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Effect of Azetidine 2-Carboxylic Acid on Ion Uptake and Ion Release to the Xylem of Excised Barley Roots[1]

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ABSTRACT

Azetidine 2-carboxylic acid (AZ) was used as an analog of proline to investigate further the relationship between protein synthesis and ion transport. AZ does not inhibit protein assembly, but the proteins formed are ineffective as enzymes. At relatively low concentrations (50 μM) AZ was a potent inhibitor of release of ions to the xylem of excised roots of barley (*Hordeum vulgare* L.) and intact plants. Uptake to the root was also inhibited but to a lesser degree. A procedure was introduced for estimating unidirectional fluxes from measurements of net tracer uptake, net transport to the xylem, and net efflux from the roots. It was shown that inhibition of release to the xylem was not caused by reduction in influx at the plasmalemma or to stimulation of influx to the vacuoles. It was suggested that AZ was acting on the process of release from symplast to the xylem. The action of AZ is compared with similar effects on ion transport produced by *p*-fluorophenylalanine, cycloheximide, and abscisic acid.

A previous publication has shown that the amino acid analog *p*-FPA[2] inhibited transport of ions across the root to the xylem (18). Influx to the root was not inhibited and, consequently, there was an increase in accumulation of tracer in vacuoles of the root cells. The inhibition occurred inward of the endodermis and hence was suggested to be due to a block in release from cells in the stele, and not to inhibition of movement in the symplasm. This view of action of FPA on transport across the root is consistent with current views on symplasmic transport (19) as a passive process, involving active transport at entry to or exit from the symplasm (4, 10). Inhibition was suggested to result from production of nonsense protein incorporating the analog instead of phenylalanine.

The purpose of the present investigation was to support the interpretation of the results with *p*-FPA by showing that a different amino acid analog produced a similar inhibition of ion transport to the xylem. Azetidine 2-carboxylic acid acts as an analog of proline, that has been shown to compete with L-proline for incorporation into protein but without inhibiting protein synthesis (13). The results have a wide relevance in interpretation of the effect of inhibitors of protein synthesis on ion transport across the root and the inhibition of ion release to the xylem produced by ABA (3).

MATERIALS AND METHODS

Plant Material. Excised roots of barley (*Hordeum vulgare* L. cv. Dampier) were used in the experiments. Seedlings were grown on stainless steel gauze, with the roots in aerated 0.5 mM $CaSO_4$ at pH 5.5 for 4 days then transferred to 0.67 or 5 mM KCl + 0.5 mM $CaSO_4$ for 24 hr to load the cell vacuoles with KCl. These roots are referred to as "$CaSO_4$/KCl" roots. For efflux studies, this solution contained ^{36}Cl so that the roots contained labeled Cl^-. Roots grown on 0.67 mM KCl contained 50 μmol g_{FW}^{-1} and roots from 5 mM KCl contained 80 μmol g_{FW}^{-1} Cl^-. Barley roots were also grown in the dark in an aerated nutrient solution at pH 5.5 containing 10 mM KNO_3, 2 mM $Ca(NO_3)_2$, 3 mM $MgSO_4$, 0.8 mM $NH_4H_2PO_4$, Fe-EDTA to give 90 μM Fe, and trace elements according to Arnon and Hoagland (1). These are referred to as "nutrient roots."

Solutions. Solutions of KCl for use in experiments also contained 0.5 mM $CaSO_4$ at pH 5.5.

Measurements of Uptake and Transport across the Root. Tracer transported into the symplasm of the root is assumed either to be retained in cell vacuoles or to be transported across the root to the xylem or to be returned to the solution if there is efflux from the symplasm.

Measurement of tracer content of the roots at different times can be used to estimate the rate of accumulation in the vacuoles, referred to here as Φ^*_{ov}. Roots were put into 5 mM KCl labeled with ^{36}Cl or ^{86}Rb, and samples removed at intervals. Samples were rinsed for a standard time of 2 min in ice-cold 5 mM KCl to remove free space tracer, though it is probable that a small amount of tracer would also be removed from the symplasm by this treatment. The slope of the content of the tissue plotted against time was used to estimate Φ^*_{ov}. There are some limitations in this procedure, but they do not detract from the usefulness of the measurements in the present experiments. The basis for the indeterminancy in Φ^*_{ov} is that part of the tracer in the tissue will be in the symplasm and in the xylem vessels. However, the major amount after about 1 hr will be in the vacuoles, which thereafter will represent the major change in content of the tissue and then the slope approximates to Φ^*_{ov}.

Transport across the root to the xylem (Φ^*_{ox}) was measured using excised roots set up with 5 to 7 cm of the apical end of the root in labeled solution and about 0.5 cm of the cut, basal end of the root in unlabeled solution. This unlabeled solution was removed periodically to determine transport from the root in the xylem exudate. Details of the system are described elsewhere (14). The main uncertainty in Φ^*_{ox}, as in all experiments to measure exudation of solute, is that the amount of tracer released from the cut end was transported to the xylem at some earlier time dependent on the rate of flow of exudate in the vessels. The time to clear the vessels of barley roots has been estimated at about 3 min cm^{-1} root (14), *i.e.* about 15 min in the present experiments.

The sum of content of the root and the cumulative total exported from the xylem can be used to estimate the net tracer influx. The total content will be underestimated by the small amount removed from the symplasm in a 2-min rinse by ice-cold KCl, but otherwise is not affected by the uncertainties in Φ^*_{ov} and Φ^*_{ox}.

[1] This work was supported by the Australian Research Grants Committee and by a University of Sydney Research Grant.

