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Action of Inhibitors of Ammonia Assimilation on Amino Acid Metabolism in *Hordeum vulgare* L. (cv Golden Promise)[1]

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ABSTRACT

Barley (*Hordeum vulgare* L. cv Golden Promise) plants were grown in a continuous culture system in which the root and shoot ammonia and amino acid levels were constant over a 6-hour experimental period. Methionine sulfoximine (MSO), 1 millimolarity when added to the culture medium, caused a total inactivation of root glutamine synthetase with little effect on the shoot enzyme. Root ammonia levels increased and glutamine levels decreased, irrespective of whether the plants were grown in 1 millimolar nitrate or 1 millimolar ammonia. Levels of glutamate, aspartate, serine, threonine, and asparagine all increased. There was little alteration in the amino acid and ammonia levels in the shoot, suggesting that MSO is not rapidly transported.

The addition of azaserine (25 micrograms per milliliter) to nitrate-grown plants caused a rapid increase in root ammonia, glutamine, and serine levels with a corresponding decrease in glutamate, aspartate, and alanine. Glutamine levels also increased in the shoot.

The *in vivo* effect of MSO and azaserine was as would be predicted by their known *in vitro* inhibitory action if the glutamine synthetase/glutamate synthase pathway of ammonia assimilation was in operation.

L-Methionine-*S*-sulfoximine has been examined extensively by Meister and his colleagues as an inhibitor of mammalian GS[2] (L-glutamate:ammonia ligase [AMP forming]; EC 6.3.1.2) (12). MSO has also been shown to be a potent inhibitor of bacterial (26, 27) and plant (8, 26) GS. MSO has been used to demonstrate the evolution of ammonia in *Anabaena cylindrica* (22), *Chlamydomonas rheinhardii* (4), *Lemna minor* (19, 21) rice (1), *Datura* (9, 16), and spinach (6, 15). Such observations have been taken as one piece of evidence to show that the GS/glutamate synthase pathway (14) was operating. However, in only one higher plant (*L. minor*, 19, 21) have the studies been carried out in sufficient detail for quantitative assessments to be made as to the proportion of ammonia assimilated via the GS/glutamate synthase pathway. In *Candida utilis*, ^{15}N-labeling studies (20) have shown that the primary assimilation of ammonia is catalyzed by glutamate dehydrogenase. Moreover, although MSO caused a rapid decrease in GS activity and a concomitant decrease in glutamine level, there was no evidence of ammonia accumulation (21).

O-Diazoacetyl-L-serine (azaserine) is an irreversible inhibitor of a wide range of enzymes that catalyze glutamine amide transfer reactions (3) including plant glutamate synthase (EC 2.6.1.53) (25). The compound has no action on plant glutamate dehydrogenase or glutamine synthetase (8). Early studies on the green algae *Scenedesmus* and *Chlorella* using azaserine and the closely related compound 6-diazo-*S*-oxo-L-norleucine, by Van der Meulen and Bassham (24) showed that there was a decrease in the glutamate and increase in glutamine and α-ketoglutarate levels. These data were later interpreted as being consistent with the operation of the GS/glutamate synthase pathway (13). Addition of azaserine to *L. minor* caused an increase in glutamine content, a rapid decrease in glutamate, and small increase in ammonia. In plants incorporating $^{14}CO_2$, azaserine caused a decrease in label incorporation into glutamate and an increased labeling of glutamine and α-ketoglutarate (21). These results were again consistent with the assimilation of ammonia via the GS/glutamate synthase pathway.

In the previous paper (5), we proposed a model for the assimilation of [^{15}N]nitrate and ammonia by barley (*Hordeum vulgare*) seedlings grown under steady state conditions. The model was complicated by tissues which contained heterogeneous populations of cells. In the studies described in this paper, the responses of barley seedlings growing on nitrate or ammonia under 'steady state' conditions to the addition of MSO or azaserine have been examined. Changes in the pool sizes of nitrogen compounds were analyzed in order to make quantitative assessments of the proportion of ammonia assimilated in barley root tissue via the GS/glutamate synthase pathway.

