Plant Physiol. (1972) 49, 138–141

Influence of Ammonium and Nitrate Nutrition on Enzymatic Activity in Soybean and Sunflower[1]

Received for publication June 7, 1971

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ABSTRACT

Under conditions of controlled pH, nitrate and ammonium are equally effective in supporting the growth of young soybean (*Glycine max* var. Bansei) and sunflower (*Helianthus annuus* L. var., Mammoth Russian) plans. Soybean contains an active nitrate reductase in roots and leaves, but the low specific activity of this enzyme in sunflower leaves indicates a dependency upon the roots for nitrate reduction. Suppression of nitrate reductase activity in sunflower leaves may be due to high concentrations of ammonia received from the roots. Nitrate reductase activity in leaves of nitrate-supplied soybean and sunflower follows closely the distribution of nitrate reductase. For the roots of both species, glutamic acid dehydrogenase activity was greater with ammonium than with nitrate. The glutamic acid dehydrogenase of ammonium roots is wholly NADH-dependent, whereas that of nitrate roots is active with NADH and NADPH. In leaves, an NADPH-dependent glutamic acid dehydrogenase appears to be responsible for the assimilation of translocated ammonia and ammonia formed by nitrate reduction.

In soybean roots ammonia is actively incorporated into amides, much of which remains in the roots. Sunflower roots are less active in amide formation but transfer much of it, together with ammonia, into the shoots. Glutamine synthetase activity in leaves is 20- to 40-fold lower than in roots.

Glucose-6-phosphate dehydrogenase activity appears to be correlated with the activity of the nitrate reducing system in roots, but not in leaves.

Although it is well established that absorbed nitrate is converted to ammonium before assimilation into the organic form, it has long been recognized that ammonium and nitrate as sources of external nitrogen differ in their effects on the growth and chemical composition of plants (19). Recent studies have indicated that ammonium and nitrate nutrition exert contrasting effects on growth (2, 24, 33), enzyme activity (9, 28), free amino acid composition of root exudate (30) and root tissue (25), and organic acid-mineral ion composition (13). Preliminary observations in this laboratory indicated further that the different forms of nitrogen supplied to higher plants may lead to differences in the levels of pyridine and adenine nucleotides, substances known to play a role in the enzymatic assimilation of ammonium and nitrate. An understanding of the significance of these nucleotide differences required, therefore, that information be obtained on the effect of ammonium and nitrate on the activity of nitrate reductase, nitrite reductase, glutamic acid dehydrogenase, and glutamine synthetase. This report deals with such an investigation. In view of indications of a stimulatory effect of nitrate utilization on the pentose phosphate pathway of respiration (4, 28), the activity of G6PDH[2] was also examined in this study. An accompanying paper (31) reports on the influence of ammonium and nitrate on the level of those nucleotides that are associated with inorganic nitrogen assimilation.

MATERIALS AND METHODS

Plant Culture. Sunflower was selected as one of the test plants in this study because its nitrate-reducing capacity is restricted nearly wholly to its roots. Furthermore, certain aspects of the nitrogen metabolism of this plant have been previously investigated in this laboratory (30). Soybean was selected as a contrasting test plant in view of its known nitrate-reducing capacity in both roots and leaves (7).

In initial experiments sunflower seeds (*Helianthus annuus* L. var. Mammoth Russian) and soybean (*Glycine max* var. Bansei) were germinated in filter-paper lined beakers and were transferred as 5-day-old seedlings to sand-filled paraffined cups as previously described (30). Culture solution lacking in nitrogen (29) was provided for the first 10 days of growth. The cotyledons were then removed, and culture solution containing either 10 mM NH_4Cl or 10 mM KNO_3 was added twice a day for the final 9 days of growth before harvest. Examination of the drippings passing through the glass wool covered perforations in the bottom of the cups on the final day of growth revealed that ammonium solutions, originally at pH 6.3, fell to pH 5.3 while nitrate solutions fell to pH 6.1. In order to minimize the effect of pH differences on the biochemical characteristics of ammonium-supplied and nitrate-supplied plants, an apparatus was devised in which culture solution was delivered to the roots of plants in a continuous flow system. Five-day-old seedlings were transferred to the apparatus previously described (32), and culture solution was passed over the roots in a continuous flow for the remainder of the 19-day growth period. Under these conditions of growth, nitrate solutions maintained their original pH of 6.3 during passage over the roots while ammonium solutions experienced no more than a decrease of 0.1 pH unit.