[2] Abbreviations: AZ: L-azetidine 2-carboxylic acid; CCCP: carbonyl cyanide *m*-chlorophenyl hydrazone; CHM: cycloheximide; FPA: fluorophenylalanine; g_{FW}: gram fresh weight.

Efflux of tracer was measured from roots either to the external solution via the cortex or through the cut end via the xylem. Labeled tissue was kept in unlabeled solution for about 2 hr before use to reduce the specific radioactivities in free space and cytoplasm from that in the labeling solution, so that tracer lost from the tissue originated in the vacuoles (at specific radioactivity s_v). Efflux to the xylem is referred to as Φ^*_{vx} and to the solution via the cortex as Φ^*_{vo}. The apparatus used has been described previously (14), but essentially consisted of two chambers so that the apical 5 cm of the roots could be set up in one end, the cut end (about 0.5 cm) in the other. The chambers were separated by barriers, into which the roots were sealed with silicone grease.

The tracer measurements of uptake (or efflux) can be related to unidirectional fluxes if assumptions are made about the relationship of phases in the root (14). The unidirectional fluxes are shown here as ϕ with subscript to denote the direction; ϕ_{oc} is the flux between solution and cytoplasm (or symplasm) and ϕ_{co} the flux in the opposite direction, ϕ_{cv}, ϕ_{vc} are fluxes across the tonoplast between symplasm and vacuole, and ϕ_{cx} is the flux into the xylem exudate. Specific radioactivities are given as s_o, s_c, s_v in solution, cytoplasm, and vacuoles, respectively. The quantities Φ^*_{ov}, Φ^*_{ox}, Φ^*_{vo}, and Φ^*_{vx} are respectively:

$$\Phi^*_{ov} = \phi_{cv} \cdot s_c = \phi_{cv} \cdot \phi_{oc} \cdot s_o / (\phi_{co} + \phi_{cv} + \phi_{cx}) \quad (1)$$

$$\Phi^*_{ox} = \phi_{cx} \cdot s_c = \phi_{cx} \cdot \phi_{oc} \cdot s_o / (\phi_{co} + \phi_{cv} + \phi_{cx}) \quad (2)$$

$$\Phi^*_{vo} = \phi_{co} \cdot \phi_{vc} \cdot s_v / (\phi_{co} + \phi_{cv} + \phi_{cx}) \quad (3)$$

$$\Phi^*_{vx} = \phi_{cx} \cdot \phi_{vc} \cdot s_v / (\phi_{co} + \phi_{cv} + \phi_{cx}) \quad (4)$$

For convenience, s_o can be set equal to 1 and s_v expressed relative to s_o; s_v can be estimated from the amount of Cl^- and its tracer in the tissue at the end of the experiment. The net tracer influx, estimated as the sum of Φ^*_{ov} and Φ^*_{ox}, is equal to $(\phi_{oc} - \phi_{co} \cdot s_c)$, which is equivalent to $\phi_{oc}(\phi_{cx} + \phi_{cv})/(\phi_{co} + \phi_{cv} + \phi_{cx})$. When information on both tracer uptakes and both tracer effluxes is available then the individual fluxes can be estimated.

Rates of transport are expressed relative to fresh weight of tissue and the specific radioactivity of the solution (14). The average least significant difference in uptake experiments (Φ^*_{ov}) was 6% and in transport (Φ^*_{ox}) to the cut end of the root, 12%. These values should be assumed where no limits are quoted.

Other Procedures. Leucine incorporation and uptake were determined as described previously (19).

ATP and ADP were extracted according to a procedure of R. Reid (personal communication). Root tissue was frozen rapidly in liquid freon near the temperature of liquid N_2. The frozen tissue was transferred to liquid N_2 in a polyethylene tube, ground with a Teflon pestle, and allowed to thaw in 5 ml of 0.4 M H_2SO_4 containing 10 mM 8-hydroxyquinoline. After standing at 0 C for 4 hr, the homogenate was centrifuged to remove particulate matter, and ATP was assayed in the supernatant after diluting it with an equal volume of water. ATP was determined by the firefly luciferase method. Extract from 50 mg of dried firefly lanterns was reconstituted with 5 ml of water and diluted 3-fold with assay buffer (25 mM HEPES, 5 mM $MgSO_4$, 0.1 mM NaH_2PO_4, pH 7.4). The assay mixture consisted of 3 ml assay buffer, 30 μl of firefly extract and 20 μl of samples. The assay was performed at 8 C and luminescence was measured over 6 sec in a Packard Tri-Carb model 3375 liquid scintillation spectrometer. In separate samples, ADP was converted to ATP using pyruvic kinase and P-enolpyruvate, then ATP assayed as above. The difference between the two estimates of ATP was equivalent to ADP. The method was checked with added ADP.

Nitrate reductase was assayed as described previously (21).

L-Azetidine-2-carboxylic acid, firefly extract (F1E-50), and L-proline were obtained from Sigma. L-[1-^{14}C]Leucine was obtained from Radiochemical Centre, Amersham, U.K.

RESULTS

Transport across the Root. Azetidine 2-carboxylic acid was found to be a potent inhibitor of transport of K^+ and Cl^- across barley roots to the xylem.

Nutrient-grown barley roots were set up to measure ^{36}Cl transport from the cut end (Φ^*_{ox}). When tracer was added to the roots, the rate of transport of ^{36}Cl from the cut end increased to a maximum of about 1.5 μmol g_{FW}^{-1} hr^{-1}. After 1.5 hr in labeled KCl, the solution was replaced by one of the same concentration and specific radioactivity for ^{36}Cl, containing either 50 μM or 100 μM AZ or 100 μM L-proline. Samples of roots were left in labeled KCl as controls (Fig. 1). There was a lag of about 40 to 60 min in development of inhibition of Φ^*_{ox} and then Φ^*_{ox} decreased exponentially with a half-time of about 50 min. There was some variability in this lag time and in the time for 50% decrease between different experiments but the large degree of inhibition (above 90% of Φ^*_{ox}) was usual.

