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Glutamine and Asparagine as Nitrogen Donors for Reductant-Dependent Glutamate Synthesis in Pea Roots

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Glutamine, in the presence of α-oxoglutarate, stimulates nicotinamide nucleotide oxidation by crude extracts of pea roots and leads to a reductant-dependent formation of glutamate. Commercially available asparagine also stimulates nicotinamide nucleotide oxidation in the presence of α-oxoglutarate, but the reaction causing the stimulation can occur in the absence of a reductant, is inhibited by transaminase inhibitors, and is additive to the glutamine reaction. The asparagine used was found to be contaminated with aspartate. Repurified asparagine, chromatographically free of aspartate, did not stimulate the rate of nicotinamide nucleotide oxidation, and it is probable that the original stimulation was due to aspartate contamination. It is concluded that pea-root glutamine (amide)–α-oxoglutarate aminotransferase (glutamate synthase), in common with the enzyme in leaves, is specific for glutamine as the N donor and α-oxoglutarate as the N acceptor. The significance of the enzyme in conjunction with glutamine synthetase in the assimilation of nitrate by roots is discussed.

Although it has been generally considered that the major reaction responsible for the assimilation of NH_3 into amino acids is the reductive amination of α-oxoglutarate, catalysed by glutamate dehydrogenase, recent work has shown that the reactions catalysed by the enzymes glutamine synthetase (EC 6.3.1.2)

$$\text{L-Glutamate} + NH_3 + ATP \xrightarrow{Mg^{2+}} \text{L-glutamine} + ADP + P_i + H_2O \quad (1)$$

and glutamate synthase [glutamine (amide)–2-oxoglutarate aminotransferase (EC 2.6.1.53)]

$$\text{L-Glutamine} + \alpha\text{-oxoglutarate} \xrightarrow{+[2H]} 2\,\text{L-glutamate} \quad (2)$$

provide an alternative route (Brown *et al.*, 1973; Lea & Miflin, 1974).

In micro-organisms, glutamate synthase is a nicotinamide nucleotide-linked enzyme, using either NADH or NADPH according to species (Tempest *et al.*, 1970; Nagatini *et al.*, 1971). Dougall (1974) and Fowler *et al.* (1974) have reported nicotinamide nucleotide-dependent enzymes in higher-plant tissue cultures, and Lea & Miflin (1974, 1975) have demonstrated the presence of a ferredoxin-dependent enzyme in pea chloroplasts and algae. Fowler *et al.* (1974) also noted that the enzyme from non-green tissues of higher plants differs from most of the bacterial enzymes in using both NADH and NADPH as electron donor, and from both the chloroplast and bacterial enzymes in using asparagine as well as glutamine as a N donor. Fowler *et al.* (1974) found that their enzyme preparations used oxaloacetate as an alternative amino acceptor to α-oxoglutarate.

In a preliminary search for ferredoxin-dependent glutamate synthase we were able to demonstrate its activity in pea roots and carrot tissue culture. However, in contrast with the results of Dougall (1974) and Fowler *et al.* (1974), we found that asparagine was ineffective as the N donor in place of glutamine. The present paper gives the results of further investigations into glutamate synthase activity in pea roots, particularly the relationship of the activities assayed by measuring the formation of glutamate to those measured by following the oxidation of nicotinamide nucleotides.

Methods

Peas (*Pisum sativum* cultivar Meteor) were sterilized in sodium hypochlorite solution, germinated and cultured over aerated water for 4–6 days. In some experiments, 10mM-KNO_3 was added after 3 days, and in others, roots were excised and preincubated for 4h in 10mM-KNO_3, as described by Fowler *et al.* (1974). No major differences in activity were noted between the two procedures. Roots were ground in 100mM-Tricine [*N*-tris(hydroxymethyl)-methylglycine], pH7.5, 5mM-EDTA and 12.5mM-2-mercaptoethanol. In some experiments, glycylglycine buffer was used with similar results. All Tricine buffers were made up at slightly above the required strength, adjusted to pH7.5 with a solution of KOH, and then diluted to the correct volume. The filtered homogenate was centrifuged at 7000*g* for

5min, the supernatant was adjusted to 50% saturation with $(NH_4)_2SO_4$ and the resulting precipitate collected. This was dissolved in 50mM-Tricine, pH7.5, containing 12.5mM-mercaptoethanol, and passed through a column (2cm×5cm) of Sephadex G-75, with the same buffer as eluent. Such preparations have been stored at −18°C for 1–2 weeks without noticeable loss of activity.

