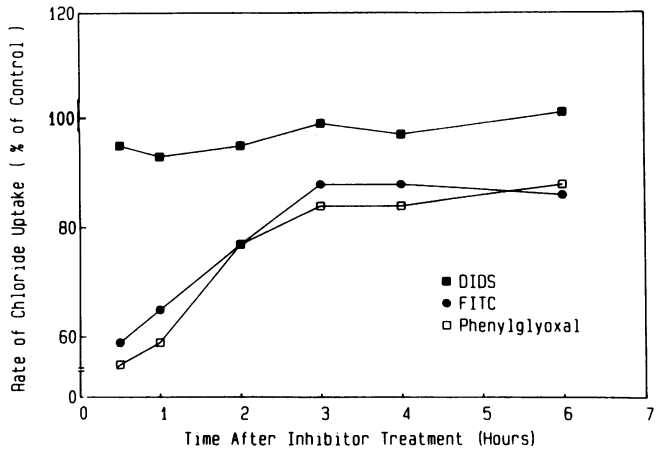

FIG. 6. Effect of DIDS, FITC, and phenylglyoxal on chloride uptake in corn roots. The $CaSO_4$ pretreated root segments were treated with 1 mM inhibitor solution for 1 h. Uptake was studied in the absence of the inhibitor. The rate of chloride uptake for the control was 0.18 μmol/(g roots/10 min).

GENERAL DISCUSSION

This study demonstrates that distinctly different mechanisms are involved in nitrate and chloride transport in corn roots. The apparently covalent nature of the reaction of phenylglyoxal and FITC with amino acid residues should make it possible to label the anion transporting polypeptides in corn roots and identify them by gel electrophoresis. FITC and radiolabeled phenylglyoxal have both been successfully used to specifically label and identify the anion transporter of human red blood cells (3, 9). When both intra- and extracellular pH was alkaline, an indiscriminate labeling with [^{14}C]phenylglyoxal was observed (3). Selective modification of the anion transporter could, however, be achieved by treating the red cell ghosts with a neutral or acidic internal pH in an alkaline medium. This selective modification probably resulted from deprotonation of only the exofacial phenylglyoxal-accessible arginyl residues of the anion transporter. Efforts to label such a polypeptide in corn root plasma membranes may be hampered by the difficulty of preparing sealed, right-side-out vesicles. The availability of such vesicles may make it possible to radiolabel the nitrate and the chloride carriers and to distinguish between them based on different inhibitor sensitivities.

Since anion transport through the plasma membrane may be energized by an ATPase-generated, free-energy gradient for proton, it is conceivable that the reagents used in this research indirectly effect anion transport by inhibiting the H^+-ATPase. For example, 1 to 10 mM phenylglyoxal rapidly inhibited the fungal H^+-ATPase apparently be reacting with an arginine residue at or near the catalytic site (15). Treatment of the corn root plasma membrane fraction with 1 mM FITC or 1 mM phenylglyoxal for 30 min inhibited ATPase activity by 85 and 35%, respectively (RT Leonard, unpublished results). However, we contend that plasma membrane H^+-pumping activity was not significantly inhibited by phenylglyoxal and did not play a role in the effect of phenylglyoxal on nitrate (or chloride) transport in root tissues. The time required for observation of inhibition was relatively short (Fig. 3), and it is unlikely that phenylglyoxal accumulated in the cytoplasm of root cells at a concentration (millimolar) sufficient to react at the catalytic site of the ATPase and inhibit H^+-pumping activity. Additionally, FITC was more effective than phenylglyoxal at inhibiting ATPase activity of the plasma membrane fraction, yet it did not inhibit nitrate transport (Fig. 2). We conclude that phenylglyoxal inhibited nitrate transport by interacting with arginyl residues of polypeptides which are accessible at the outer surface of the plasma membrane.

We found that the presence of excess nitrate in the uptake solution protected nitrate uptake from inhibition by phenylglyoxal (data not shown). This observation is consistent with earlier reports on adenylate kinase and creatine kinase, where the presence of the substrate strongly inhibited the modification of the essential arginyl residue by phenylglyoxal (2, 5). This provides an additional tool for identification of the nitrate carrier in that the presence of excess nitrate may inhibit the modification of the carrier by radiolabeled phenylglyoxal. Further, the apparently short half-lives of the nitrate and the chloride carriers should make it possible to label such polypeptides in the intact roots and study the rates of their degradation. The information so obtained, in conjunction with phenylglyoxal and FITC labeling, should be useful in narrowing down the number of candidates for nitrate and chloride carriers.

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