Table I summarizes a number of experiments like that in Figure 1, when AZ concentration was varied. Substantial inhibition of Φ^*_{ox} occurred above 10 μM AZ, with Φ^*_{ox} falling to less than 5% of the control value, which ranged from 1.30 to 2.05 μmol g_{FW}^{-1} hr^{-1}. Transport of both ^{86}Rb and ^{36}Cl was inhibited by AZ. Inhibition of one ion should lead to reduced transport of any passively moving counter ion, and also to reduction in volume exudation, which in turn, would reduce transport out of the root of any other flow-dependent export.

Figure 1 showed that 100 μM proline produced no inhibition of transport. Table I gives results of other similar experiments in which inhibition of Φ^*_{ox} in L-proline was only found at concentrations of 1 to 5 mM, much higher than the concentrations at which AZ was effective.

Table I shows that Φ^*_{ox} was reduced by 45% in a mixture of 50 μM AZ and 50 μM proline compared with 95% when 50 μM AZ was present alone. The reduced inhibition produced by the mixture is consistent with AZ acting as an analog of proline. The degree of inhibition corresponded to a concentration of AZ between 1 and 10 μM, *i.e.* L-proline appeared to compete effectively with AZ for uptake or incorporation. Further interactions between AZ and proline are given in Table II and V.

Figures 2 and 3 show estimates of net tracer influx for tissue in

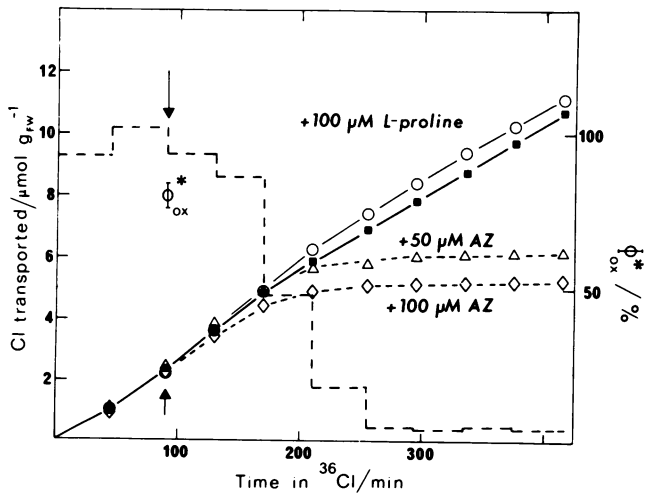

FIG. 1. Inhibition of transport from the xylem by AZ. The symbols show cumulative transport of ^{36}Cl from the cut end of excised barley roots in 5 mM KCl. At 90 min (arrows) there was introduced: (△) 50 μM AZ; (◇) 100 μM AZ; (○) 100 μM L-proline. (■) was unchanged. The histogram of rates of transport (Φ^*_{ox}) from the data for treatment with 100 μM AZ, plotted as a percentage of the rates in untreated roots, shows the time course of development of inhibition more clearly than the cumulative content.

Table I. *Effect of varied concentrations of AZ and L-proline on the rate of transport of ^{36}Cl or ^{86}Rb (Φ^*_{ox}) expressed as percentage of control rates after 4 hr treatment.*

	Rates of transport (% of control)							
	Concentration of AZ or proline added (μM)							
	1	10	50	100	500	1000	5000	(50 + 50)
Addition of:								
AZ, ^{36}Cl	88	12	5	2	0.5	0.2		
AZ, ^{86}Rb			5					
L-proline, ^{36}Cl			100	95	93		49	
Mixture of AZ and L-proline, ^{36}Cl								55

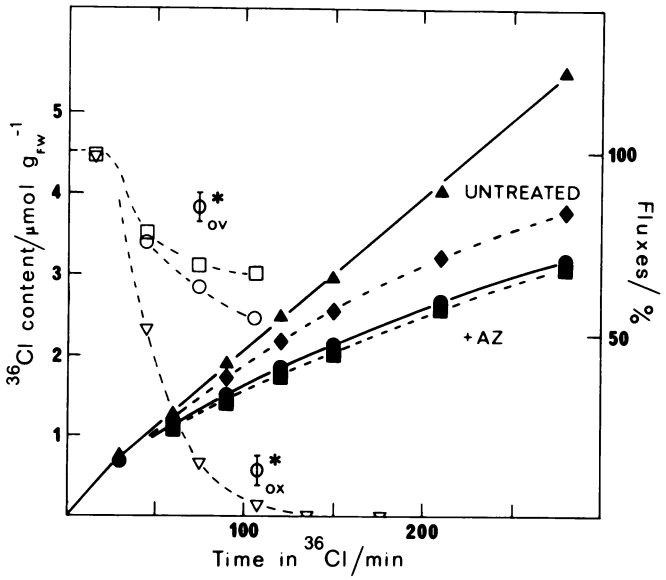

FIG. 2. Effect of AZ on tracer uptake and accumulation in barley roots. Amounts of ^{36}Cl accumulated from 0.67 mM KCl by excised barley roots (◆) and the total tracer uptake (▲) calculated as the sum of accumulation and cumulative transport from the cut end (as in Fig. 1). (■) and (●) are data for accumulation and total uptake in the presence of 50 μM AZ added at zero time. Open symbols are rates of accumulation (□), total uptake (○), and transport from the xylem (▽) in AZ all relative to values in untreated roots.