MATERIALS AND METHODS

Plant Material. Barley (*Hordeum vulgare* L. cv Golden Promise) plants were grown in a 'continuous flow' water culture system as described in the accompanying paper (5). In order for valid interpretations to be made from experiments investigating the effects of enzyme inhibitors on the nitrogen assimilation of barley, it was important that any changes observed in the amino acid pools in the tissues were due only to the effects of the inhibitor. By choosing 9-d-old barley seedlings which were of uniform size, receiving no nitrogen from the seed and utilizing a 100-liter reservoir of either 1 mM $NaNO_3$ or 1 mM NH_4Cl, steady state levels of soluble amino acids were obtained over a 6-h period. On the ninth day of growth, one group of plants was transferred into a nutrient solution containing 1 mM MSO, while the culture solution of 'control' plants was changed for a nutrient solution to which no inhibitor had been added. Plants were sampled over a 6-h period after the addition of the inhibitor, 0.5 g root and shoot samples being taken for GS extraction and assay and 1.5 g root

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[2] Abbreviations: GS, glutamine synthetase; MSO, methionine sulfoximine.

and shoot samples being taken for the analysis of ammonia and amino acids.

Glutamine Synthetase Assays. Root or shoot tissue was extracted in 5 ml buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 1 mM mercaptoethanol, 1 mM reduced glutathionine, 10 mM magnesium sulfate, 5 mM glutamate, 10% ethylene glycol, and 2% Polyclar AT. Activity was determined by the Mn^{2+}-dependent transferase assay (18), incubating 0.1 ml of extract for 20 min at 30°C.

Analysis of Nitrogenous Constituents. Tissues were extracted in a methanol-chloroform-water mixture as described previously (5). Samples were evaporated down at 20°C, taken up in 0.2 M lithium citrate buffer, pH 2.2, and analyzed on an LKB Biocal BC100 amino analyzer equipped with Aminex A6 ion exchange resin. Nitrate was measured by a modification of the method of Mann *et al.* (11) and ammonia by the method of McCullough (10).

RESULTS

Effect of MSO on Nitrate-Grown Barley Plants. The addition of 1 mM MSO to nitrate-grown barley plants brought about a rapid inactivation of GS in the root, no activity being detectable in the root after 2 h (Fig. 1A). The loss of activity was, however, much slower in the shoot, 60% remaining after 6 h. The GS activities of both the root and shoot of the control plants remained constant. Accompanying the sharp decline in GS activity in the root, there was a rapid accumulation of ammonia at a rate of 0.93 μmol/h·g fresh weight (Fig. 1B). The root glutamine content rapidly decreased, but appeared to level off at around 0.25 μmol/g fresh weight (Fig. 2A). The tissue concentration of glutamate in the root showed an initial increase from 1.2 to 1.7 μmol/g fresh weight but subsequently decreased to 1.5 μmol/g fresh weight after 6 h. The changes in glutamine and glutamate concentrations were accompanied by increases in the root tissue concentrations of aspartate, alanine, and serine, whereas the concentration of asparagine remained relatively constant (Fig. 2B).

The effects of MSO on the pool sizes of nitrogen compounds in the shoot were much less marked, the tissue ammonia content increasing only slightly after 4 h (Fig. 1B). The shoot tissue concentrations of the amino acids remained relatively constant. During the 6-h period, ammonia and amino acid levels of both roots and shoots of the control plants remained constant.

Effects of MSO on Ammonia-Grown Barley Plants. The addition of 1 mM MSO to ammonia-grown plants again caused a rapid loss of GS activity in the root, there being no detectable activity after 2 h although in this case the initial rate of GS activity was 3.3 μmol/min·g fresh weight. Again, the loss of GS activity in the shoot was much slower, 54% of the initial activity still being detectable after 6 h. The initial ammonia concentration in the roots of ammonia grown barley plants (3.2 μmol/g fresh weight) was much higher than that of nitrate-grown plants. The addition of MSO caused a rapid accumulation of ammonia in the root after a 1-h lag at a rate of 1.5 μmol/h·g fresh weight, which was approximately linear for the 5 h tested.

The initial concentration of glutamine (5 μmol/g fresh weight) in the roots was also considerably higher than that of the nitrate-grown plants, and the addition of MSO again caused a rapid decrease in glutamine content, although this decrease started to level out as the concentration approached 2 μmol/g fresh weight. After an initial rise, the glutamate level subsequently decreased to 3 μmol/g fresh weight. Again, as in the previous experiment on nitrate-grown plants, these changes were accompanied by a marked increase in the root aspartate concentration, whereas the total levels of serine, threonine, and asparagine showed only slight increases. In the shoots over the experimental 6-h period, the ammonia and amino acid levels remained essentially constant.