Assays for glutamate synthase

Method 1. The nicotinamide nucleotide-oxidation assay involved incubating the enzyme at 30°C in a cuvette, together with 5mM-glutamine (or -asparagine), 5mM-α-oxoglutarate, 0.16mM-NAD(P)H and 50mM-Tricine, pH7.5, in a total volume of 1ml. The oxidation of the nicotinamide nucleotides was monitored continuously by following the decrease in E_{340}.

Method 2. The glutamate synthesis assay reaction mixture contained the enzyme together with 5mM-glutamine, 5mM-α-oxoglutarate, and either 0.1mg of Methyl Viologen or 0.1mg of sugar-beet ferredoxin or 1.5mM-NAD(P)H. Methyl Viologen and sugar-beet ferredoxin were reduced by adding 50μl of a freshly prepared solution of $Na_2S_2O_4$ and $NaHCO_3$, both 8mg/ml. The final reaction volume was 0.7ml. The reaction was terminated by adding 1ml of ethanol, and after mixing and centrifugation, a portion of the supernatant was spotted on Whatman no. 4 chromatography paper and subjected to descending chromatography with aq. 75% (w/w) phenol (in the presence of NH_3 vapour) as solvent. When asparagine was present in the reaction mixture, no hot air was used during the spotting procedure, in order to prevent a non-enzymic reaction occurring on the paper. The amino acids were measured by the technique of Atfield & Morris (1961). Authentic glutamate was spotted on the papers, both as a marker and as an internal standard for colour development.

Protein was determined by the method of Heepe *et al.* (1951).

Chemicals

Ferredoxin was obtained from sugar beet by a modification of the method of Hall *et al.* (1972). L-Glutamine, α-oxoglutarate, NADH, NADPH and amino-oxyacetate were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Asparagine was obtained both from Sigma and from BDH, Poole, Dorset, U.K. Azaserine was obtained from Calbiochem, London W.1, U.K. Albizziine (L-2-amino-3-ureidopropionic acid) was isolated from *Acacia armata* by the method of Seneviratne & Fowden (1968).

Results

Initial experiments using the glutamate synthesis assay (Method 2) (Table 1) showed that whereas glutamine can donate its N to α-oxoglutarate to form glutamate in a reductant-dependent reaction, little or no reductant-dependent synthesis occurs with asparagine. In the glutamine reaction, a range of electron donors can support the activity. Although not shown in Table 1, it was found that oxaloacetate cannot substitute for α-oxoglutarate as the N acceptor. The absence of any measurable glutamate formation in the presence of glutamine alone suggested that the glutaminase activity of the preparations was low.

In contrast, glutamine and asparagine stimulate the rate of nicotinamide nucleotide oxidation in the presence of α-oxoglutarate (Table 2). NH_3 was also effective, indicating the presence of glutamate dehydrogenase in the extracts. Oxaloacetate caused a

Table 1. *Rates of glutamate synthesis by pea root extracts*

Pea root extracts (300μl, containing about 1.2mg of protein) were incubated with the standard reaction mixture, except that the electron donors were varied, and in two cases glutamine was replaced by asparagine. The mixture was incubated at 30°C for 20min. The glutamate formed was determined as described in the Methods section.

N donor (5mM)	Nitrogen acceptor	...	Glutamate synthesis (nmol/min per mg of protein) α-Oxoglutarate	None
	Electron donor			
Glutamine	None		3.5	1.0
	Reduced Methyl Viologen		11.2	1.2
	Reduced ferredoxin		23.8	—
	NADH		21.4	1.5
Asparagine	None		8.4	—
	NADH		6.2	—

Table 2. *Rates of nicotinamide nucleotide reduction in the presence of various electron acceptors*

Pea root extracts (50 μl, containing about 0.2 mg of protein) were incubated in Tricine buffer with various N donors, N acceptors and either NADPH or NADH as electron donor. The final concentration of all the N donors and acceptors was 5 mM. The initial rates of nicotinamide nucleotide oxidation at 30°C were recorded.