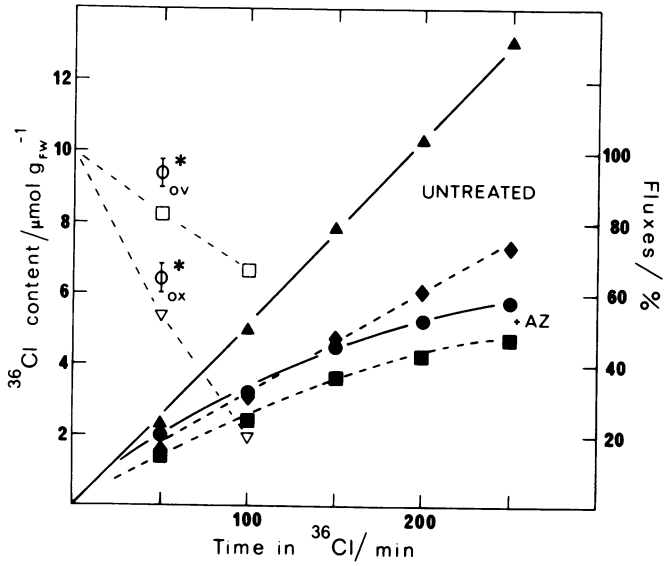

FIG. 3. Effect of AZ on tracer uptake and accumulation in barley roots in 5 mM KCl. Symbols as in Figure 2, but different scale.

0.67 and 5 mM KCl. In both cases the net uptake of tracer to untreated roots was linear over several hours, as found previously (14), but it was inhibited from about 30 to 60 min after addition of AZ. Since net tracer uptake is equal to ($\phi_{oc} - \phi_{co} \cdot s_c$) the inhibition could be due to reduction in ϕ_{oc} or increase in ϕ_{co}. Both fluxes appear to contribute to the reduction observed.

The rate of accumulation (Φ^*_{ov}) was also reduced, but to a smaller extent. In 0.67 mM KCl the reduction was from 0.55 to 0.48 μmol g_{FW}^{-1} hr^{-1} and in 5 mM KCl from about 1.8 to 0.8 μmol g_{FW}^{-1} hr^{-1}. There was some variability in the degree of inhibition of Φ^*_{ov} between experiments, which possibly reflects the sensitivity of Φ^*_{ov} to ϕ_{oc} and ϕ_{cv} as well as to ϕ_{cx}. Inhibition of Φ^*_{ov} was found both for ^{36}Cl and ^{86}Rb and using either nutrient-grown or $CaSO_4$/KCl roots.

For comparison with the effect of AZ on Φ^*_{ox}, the percentage reduction of net tracer influx and of Φ^*_{ox} is shown in Figures 2 and 3 for the first 2 hr of measurement. The general conclusion is that inhibition of each of these quantities occurred at about the same time after addition of AZ.

Ions transported to the xylem of barley roots have been shown to be derived both from cell vacuoles and from the external solution (7). To test that AZ also inhibited transport Cl^- from vacuoles to the xylem, $CaSO_2$/KCl roots were prepared containing labeled ^{36}Cl and then net efflux of tracer measured from the cut end (Φ^*_{vx}) and to the solution around the root (Φ^*_{vo}). Roots were rinsed in unlabeled KCl for 2 hr before the start of measurements to eliminate exchange from the free space and cytoplasmic phase. Figure 4a shows the inhibitory effect of 50 μM AZ on Φ^*_{vx}, which was reduced by about 93%, after a lag of about 30 to 50 min. Figure 4b shows that there was a stimulation of Φ^*_{vo} by about 1 μmol g_{FW}^{-1} hr^{-1} which was evident during the first sampling period. This increase in Φ^*_{vo} is shown below to be due to an increase in ϕ_{co}. Note that there was no stimulation of total tracer efflux from the tissue ($\Phi^*_{vo} + \Phi^*_{vx}$).

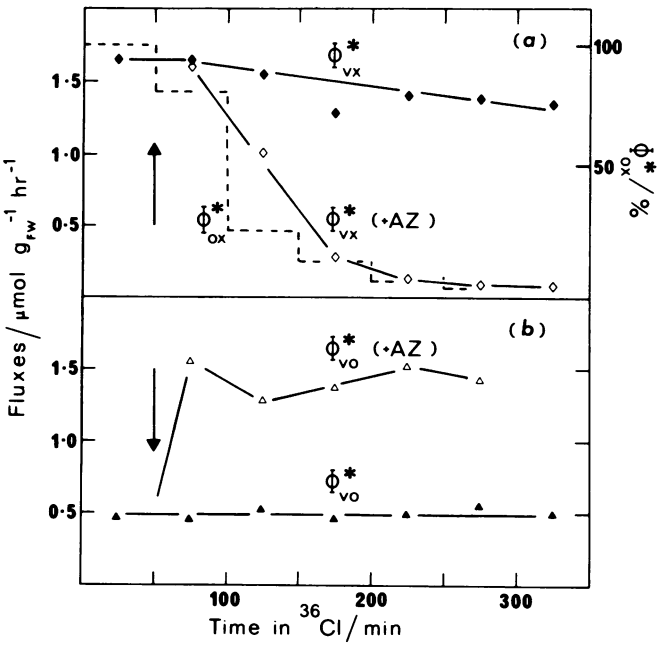

FIG. 4. Effect of AZ on efflux of ^{36}Cl from barley roots in 5 mM KCl. (a): Efflux was measured to the xylem (Φ^*_{vx}) from untreated roots (◆) and from roots treated with 50 μM AZ (◇) from the time shown. The histogram (- - -) shows Φ^*_{ox} determined with similar tissue and expressed relative to values in untreated roots; 100% was 1.45 μmol g_{FW}^{-1} hr^{-1}. (b): Efflux measured to the external solution (Φ^*_{vo}) from untreated roots (▲) and from roots in 50 μM AZ from the time shown (△).