Uptake of Nitrate and Ammonia. The rates of uptake of ammonia and nitrate by barley plants in the continuous flow culture system were determined in a separate experiment by measuring the decrease in the concentrations of these ions over an 8-h period. The rate of uptake of nitrate by barley was measured as 1.3 μmol/h·g fresh weight. As a substantial portion of the nitrate entering barley plants is transported to the leaves, the rate of accumulation of ammonia following MSO treatment (0.93 μmol/h·g fresh weight) suggests that a large portion if not all of the ammonia resulting from nitrate reduction in barley roots is assimilated via GS. The rate of uptake of ammonia by barley roots was measured as 1.2 μmol/h·g fresh weight. Again, the rate of ammonia accumulation following MSO treatment (1.5 μmol/h·g fresh weight) is more than adequate to account for the ammonia taken up from

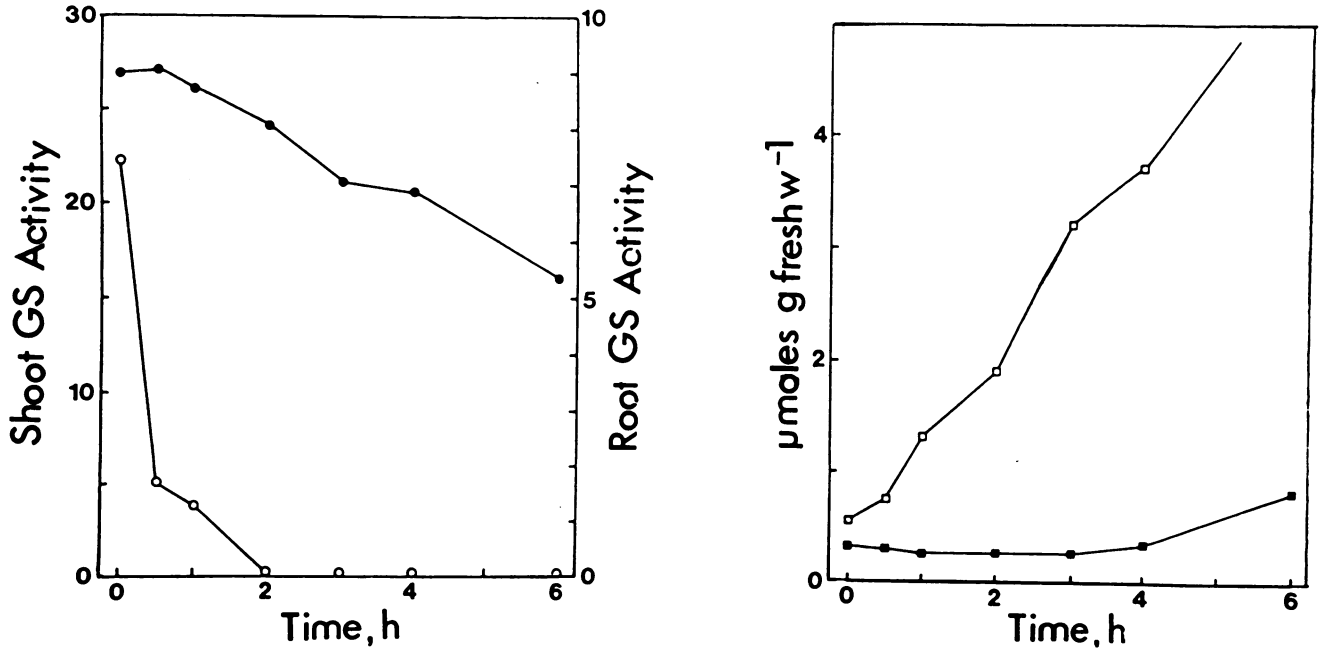

FIG. 1. Effect of 1 mM MSO added at zero time on root and shoot tissues of nitrate-fed barley plants. A, (●) Shoot GS activity; (○) root GS activity. B, (■) Shoot ammonia level; (□) root ammonia level.