In order to permit comparison with data previously obtained for sunflower (30), shoots were removed from some soybean plants on the 13th day of growth, and the collected

[1] This investigation was conducted with the aid of a research grant provided by the Research Council of Rutgers University.

[2] Abbreviations: G6PDH: glucose-6-phosphate dehydrogenase; GDH: glutamic acid dehydrogenase; GS: glutamine synthetase; NR: nitrate reductase; NiR: nitrite reductase.

exudate was analyzed for ammonia and amide content by methods previously described (30). All plants were grown in a controlled temperature chamber (26 to 27 C day, and 20 to 21 C night) under artificial light on a 14-hr photoperiod.

Harvest. At the time of harvest sunflower plants had developed three sets of opposite leaves, and soybean had produced a set of juvenile leaves and two alternate trifoliate leaves. No nodules were in evidence on soybean roots. The second set of sunflower leaves, the oldest trifoliate leaf of soybean, and, for both species, the root system of some plants grown in sand and some in the continuous flow apparatus were removed for fresh and dry weight determinations. The remainder of those plants grown by continuous flow were utilized in enzyme analyses. Midribs were removed from leaves and leaflets and roots were washed with a vigorous stream of distilled water before enzyme extraction procedures were applied.

Methods of Analysis. NR and NiR were extracted and purified through Sephadex columns by the method of Sanderson and Cocking (21). Soybean roots yielded the most vigorous nitrate-reducing preparation in the presence of 1 mM cysteine-HCl in the tris buffer extracting medium. For soybean leaf extracts, a range of cysteine-HCl concentration to 5 mM had little effect on nitrate reducing activity. Sunflower roots yielded an active NR preparation only when the concentration of cysteine-HCl in the tris buffer extracting medium was 5 mM. Assay of the enzyme was based on the procedure of Evans and Nason (7) as modified by Wallace and Pate (26). The dithionite-benzyl viologen method of Joy and Hageman (11) was utilized in the determination of NiR activity.

Homogenates for the analysis of GDH activity were prepared by the method of Ritenour *et al.* (20). After the slurry was filtered through cheesecloth, the filtrate was centrifuged at 200*g*, the sediment was discarded, and the supernatant was centrifuged at 20,000*g*. Following the procedure described by Leech and Kirk (16), the pellet was resuspended in tricine-NaOH buffer and homogenized in a Ten-Broeck ground glass homogenizer. NADH- and NADPH-dependent enzyme activity was determined for the resultant extract by the method of Bulen (3).

Glutamine synthetase-glutamyl transferase activity was determined on acetone powders prepared from leaf and root tissue by the method of Loomis (17). Although a single enzyme is believed to be responsible for both reactions (8), preliminary analyses indicated that with the reaction mixtures utilized more activity could be demonstrated with the transferase system than with the synthetase system. The reaction mixture of Loomis (17) containing acetone powder, sodium maleate buffer, glutamine, $MnSO_4$, sodium arsenate, ADP, and hydroxylamine, incubated at 38 C, was accordingly adopted. The red chelated complex of glutamyl hydroxamate with ferric ion was read at 540 nm.

The activity of G6PDH was conveniently determined utilizing the acetone powders prepared for glutamine synthetase analyses. Following the method of Clayton (5), 1 g of powder was homogenized with 20 ml of 0.1 M tris, pH 7.6, containing 0.1 M cysteine-HCl. The homogenate was passed through four layers of cheesecloth, and the debris was further extracted with 10 ml of tris buffer. Combined filtrates were centrifuged at 15,000*g* for 20 min, and the clear supernatant was adjusted to pH 7.0. G6PDH activity was determined immediately in a reaction mixture (15) containing enzyme (0.9 mg protein), 5 μmoles of glucose-6-P, 0.5 μmoles of NADP, 200 μmoles of tris, pH 7.6, and 10 μmoles of $MgCl_2$; total volume, 3 ml. The increase in absorbance at 340 nm was followed for 3 min at 25 C.