The reversibility of inhibition of Φ^*_{ox} produced by AZ was investigated by replacing AZ in the labeled solution with 100 μM L-proline after varied periods. Nutrient-grown roots set up in 5 mM KCl labeled with ^{36}Cl were treated with 50 μM AZ for 20 min, 1 hr, or 2 hr and then AZ removed by changing the solution to 5 mM KCl + 100 μM L-proline. The solution was replaced twice with 5 min between changes to remove AZ in surface layers of solution and in the free space of the roots. L-Proline was included as it had been shown to reduce the effectiveness of AZ (Table I) and it was expected to counteract any residual AZ in the free space and to speed up displacement of AZ from the cytoplasmic pools. Other sets of roots were left in AZ to check the degree of inhibition achieved. All labeled solutions contained the same specific radioactivity of Cl^-.

When AZ was removed after 20 min, there was no development of inhibition. The effect of removal of AZ after 1 hr is shown in Table II. The response of Φ^*_{ox} to AZ was analogous to that shown in Figure 1, so the results are summarized in Table II as rates of transport in roots at the times of addition and removal of AZ and at 2 and 6 hr after addition of AZ. Removal of AZ did not prevent the subsequent development of inhibition of Φ^*_{ox} even though there had been little reduction during the 1 hr in AZ. At 6 hr the rate had fallen to 0.25 μmol g_{FW}^{-1} hr^{-1} compared with 0.10 μmol g_{FW}^{-1} hr^{-1} when treated with AZ throughout. Similar results were found in a separate experiment when AZ was removed after 2 hr, though in this case the inhibition of Φ^*_{ox} was more fully developed at 2 hr than at 1 hr, and there was less difference at 6 hr between roots in AZ continuously (4% of controls) and roots removed from AZ (5% of controls).

The effect of AZ on the net amount of tracer crossing the endodermis was estimated by separating stele from cortex. Roots were excised into unlabeled 0.67 mM KCl or 0.67 mM KCl + 50 μM AZ, left for 90 min, then transferred to the same solutions labeled with ^{36}Cl. After 1 hr the roots were removed and rinsed in ice-cold 0.67 mM KCl for 2 min, then the cortex stripped from the stele using a pair of forceps. The steles were

Table II. *Effect of removal of 50 μM AZ after 1 hr on Φ^*_{ox}, in comparison with untreated roots and roots kept in AZ.*

See text for details.

Time (min)	Values of Φ^*_{ox} (μmol g_{FW}^{-1} hr^{-1}) Control	+ 50 μM AZ at 80 min	AZ removed at 140 min
80	1.36	1.15	1.30
140	1.40	1.05	1.30
200	1.30	0.50	0.60
440	0.85	0.10	0.25

rinsed briefly (2 sec) in ice-cold 0.67 mM KCl, their lengths measured and tracer content determined. The amount of ^{36}Cl is expressed relative to the weight of the intact roots by combining the total length of steles in the sample and the ratio of length to fresh weight for intact roots (14 mg g_{FW}^{-1}). Table III shows that net accumulation of ^{36}Cl in the root (measured from separate samples of intact roots) was reduced by about 40%.

The content of the steles was reduced from 0.13 to 0.10 μmol g_{FW}^{-1} (about 25% reduction). Over the same period of 1 hr the amount of tracer transported from the cut end (determined in separate experiments) was about 10% of that in the control roots. The net tracer transport across the endodermis can then be estimated at 0.32 μmol g_{FW}^{-1} in control and 0.12 μmol g_{FW}^{-1} in AZ treated roots over this period. This result is similar to that found for FPA (18) and for ABA (16) and is considered to support the view that the site of inhibition of Φ^*_{ox} is not at the endodermis but within the stele.

Measurements were made of the effect of AZ on intact, transpiring plants to test the possibility that reduction in volume flow from excised roots due to decreased permeability of the root to water caused the inhibition of ion release to the xylem. After 3 hr in either 0.67 mM KCl or 0.67 mM KCl + 50 μM AZ, intact seedlings, 7 days old, were transferred to the same solutions labeled with ^{36}Cl. After 90 min the plants were rinsed for 2 min in ice-cold 0.67 mM KCl, then the ^{36}Cl content of roots and shoots measured. The content of the roots was 0.71 ± 0.05 and 1.37 ± 0.13 μmol g_{FW}^{-1} in AZ and untreated plants, respectively (means ± SE, four replicates). The corresponding content of the shoots was 0.07 ± 0.01 and 0.87 ± 0.07 μmol g_{FW}^{-1}, respectively (relative to root weight). There is clearly a strong reduction in transport to the shoot AZ (92%). Measurements of transpiration from similar plants showed that water flow relative to root weight was 360 mg g_{FW}^{-1} hr^{-1} in AZ and 350 mg g_{FW}^{-1} hr^{-1} in untreated plants over the period 3-4.5 hr after introduction of AZ. Exudation rate from barley seedlings is about 120 mg g_{FW}^{-1} hr^{-1}.

Estimation of Unidirectional Fluxes. Unidirectional fluxes can be estimated from the measurements of Φ^*_{ov}, Φ^*_{ox}, Φ^*_{vo}, and Φ^*_{vx}, together with knowledge of the specific radioactivity in the vacuole. The equations given above (equations 1-4) relate these tracer uptakes and effluxes to the unidirectional fluxes. If it is assumed that changes in the content of the cytoplasmic phase are small, conservation of Cl^- then requires that net influx = net efflux for the cytoplasm, *i.e.*:

$$\phi_o = \phi_{co} + \phi_{cx} + (\phi_{cv} - \phi_{vc})$$

or

$$\phi_{oc} + \phi_{vc} = \phi_{co} + \phi_{cx} + \phi_{cv}$$

Table III. *Effect of AZ on transport of ^{36}Cl to the stele.* Tracer applied between 90 and 150 min from excision and start of AZ treatment.