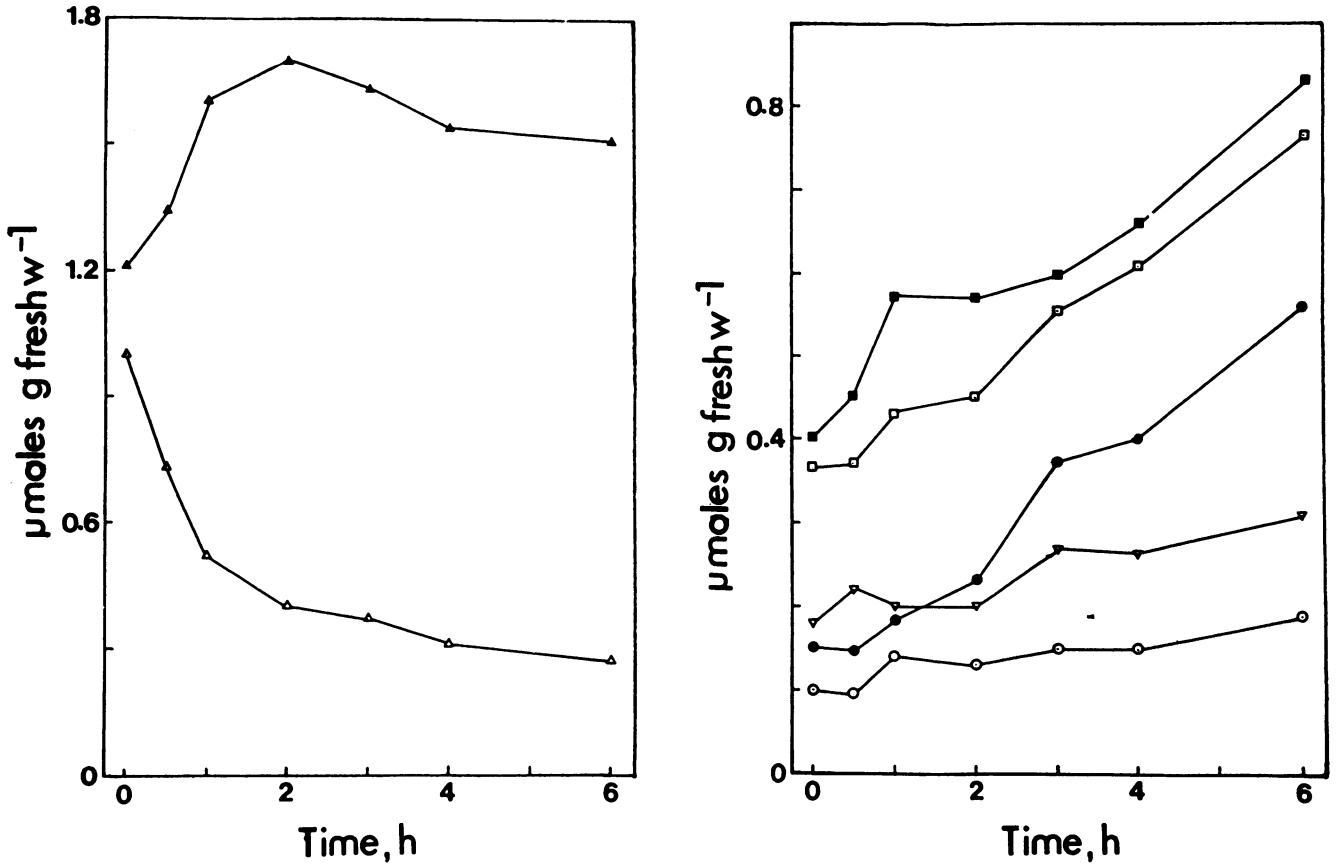

FIG. 2. Effect of 1 mM MSO added at zero time on the amino acid levels in the roots of nitrate-fed barley plants. A, (△) Glutamine; (▲) glutamate. B, (■) Aspartate; (□) serine; (▽) alanine; (●) threonine; (○) asparagine.

the medium, suggesting that ammonia is assimilated entirely via GS.

The effect of MSO on the uptake of nitrate and ammonia was examined using 50 barley plants in 400 ml of either 0.5 mM $NaNO_3$ or 0.5 mM NH_4Cl. The rate of disappearance of nitrate from the medium was linear over a 4.5-h period and MSO (1 mM) had no effect on the rate of uptake. Ammonia uptake from this 'static' culture was nonlinear with time. MSO-treated plants showed the same rate of ammonia uptake over the first hour but there was an apparent cessation of uptake after this time.