		Oxidation (nmol/min per mg of protein)	
	Electron donor ...	NADH	NADPH
N donor	N acceptor		
None	None	4.0	4.0
None	α-Oxoglutarate	4.0	—
Glutamine	None	4.0	—
Glutamine	α-Oxoglutarate	13.7	7.6
NH_3	α-Oxoglutarate	27.0	8.0
Asparagine	α-Oxoglutarate	16.1	7.2
Asparagine	None	4.8	—
None	Oxaloacetate	>1000*	49.4
Glutamine	Oxaloacetate	—	51.4

* Under these conditions the addition of oxaloacetate caused almost immediate oxidation of the NADH.

Table 3. *Effect of various inhibitors and treatments on the relative rates of NADH oxidation in the presence of various nitrogen donors*

Pea root extracts (50 μl, containing about 0.2 mg of protein) were incubated in the standard assay mixture with different N donors (all 5 mM final concentration). One portion of the extract had been previously heated to 52°C for 10 min and then returned to about 4°C, before assay. Inhibitors, adjusted to pH 7.5 where necessary, were added to give the final concentrations indicated below. The initial rates of NADH oxidation at 30°C were recorded and those obtained with the different treatments are expressed as a percentage of the control rate.

	Initial rate of NADH oxidation (% of control)		
N donor ... Treatment	Glutamine	Asparagine	Ammonia
Heat (52°C for 10 min)	9	90	90
+Amino-oxyacetate (1 mM)	95	0	93
+Albizziine (10 mM)	11	92	100
+Azaserine (10 mM)	2	93	100

rapid oxidation of all the NADH, showing that the extract contained a highly active malate dehydrogenase. This prevented the testing of the effect of glutamine or asparagine on NADH oxidation in the presence of oxaloacetate. However, it was possible to measure their effects on NADPH oxidation, but no stimulation of the rate was observed. In general, the nitrogenous compounds cause a higher rate of oxidation of NADH than of NADPH.

Table 4. *Additive effect of asparagine and glutamine on NADH oxidation by pea root extracts*

Pea root extracts (50 ml, containing about 0.3 mg of protein) were incubated in the standard reaction mixture together with different N donors as indicated. Initial rates of NADH oxidation at 30°C were recorded, and are expressed below. The calculated additive rate is the sum of the stimulation of the oxidation on the addition of asparagine, the stimulation with 5 mM-glutamine, and the rate in the absence of an N donor (6.3+4.2+2.0).

N donor	Rate (nmol/min per mg of protein)
None	2.0
Asparagine (5 mM)	8.3
Glutamine (5 mM)	6.2
Glutamine (10 mM)	6.7
Glutamine (5 mM)+asparagine (5 mM)	12.2
Calculated additive rate	12.5

Table 3 reports the effect of various treatments on NADH oxidation, and shows quite clearly the different characteristics of asparagine- and glutamine-dependent reactions. Whereas glutamate dehydrogenase is relatively unaffected by all treatments, the asparagine-stimulated rate is severely inhibited by amino-oxyacetate, and the glutamine-stimulated rate by glutamine analogues and heat treatment of the extract. The differential inhibition by the glutamine analogues albizziine and azaserine, which should block the glutamine-binding site on the enzyme, suggests that asparagine is not being utilized by the same enzyme site to produce nicotinamide nucleotide oxidation. Further evidence that asparagine is being utilized by a different enzyme is given in Table 4, which shows that the activity in the presence of asparagine is additive to that obtained with saturating concentrations of glutamine.

Since amino-oxyacetate inhibited asparagine-dependent NADH oxidation (Table 3), and was a potent inhibitor of the aspartate transaminase activity present in the crude extracts, this transaminase, coupled to malate dehydrogenase activity also present in the extracts, could possibly be responsible for the observed nicotinamide nucleotide oxidation. Aspartate could arise in the reaction mixture either by the action of asparaginase

$$\text{Asparagine} \longrightarrow \text{aspartate} + NH_3 \qquad (3)$$

or by contamination of the asparagine. Any aspartate present would subsequently be converted by aspartate transaminase to give oxaloacetate. Oxaloacetate