Protein in enzyme preparations was estimated by the method of Lowry *et al.* (18).

Table I. *Enzyme Activity of 19-day-old Soybean and Sunflower Plants as Affected by Ammonium and Nitrate Nutrition*

	NR	NiR	NADH-GDH	NADPH-GDH	GS	G6P-DH
	nmoles of substrate changed per mg protein per hr					
Soybean						
Leaves NH_4^+	0[1]	45	96	118	510	311
Leaves NO_3^-	208	632	168	800	455	466
Roots NH_4^+	0		2080	20	12400	1120
Roots NO_3^-	272		720	430	20300	1790
Sunflower						
Leaves NH_4^+	0	28	52	502	150	230
Leaves NO_3^-	16	90	46	640	135	233
Roots NH_4^+	0		3600	25	4680	985
Roots NO_3^-	960		1690	205	2440	1485

[1] Values are the averages of three determinations per sample.

RESULTS AND DISCUSSION

Growth determinations and enzyme analyses were repeated in at least three separate experiments. Minor variations occurred from experiment to experiment; the results reported are of one trial and are typical of all others.

Plant Growth. When plants were grown in sand, leaf and root growth of both sunflower and soybean were greater with nitrate than with ammonium as the source of nitrogen. That this difference is primarily due to the effect of the low pH developed in ammonium solutions is indicated by the fact that ammonium and nitrate proved equally effective when supplied by the continuous flow technique. Observations on the leaf appearance of plants grown at pH 6.3 in the continuous flow system gave no evidence of the frequently reported symptoms of ammonium-toxicity. In a review dealing with the ability of different plant species to grow in ammonium or nitrate, McKee (19) was careful to point out that any conclusions must be regarded as tentative in view of the absence in most studies of efforts to maintain the hydrogen ion concentration of the culture solutions. The results of the present study add substance to this comment.

Nitrate Reductase and Nitrite Reductase. In a comparison of the nitrogen metabolism of cocklebur (*Xanthium pennsylvanicum*) with that of the field pea (*Pisum arvense*), Wallace and Pate (27) concluded that each exemplified a group of plants with a characteristic distribution of nitrate-reducing capacity. Whereas both the leaves and roots of the field pea displayed vigorous NR activity, such activity in *Xanthium* was restricted wholly to the leaves. Soybean appears to resemble the field pea in that the present (Table I) and previous (7) studies give evidence of active nitrate reduction centers in both roots and leaves of plants grown with nitrate. Sunflower, however, falls into neither of the Wallace and Pate categories in that nitrate-reducing activity, as previously reported by Cresswell (6), is nearly completely absent from the second pair of leaves of 19-day-old plants (Table I). Variation in the cysteine concentration of the extracting medium had no effect on the nitrate-reducing activity of Sephadex column fractions and determinations of NR activity made on both the oldest and youngest of the three pairs of leaves revealed similar low values. Even if activity is calculated on the basis of fresh weight, the leaves are found to have only a small part of the total NR activity of the plant. It is clear that young sun-

Table II. *Ammonia, Glutamine, and Asparagine in the Exudate of 13-day-old Soybean and Sunflower as Affected by Ammonium and Nitrate Nutrition*

	Soybean		Sunflower[1]	
	NH_4^+	NO_3^-	NH_4^+	NO_3^-
	μg per ml exudate			
Ammonia	24	27	206	317
Glutamine	2220	335	2068	796
Asparagine	484	246	690	137

[1] Data on sunflower exudate taken from Weissman (30).

flower plants are nearly wholly dependent upon their roots for nitrate utilization.