Effect of Azaserine on Nitrate-Grown Barley Plants. The addition of 25 µg/ml azaserine to the continuous culture medium caused a rapid accumulation of ammonia in the roots, the rate of accumulation reaching approximately 0.71 µmol/h·g fresh weight after 30 min (Fig. 3A). The root glutamate content rapidly decreased and after 2.5 h leveled off at approximately 0.15 µmol/g fresh weight; the glutamine content, however, rapidly increased from 0.77 to 1.76 µmol/g fresh weight over the 6-h time course (Fig. 3B). The changes in the root glutamine and glutamate levels were accompanied by decreases in the root tissue concentrations of aspartate and alanine (Fig. 4A), while the concentration of serine was markedly increased (Fig. 4B). The root concentrations of glycine, threonine, and asparagine also showed slight increases.

The addition of azaserine caused a small increase in the tissue ammonia concentration in the shoot (Fig. 3A). The shoot tissue concentration of glutamate showed a small decrease, whereas that of glutamine showed a small increase. The concentrations of the other amino acids remained relatively constant (data not shown). The levels of ammonia and amino acids in the control root and shoot tissue remained constant over the 6-h time course.

DISCUSSION

The addition of 1 mM MSO to barley plants growing under steady state conditions caused a rapid deactivation of root GS as would be expected from its known strong *in vitro* inhibitory action on GS isolated from a wide range of sources (8, 12, 26, 27). It can be seen that although MSO is able to penetrate into root tissues it is not rapidly transported into the shoot; this may be due to the fact that xylem transport appears to stop shortly after MSO feeding to the root.

In both nitrate- and ammonia-fed plants, there was a rapid accumulation of ammonia immediately after the introduction of MSO, suggesting that GS was operating in the first step of assimilation. As glutamate dehydrogenase is present in barley roots at a level three times higher than that of GS (5), and is not inhibited by MSO (2, 16), it might be expected to operate at the high tissue levels of ammonia present after the addition of MSO (9 mM in the ammonia-fed plants). However, the rate of ammonia accumulation in both sets of plants was approximately linear, suggesting that no other mechanism of assimilation was beginning to operate even at the higher ammonia concentrations. Although it could be proposed that MSO has a secondary inhibitory action on either the production of NADH or α-ketoglutarate, no such inhibition was obtained in *C. utilis (21) or Peltigera aphthosa* (17), where fungal glutamate dehydrogenase operates.

Since glutamate is not only a precursor but also a product of glutamine in the GS/glutamate synthase pathway, it might be expected that the inactivation of GS in a tissue would lead to an initial increase in the level of glutamate followed by a decrease as the rapidly depleting concentration of glutamine would limit the rate of glutamate synthesis via the glutamate synthase reaction. The changes in glutamine and glutamate levels exhibited by both nitrate- and ammonia-grown plants are entirely consistent with the operation of the GS/glutamate synthase pathway.

The changes of the glutamate and glutamine levels of nitrate-grown barley plants in response to the addition of azaserine were