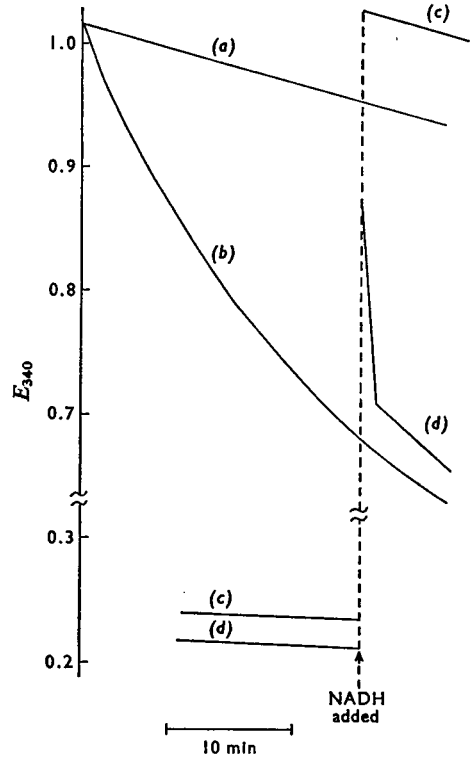

Fig. 1. *Effect of time of addition of NADH on the stimulation by asparagine of the oxidation rate in the presence of α-oxoglutarate*

Pea root extracts (50μl, containing about 0.2mg of protein) were added to 50mM-Tricine buffer containing 5mM-α-oxoglutarate, and also either (*a*) 0.12mM-NADH, (*b*) 5mM-asparagine and 0.12mM-NADH, (*c*) nothing, or (*d*) 5mM-asparagine. The cuvettes were incubated at 30°C and their E_{340} was monitored continuously. After 20min incubation, 120nmol of NADH was added to cuvettes (*c*) and (*d*).

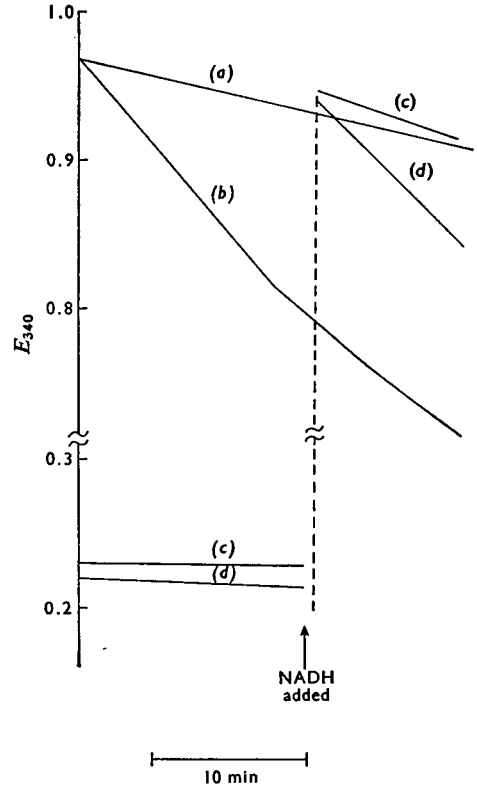

Fig. 2. *Effect of time of addition of NADH on the stimulation by glutamine of the oxidation rate in the presence of α-oxoglutarate*

Pea root extracts (50μl, containing about 0.2mg of protein) were added to 50mM-Tricine buffer containing 5mM-α-oxoglutarate, and also either (*a*) 0.12mM-NADH, (*b*) 5mM-glutamine and 0.12mM-NADH, (*c*) nothing, or (*d*) 5mM-glutamine. The cuvettes were incubated at 30°C and their E_{340} was monitored continuously. After 20min incubation 120nmol of NADH was added to cuvettes (*c*) and (*d*).

could also arise if asparagine transamination occurred, and the α-oxosuccinamate produced broke down subsequently to oxaloacetate (Meister, 1965):

$$\text{Asparagine} + \alpha\text{-oxoglutarate} \rightarrow \text{glutamate} + \alpha\text{-oxosuccinamate}$$

$$\alpha\text{-Oxosuccinamate} \xrightarrow{H_2O} \text{oxaloacetate} + NH_3 \quad (4)$$

If any of these reactions were taking place to give oxaloacetate, they would occur in the absence of a reductant. Also, since malate dehydrogenase is much more active than asparagine-dependent NADH oxidation (Table 2), the production of oxaloacetate should be the limiting step in any coupled reaction. Thus preincubation of the reactants in the absence of NADH would lead to a build-up of oxaloacetate, giving a fast initial oxidation rate on the subsequent addition of NADH. These results were observed when the hypothesis was tested (Fig. 1). The amount of rapid NADH oxidation corresponded to that which would have occurred if NADH had been present from the beginning and was measured in the control cuvette which contained NADH throughout. After the rapid phase of oxidation, the rate declined to that of this control. The results in Fig. 1 contrast with those in Fig. 2, which are from a similar experiment with glutamine as the N-donor. In this experiment there was no rapid oxidation of NADH added after preincubation of the rest of the reactants, and the initial rate of oxidation was the same as the control.