Limited as it may be, however, a definite amount of NR activity appears to be present in sunflower leaves. This suggests that a process of enzyme inhibition or of one limiting synthesis, rather than a basic inability to synthesize the enzyme, is operative in this plant part. Joy (9), noting reduced NR activity in *Lemna* plants pretreated with ammonia before transfer to a nitrate medium, concluded that ammonia represses the nitrate-induced synthesis of NR. In a previous study (30) on sunflower, it was noted that whereas the concentration of ammonia in root exudates of 11-day-old decotyledonized plants was 12 μg per ml of exudate, it rose to 15 μg after an application of 10 mM KNO_3 on the 11th day and 317 μg after 2 more days on nitrate treatment. Such a concentration of ammonia, representing 27% of the total nitrogen moving out of the roots, may well be responsible for the end-product repression of NR in sunflower leaves. That no such repression of NR activity occurs in soybean leaves may be due to the limited amount of ammonia entering these tissues. The data of Table II indicate that the ammonia concentration in exudates of comparable soybean plants is only one-tenth that found in sunflower.

The pattern of NiR activity in the leaves of nitrate-supplied soybean and sunflower plants follows closely the distribution of NR in these tissues. In soybean, vigorous enzyme activity is present (Table I) at a level approximately six times greater than that for NR; for sunflower leaves NiR activity is similarly greater than NR but occurs at such a low level of activity as to suggest again the absence of any significant system of nitrate assimilation.

Beevers and Hageman (1) have noted the limited demonstration of NiR in root tissue; in the present study NiR activity in roots was so low and the results so erratic as to preclude the presentation of data in Table I. Klepper and Hageman (14), employing the same method utilized in this study, were unable to demonstrate NiR activity in apple roots even though this tissue is known to metabolize nitrate to the organic form. Indirect evidence for the presence in soybean and sunflower roots of a system capable of converting nitrite to ammonia comes from (*a*) the present study in which vigorous NR activity has been demonstrated in both these tissues and from (*b*) past studies (30) indicating an increase in the ammonia content of root exudates of plants provided with nitrate. The inability of the dithionite-benzyl viologen assay system to detect root NiR in this study and others (14) remains to be explained.

Glutamic Acid Dehydrogenase. From the data of Table I it may be reasonably concluded that the GDH of soybean and sunflower roots plays a significant role in the assimilation of ammonia formed by the reduction of nitrate or absorbed as such from ammonium culture solutions. For both species enzyme preparations extracted from ammonium-supplied roots displayed a higher level of activity than those from nitrate roots. However, the enzyme of ammonium roots was active only in the presence of NADH while nitrate-root preparations displayed activity with both NADH and NADPH. Joy and Pahlich (12) have found evidence in pea roots of a single GDH existing in two forms, each with synthetic capability; one utilized NADH and the other NADPH. The observations of the present study that the GDH of ammonium roots is wholly NADH-dependent while the preparation extracted from nitrate roots is active with both NADH and NADPH is suggestive of the presence of two distinct enzymes in the nitrate-supplied roots of soybean and sunflower. The reported inhibition of NADH-GDH activity by EDTA in the extracting medium and the relative insensitivity of NADPH-GDH (10) may also be indicative of two distinct enzymes. It is not clear whether the different pattern of NADH- and NADPH-dependent GDH in ammonium and nitrate roots is directly related (*i.e.*, an inductive response) to the different sources of nitrogen. Additional determining factors may be the accumulation of amino acids in the metabolic pool (23) and the availability in root tissue of reducing power in the form of NADH and NADPH. The question is further examined in an accompanying study (31).