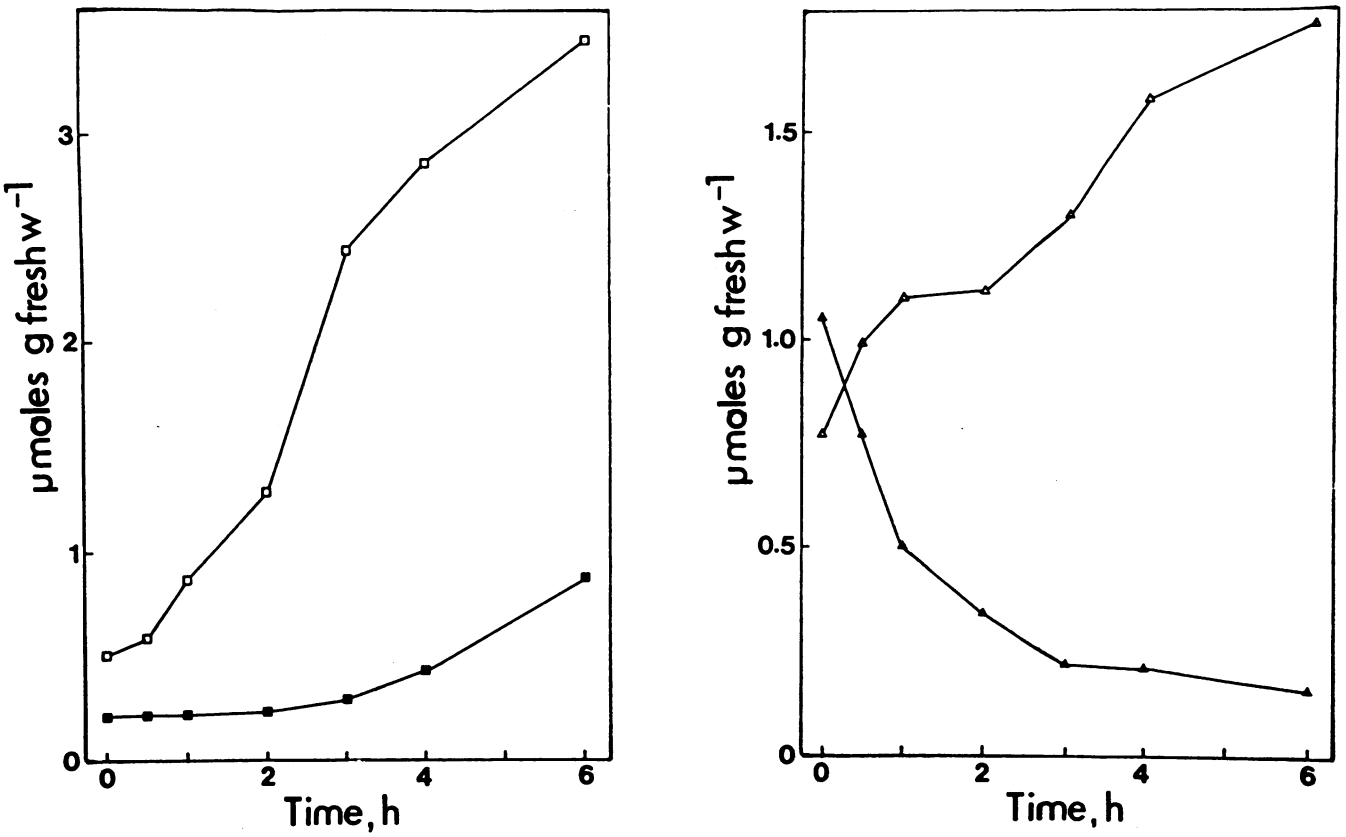

FIG. 3. Effect of azaserine (25 µg/ml) added at zero time on the roots and shoots of nitrate-fed barley plants. A, (■) Shoot ammonia; (□) root ammonia. B, (△) Root glutamine level; (▲) root glutamate level.