	^{36}Cl content ($\mu mol\ g_{FW}^{-1}$)		Transport to xylem (Φ^*_{ox})
	Whole roots	Steles	($\mu mol\ g_{FW}^{-1}\ hr^{-1}$)
Control	0.91 ± 0.03 (4)	0.13 ± 0.01 (4)	0.19
AZ (50 μM)	0.55 ± 0.05 (4)	0.10 ± 0.01 (4)	0.02

Hence, referring to equations 1 through 4, it can be shown that

$$\phi_{cx} = \Phi^*_{ox} + \Phi^*_{vx}/s_v$$

$$\phi_{co} = \phi_{cx} \cdot \Phi^*_{vo}/\Phi^*_{vx}$$

$$\phi_{cv} = \phi_{cx} \cdot \Phi^*_{ov}/\Phi^*_{ox}$$

Substituting for these fluxes in Φ^*_{vo} or Φ^*_{ov} then allows calculation of ϕ_{vc} and ϕ_{oc}. Note that information on both tracer uptake and efflux is needed for these calculations, and that s_v must be adjusted to the changing tracer content of the tissue during efflux.

Figures 5 and 6 show some estimates of the effect of AZ on unidirectional fluxes. Roots grown on 5 mM KCl or KCl labeled with ^{36}Cl were used to estimate Φ^*_{ov}, Φ^*_{ox}, Φ^*_{vo}, and Φ^*_{vx}. These estimates were made by plotting cumulative amounts of tracer transported into or lost from the tissue and drawing tangents to curves fitted to the points. Each point used for the cumulative graphs was the mean of three separate determinations.

Values of fluxes ϕ_{cx} and ϕ_{oc} in untreated roots were nearly constant over the 200 min, having mean values of 2.9 ± 0.03 and 3.7 ± 0.1 $\mu mol\ g_{FW}^{-1}\ hr^{-1}$ (± SE), respectively. The influx to the vacuole, ϕ_{cv}, fell during the experiment from 4.4 to 2.7 $\mu mol\ g_{FW}^{-1}\ hr^{-1}$. These results show that AZ produces strong inhibition of ϕ_{cx} (to 95%), as would be expected from its action on Φ^*_{ox} and Φ^*_{vx}. There was less relative reduction in ϕ_{oc}, and further, its absolute reduction at 50 and 100 min was less than that in ϕ_{cx} by about 0.6 and 0.5 $\mu mol\ g_{FW}^{-1}\ hr^{-1}$, respectively. This implies that reduction in ϕ_{cx} was not entirely a consequence of reduction in ϕ_{oc}. The influx to the vacuole was less affected by AZ than either ϕ_{cx} or ϕ_{oc}; the slight increase at 50 min was found in other similar experiments. Over longer times the reduction observed in ϕ_{cv} could be a direct effect of AZ through its action on metabolism but could also be a consequence of the reduction in ϕ_{oc}.

The efflux from the tissue, ϕ_{co}, is shown separately in Figure 6 with the absolute values of ϕ_{cx}. The efflux from untreated roots was constant at 0.90 ± 0.03 $\mu mol\ g_{FW}^{-1}\ hr^{-1}$, which has been shown as the value at zero time. The reduction in ϕ_{cx} was accompanied by an increase in ϕ_{co}, as expected from the changes in Φ^*_{vo} already given in Figure 4.

Action of AZ on Metabolism in the Root. AZ is reputed to act as an analog of L-proline (13), *i.e.* AZ can be incorporated into protein or short chain polypeptides in place of L-proline, when these compounds are consequently nonfunctional in their normal roles. It would be expected then that AZ would not inhibit the rate of amino acid assembly, apart from indirect effects due to feedback from the resultant, nonfunctional polypeptides. However, newly synthesized protein should not show enzyme activity.

Table IV gives data for incorporation of L-[^{14}C]leucine into

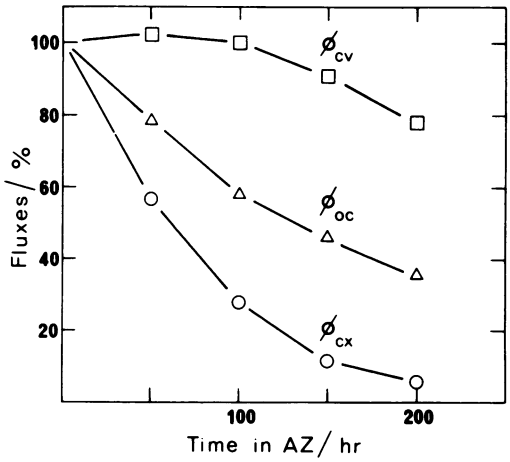

FIG. 5. Effect of AZ on unidirectional fluxes ϕ_{oc}, ϕ_{cx} and ϕ_{cv} in 5 mM KCl. Fluxes shown relative to values in untreated roots (see text) and plotted against time from addition of AZ.