Compared to the level of activity in soybean roots, NADH-dependent GDH is relatively inactive in the leaves of ammonium- and nitrate-supplied soybean plants (Table I). NADPH-dependent GDH extracted from nitrate leaves shows vigorous activity, however, and would appear to be involved with the assimilation of ammonia produced in nitrate reduction. Recent findings by Leech and Kirk (16) point to the presence in chloroplasts of an NADPH-dependent glutamate dehydrogenase responsible for *in vivo* ammonia assimilation. It is not clear whether this enzyme is restricted in its function to that ammonia produced via NiR, itself presumably located in or on chloroplasts (20), or whether it may assimilate as well ammonia brought into the leaves from the roots via the transpiration stream. The data on NADH- and NADPH-GDH activity in sunflower leaves suggest that the latter may be the case. Sunflower leaves in these experiments, both those of ammonium plants and nitrate plants, receive a continual supply of ammonia from the roots (30). The vigorous NADPH-GDH activity and low NADH-GDH activity of ammonium and nitrate sunflower leaves (Table I) suggests that both translocated ammonia and the small amount of ammonia formed by nitrate reduction in nitrate leaves are assimilated primarily by chloroplastic NADPH-GDH.

Glutamine Synthetase. The observation that GS extracts from soybean roots are three to eight times more active than those extracted from sunflower (Table I) may provide an explanation for the already noted low concentration of ammonia in soybean exudates. Also to be noted is that despite greater GS activity in soybean roots than in sunflower, glutamine concentration in soybean exudates is no greater than that found for sunflower (Table II). The asparagine content of the exudates of the two plants is also similar. Thus it would appear that in soybean roots large quantities of ammonia, absorbed as such or formed from nitrate, are rapidly assimilated to the amide form and that much of it, rather than entering the transpiration stream, is metabolized, stored, or utilized in protein synthesis. Sunflower roots, however, are less active in the assimilation of ammonia to the amide form and appear to send a larger proportion of their glutamine and asparagine, together with unassimilated ammonia, into the shoot system.

Soybean and sunflower differ further with regard to GS activity in that for soybean, enzyme activity is greater with nitrate in the culture solution than it is for ammonium, while

for sunflower, the opposite is the case (Table I). For higher plants, little in the way of direct data is available with regard to the various nitrogeneous parameters that might affect GS activity. Shapiro and Stadtman (22) cite studies with microorganisms indicating the importance of ammonium in the culture medium and glutamine concentration in the cell as factors determining GS activity. In the present study, data for sunflower indicating a higher concentration of ammonia in nitrate-supplied roots (Table II) is suggestive of an ammonium-influenced GS system. For soybean roots, however, where no correlation is evident between GS activity and ammonium concentration, high glutamine concentration in the tissue may be responsible for the limited activity of GS in roots supplied with ammonium.

No difference in GS activity could be detected in the leaves of plants grown with either ammonium or nitrate in the culture solution (Table I). Although enzyme activity in soybean leaves was somewhat greater than that found in sunflower, the general level of activity in leaves, whether measured as a synthetase or as a transferase, was 20- to 40-fold lower than that found in root tissue. Thus it would appear that the large quantities of ammonia entering the leaves of sunflower or formed by nitrate reduction in the leaves of soybean, together with the quantities of glutamine entering the leaves of both plants via the transpiration stream, utilize in their assimilation and metabolism enzyme systems other than GS.

The influence of the adenine nucleotides on GS activity is examined in an accompanying study (31).

Glucose-6-Phosphate Dehydrogenase. Butt and Beevers (4) noted an increased metabolism of carbohydrate via the pentose phosphate pathway in maize roots supplied with nitrite. For roots of wheat seedlings treated with nitrate in place of ammonium, a 2-fold increase in G6PDH has been reported (28). These observations are supported by those of the present study (Table I) that indicate, for both soybean and sunflower roots, a greater level of G6PDH activity in nitrate plants than in ammonium plants. The proposal (4) that nitrite reduction in roots utilizes the NADPH generated by the oxidation of glucose-6-P is in keeping with the data of this study and will be further examined in an accompanying paper (31). For soybean leaves, the absence of any effect of nitrate on G6PDH activity (Table I) suggests that some mechanism other than the pentose phosphate pathway is responsible for the generation of the reduced NADP presumably required for nitrite reduction (1) in this tissue.

Acknowledgments—The author is indebted to Miss Anne Cusack and Miss Lynn Garfield for skillful technical assistance.

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