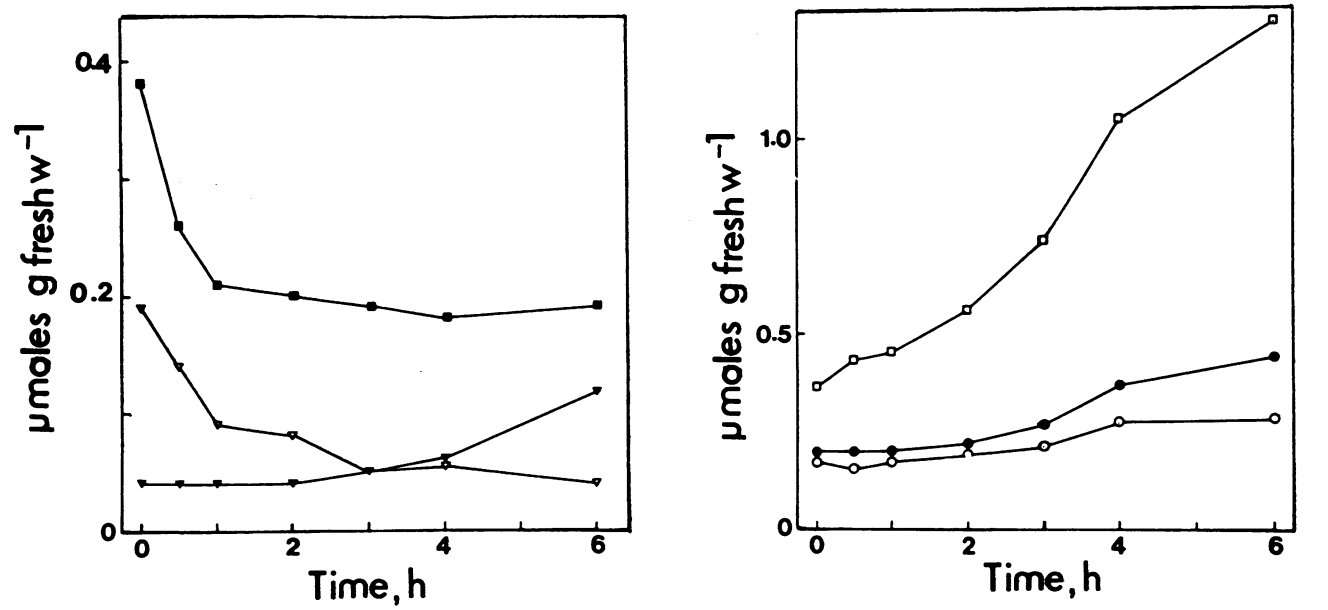

FIG. 4. Effect of azaserine (25 µg/ml) added at zero time on the amino acid levels of roots of nitrate-fed barley plants. A, (■) Aspartate; (▽) alanine; (▼) glycine. B, (□) Serine; (●) threonine; (○) asparagine.

very similar to those shown by Van der Meulen and Bassham (24) in *Scenedesmus* and Stewart and Rhodes (21) in *Lemna*. The rapid linear increase in the glutamine level and equally rapid fall in the glutamate level are again consistent with ammonia assimilation via the GS/glutamate synthase pathway. Changes in the tissue concentrations of aspartate and alanine in barley roots in response to MSO (Fig. 2B) and azaserine (Fig. 4A) to a large extent followed the changes occurring in the glutamate level. Thus, in the roots of both nitrate- and ammonia-grown plants treated with MSO, the tissue glutamate content showed an increase, followed by a small decrease. The tissue aspartate concentration increased throughout the 6-h time course. The tissue alanine content also increased in nitrate-grown roots in response to MSO. The glutamate concentration of nitrate-grown plants decreased rapidly in response to azaserine (Fig. 3B) and the levels of aspartate and alanine were also reduced. The changes in the levels of aspartate and alanine may be explained by the derivation of the amino nitrogen of these amino acids from glutamate via transamination

reactions. The changes observed in the aspartate and alanine pools of barley roots in response to MSO are in contrast to the results obtained in *A. cylindrica* (22). In the cyanobacteria, MSO caused a small increase in the internal glutamate concentration followed by a large decrease. However, the concentration of aspartate and an alanine + glycine fraction remained constant during the period of MSO feeding. The increases in threonine and serine concentrations on MSO treatment (Fig. 2B) are difficult to interpret in the light of present knowledge of amino acid biosynthesis. The dramatic increase in serine stimulated by azaserine (Fig. 3B) may indicate a second inhibitory action site for azaserine. *O*-Acetyl serine (formed by the acetylation of serine) is a major precursor in the synthesis of a number of β-substituted alanine derivatives including cysteine. The structure of azaserine is not dissimilar to that of *O*-acetyl serine, and the possibility of *O*-acetyl serine synthesis or metabolism being blocked should be considered.

Asparagine is known as a compound used to store excess reduced nitrogen in nontoxic form. In only one treatment (ammonia-grown barley treated with MSO), did the level show a slight increase (0.3 μmol/g fresh weight), whereas the ammonia level increased by over 6 μmol/g fresh weight and the aspartate level by 1 μmol/g fresh weight. In the other treatments where ammonia was evolved, there was no increase in asparagine. Such data indicate that asparagine cannot be synthesized, even under extreme conditions, directly from aspartate and ammonia as has been recently suggested (23), but needs to proceed via glutamine and the amide transfer reaction of asparagine synthetase (7).

It is interesting to note that in all the treatments where there is a rapid fall in the level of an amino acid (glutamine, Fig. 2A; glutamate, Fig. 4B; aspartate and alanine, Fig. 5A), the level does not drop to zero. In all cases, there is a leveling off of the curves during the 6-h period. This would suggest that there are pools of the amino acids that are not accessible to normal metabolism, over a short period. The existence of a large unavailable pool of glutamine (particularly in ammonia-grown plants) was suggested in the previous paper (5), and is borne out by the data presented here. An obvious site for the location of this pool is within the vacuole of the cell.

All the data presented indicate that in both the nitrate- and ammonia-fed plants, ammonia is assimilated via the GS/glutamate synthase pathway. There is no indication that ammonia may be assimilated in the presence of MSO, and no indication that any nitrogen is incorporated into any compound other than glutamine in the presence of azaserine. It would appear that under the conditions employed neither glutamate dehydrogenase nor asparagine synthetase operates in ammonia assimilation.

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