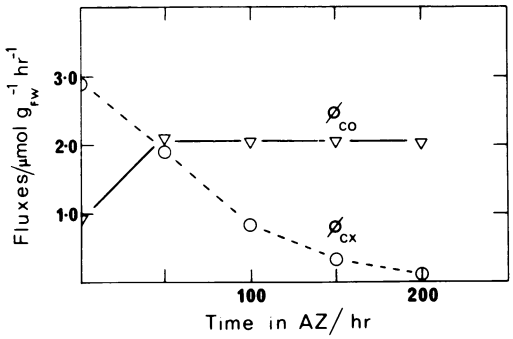

FIG. 6. Effect of AZ on transport to the xylem (ϕ_{cx}) and efflux to the solution (ϕ_{co}). Fluxes plotted against time from addition of AZ.

trichloroacetic acid-insoluble material (protein). Roots were excised and kept in 5 mM KCl with either 50 μM L-proline or 50 μM AZ. At zero time and after 100 min in proline or AZ samples were put into similar solutions containing 20 μM L-leucine. After 45 min the samples were assayed. The ratio of incorporated L-leucine to the total content of the tissue was the same in L-proline and AZ, *i.e.* compared with L-proline, AZ does not reduce L-leucine incorporation at times (100–145 min) when transport (Φ^*_{ox}) was inhibited (*e.g.* see Figs. 1 and 2). In contrast, CHM, which blocks protein assembly at the ribosomes, has been found to reduce L-leucine incorporation by 54% with 45 min in barley roots (22). Both L-proline and AZ reduced L-leucine uptake and incorporation by about 40% compared with

Table IV. *Comparison of the effect of proline and AZ on uptake and incorporation of ^{14}C-L-leucine into TCA-insoluble material.*
Each is a mean of 4 replicates, but only the SE of the individual ratios is shown.

Time	Uptake (nmol g_{FW}^{-1})	Incorporation (nmol g_{FW}^{-1})	Ratio $\frac{\text{Incorporation}}{\text{Uptake}}$
0-45 min			
50 μM L-proline	35	17	0.47 ± 0.05
50 μM AZ	44	21	0.48 ± 0.04
100-145 min			
50 μM L-proline	49	12	0.24 ± 0.005
50 μM AZ	52	12.5	0.24 ± 0.015

solutions containing 20 μM L-leucine as the only amino acid. The reduction is assumed to be due to competitive inhibition of the amino acids, though this does not affect the main purpose of the experiment described here.

To test the effectiveness of protein formed in AZ, the induction of nitrate reductase by NO_3^- was assayed, since the increased level of this enzyme is due to synthesis of new protein. Tissue pretreated with 5 mM KNO_3 for 4.5 hr showed induction of NO_3^- reductase activity which was absent in similar tissue also treated with AZ (Table V). Measurements of NO_3^- content showed that NO_3^- was taken up to the tissue, eliminating the possibility that AZ inhibited NO_3^- reductase by blocking NO_3^- uptake. The second part of Table V tests the reversibility of the inhibition produced by AZ. After 2.5 hr in 5 mM KCl + 50 μM AZ, roots were transferred to 5 mM KNO_3 or 5 mM KNO_3 + 100 μM proline. With added proline, the amount of nitrate reductase activity induced was at least 70% of that in roots not treated with AZ, but in the absence of L-proline there was no recovery of NO_3^- reductase activity. The results of Tables IV and V support the suggestion that AZ leads to formation of nonfunctional protein.

Inhibition of ion uptake and protein synthesis by CHM in barley roots was accompanied by about 19% reduction in O_2 uptake, and 45% increase in ATP level with little or no reduction in the ratio of ADP to ATP (11, 22). AZ produced a similar response, reducing O_2 uptake from 18 to 15.5 μmol g_{FW}^{-1} hr^{-1} (*i.e.* about 15%) and increasing ATP content from 59 ± 2 to 88 ± 2 μmol g_{FW}^{-1}. The ratio of ADP to ATP was 0.40 in control and 0.35 in AZ-treated roots.

Table V. *Induction of nitrate reductase and the effect of 50 μM AZ.*
(i) and (ii) are separate experiments. Paired values are duplicates; single values are means of 3 replicates for which L.S.D. (5%) = 9%.

		Nitrate reduction (NO_2^-: pmol g_{FW}^{-1} sec^{-1}) (i)	(ii)
(a)	Initial level	64	120
	After 4½ hr in KCl	56, 65	98
	" " " KNO_3	145, 180	270
	" " " KNO_3 + AZ	38 58	58
(b)	Initial level		
	2½ hr in KCl + AZ then:		
	4½ hr in KNO_3	51, 61	-
	4½ hr in KNO_3 + proline	145, 115	255

DISCUSSION

The results presented show that moderately low concentrations of AZ (10-15 μM) inhibited release of Cl^- and K^+ to the xylem exudate of excised roots. The inhibition was found either when tracer was derived from the external solution (Φ_{ox}^*) or from the cell vacuoles (Φ_{vx}^*). Transport from the xylem (Φ_{ox}^*) was inhibited in both 0.67 mM KCl and in 5 mM KCl. Inhibition of Φ_{ox}^* was accompanied by inhibition of net tracer uptake and to a much less extent by reduction in Φ_{ov}^*. Two aspects of this inhibition are considered here: the interpretation of inhibition in terms of processes involved in transport of ions across the root, and the effects of AZ on cell metabolism that could account for inhibition of transport.

Interpretation of Inhibition of Φ_{ox}^*. Calculations of unidirectional fluxes were made for roots in 5 mM KCl assuming a cytoplasmic-symplast phase in contact with the xylem and with the vacuoles. On this model, the reduction in Φ_{ox}^* was due to inhibition of ϕ_{cx}. However, there are a number of ways this inhibition could be produced, apart from action at a specific carrier contributing to ϕ_{cx}. These are by reduction in ϕ_{oc}, by stimulation of ϕ_{cv}, by blocking symplasmic transport to the stele, or by reducing water permeability of the root and so reducing the volume of water flow.

Analysis of the fluxes showed that inhibition of ϕ_{cx} was accompanied by a reduction in ϕ_{oc} and initially there was no effect or a slight increase in ϕ_{cv}. However, the absolute changes in ϕ_{oc} were less than those in ϕ_{cx} so its reduction cannot be interpreted as due to the inhibition of ϕ_{oc}. Similarly, the stimulation of ϕ_{cv} was small and found only over the first 50 min; at later times ϕ_{cv} was inhibited (Fig. 5).

Inhibition of transport in the symplast should show as a reduction of transport across the endodermis to the stele. Measurements of tracer content of the steles and of Φ_{ox}^* (Table IV) were used to show that although net transport across the endodermis was reduced, it was relatively less affected (65%) than Φ_{ox}^* (90%

inhibition). Experiments using ABA (16) and FPA (18) had produced similar evidence that in barley roots the endodermis was not a complete barrier to tracer when Φ^*_{ox} was inhibited. Using current views on the symplasm (19), the degree of inhibition across the endodermis can be interpreted as due to a change in sinks within the stele from accumulation in stelar cells plus release to the xylem in controls to accumulation in stelar cells alone in AZ.

Observation of tracer transport from the cut end of the root depends on there being a volume flow from the xylem. Inhibition of Φ^*_{ox} might then occur due to inhibition of water flow, irrespective of inhibition of ϕ_{cx}. Reduction in permeability of roots to water flow has been observed with corresponding reduction in Φ^*_{ox} produced by cytokinin (8). Changes in hydraulic conductivity of the root have also been found using ABA (5, 20), and suggested to affect solute transport to the xylem. However, the inhibition of Φ^*_{ox} has also been observed in CHM (22) and ABA (3) using intact plants in which there was continued transpiration, and in the present paper similar results are reported for AZ. Inhibition of Φ^*_{ox} in these examples cannot be due to reduced water permeability of the root.

These results support the view that inhibition of Φ^*_{ox} is due to inhibition of ϕ_{cx} by AZ. According to Davis and Higinbotham (4), release to the xylem appears to involve active transport and it would be interesting to test if the process they investigated is inhibited by AZ. It is suggested that the observed inhibition of release to the xylem takes place at xylem parenchyma cell membranes.

Mechanism of Action of AZ on Ion Transport. The compounds CHM, FPA, and AZ all prevent effective protein synthesis and also inhibit Φ^*_{ox}. CHM acts by inhibiting protein assembly at the ribosomes and AZ and FPA result in synthesis of nonsense protein by incorporation of AZ in place of L-proline or FPA in place of L-phenylalanine. Various suggestions have been made about involvement of protein synthesis in ion transport, either for synthesis of membrane or for continuing production of carriers that turn over rapidly. It is difficult to separate these explanations from those based on indirect requirements for protein involved in energy metabolism, resulting in inhibition of ion transport by reduction in energy transfer. This dilemma has been shown particularly in studies with CHM (12).

Effects of these compounds can be compared. The results presented here support views that AZ is incorporated into protein in place of L-proline resulting in nonsense proteins. This action is analogous to that of FPA. CHM, however, acts by inhibiting protein assembly at the ribosomes. All of these compounds inhibit ion release to the xylem (CHM 9, 11, 22; FPA 18, 21), although there are minor differences in the lag before inhibition develops and the degree of inhibition of Φ^*_{ov}. With FPA there was no reduction in net tracer uptake and an increase in Φ^*_{ov}. Both AZ and CHM inhibited net tracer uptake and Φ^*_{ov}. The different response of Φ^*_{ov} to FPA and AZ suggests that inhibition of entry to the cells (ϕ_{oc} and ϕ_{cv}) may be different in kind from that of ϕ_{cx}.

The simplest explanation of inhibition of Φ^*_{ox} by these compounds (18) is that ϕ_{cx} involves a carrier protein that turns over by 50% in 1 to 2 hr (the time for 50% decrease in Φ^*_{ox}). However, the inhibition of Φ^*_{ox} by AZ was not readily reversible in L-proline (Table II) although protein synthesis (measured as induction of NO_3^- reductase) recovered when AZ was replaced by L-proline.

An alternative explanation is that inhibition results from a secondary effect of inhibition of protein synthesis. CHM produces many changes to metabolism, other than inhibition of protein synthesis (12). Energy metabolism has been suggested to require proteins or polypeptides with rapid turnover as control factors (2). Either energy metabolism or protein synthesis inhibition has been shown to lead to reduction in RNA synthesis (12). Although AZ acts in a different way from CHM on protein synthesis, it appears to produce similar effects on metabolism in barley roots as seen by the small reduction in O_2 uptake and increased ATP content. The same, indirect, effect could account for inhibition of ion transport both by AZ and CHM.

This indirect effect does not seem to be inhibition of energy availability (ATP levels were increased) and an alternative hypothesis is that there are "signal" compounds in the cell that regulate the activity and integration of other processes. For example, inhibition of protein synthesis, or production of nonsense protein (in AZ) could lead to changes in the levels of compounds that inhibit other processes such as ion transport and RNA synthesis (12), both of which need to be integrated with cell or plant development. Such an explanation could be an integrative hypothesis including the effects of ABA and cytokinins on ion transport, as well as the inhibitors of protein synthesis. Both ABA (3, 15, 16) and cytokinin (8, 15) inhibit Φ^*_{ox} but their action cannot be consistently related to protein synthesis. For example, although cytokinins have been suggested to be components of tRNA, there are other situations where cytokinins are active and tRNA synthesis has stopped (*e.g.* senescence) (17). Similarly, ABA has been shown to inhibit nitrate reductase induction in mung beans (6) but we have found no effect on either nitrate reductase or protein synthesis in barley roots. The phytohormones could have a separate route of action to the levels of the signal compounds, producing the same effect on ion transport as inhibitors of protein synthesis.

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