Table 5. *Effect of commercial and repurified samples of asparagine on the rates of NADH oxidation in the presence of oxoglutarate*

Pea root extracts (50μl, containing about 0.2mg of protein) were incubated in Tricine buffer together with different sources of asparagine (5mM final concn.) as N donor and, except in one case, with α-oxoglutarate (5mM final concn.) as electron acceptor. The initial rates of NADH oxidation at 30°C were recorded.

Conditions	Rate (nmol/min per mg of protein)
No N donor	1.4
Commercial asparagine	10.4
Purified asparagine	2.2
Commerical asparagine minus α-oxoglutarate	1.2

This suggests that the glutamine reaction is reductant dependent, as shown in the glutamate synthesis assay (Table 1).

Since all the above results suggested that the oxidation of NADH in the presence of asparagine was due to the combined action of a transaminase and malate dehydrogenase, it seemed essential to ensure that the asparagine was free from aspartate. The results of enquiries suggested that commercially available asparagine could contain about 0.5% aspartate. Chromatography of large amounts of asparagine on paper in aq. phenol–NH_3 showed this to be, if anything, an underestimate. Consequently asparagine was purified by passage over Dowex 1 (X8; acetate form), which retains the aspartate, and subsequent recrystallization from ethanol. This preparation was shown to be essentially free from aspartate on rechromatography. When tested, it only stimulated NADH oxidation to a very small extent (Table 5), and it is concluded that at least 90% of the rate of oxidation observed in the presence of commercial asparagine is probably due to contamination with asparate (Table 5).

Although the results in Table 1 and Fig. 2 suggest that NADH oxidation in the presence of glutamine was not due to either the combined operation of glutaminase and glutamate dehydrogenase, or to contamination of the glutamine with NH_3, the fact that the preparations contained glutamate dehydrogenase is disturbing. Root extracts from which the majority of mitochondria were removed were therefore made by grinding roots in 0.05mM-potassium phosphate buffer, pH7.5, containing 5mM-EDTA, 12.5mM-mercaptoethanol and 0.4M-sucrose. After filtration through cheesecloth, these extracts were centrifuged at 7000*g* for 5min to sediment the plastids, which were then ruptured by resuspension in a small volume in the isolation medium without sucrose. The resuspended plastids were added back to the 7000*g* supernatant, which was subsequently centrifuged at 60000*g* for 40min to remove mitochondria. The supernatant from the centrifugation was then treated with $(NH_4)_2SO_4$ and passed over Sephadex as described in the Methods section. The glutamate dehydrogenase and glutamate synthase activities of this preparation were compared with those found in a preparation made in the usual manner from the same batch of roots. The inclusion of sucrose in the isolation medium and the high-speed centrifugation diminished the activity of glutamate dehydrogenase to considerably below that of glutamate synthase (Table 6). Thus it is impossible

Table 6. *Effect of extraction conditions on the relative rates of NH_3 and glutamine-dependent NADH oxidation in the presence of α-oxoglutarate*

Pea roots were homogenized and extracted as described in the Methods section, either in the normal way, or in a way designed to protect the integrity of the mitochondria. Most of the mitochondria were then removed by differential centrifugation and the supernatant, depleted of mitochondria, was treated with $(NH_4)_2SO_4$ and passed through Sephadex in the same way as the standard extract. Portions of the two extracts (50μl, containing about 0.2mg of protein) were then incubated in the assay medium with either glutamine or $(NH_4)_2SO_4$ (final concentration 5mM) as indicated. The initial rates of NADH oxidation at 30°C were recorded.

	NADH oxidation (nmol/min per mg of protein)	
Nitrogen donor ...	NH_3	Glutamine
Extraction technique		
Standard extraction	14.8	8.0
Mitochondria depleted	3.6	10.2

Table 7. *Stoicheiometry of the glutamate synthase reaction*

Pea root extract (200μl, containing about 0.7mg of protein) was incubated with 1mM-glutamine, 0.32mM-NADH, 5mM-α-oxoglutarate and 50mM-Tricine buffer, in a spectrophotometer cuvette for 0 or 30min at 30°C. The oxidation of NADH was determined and then ethanol was added to the reaction mixture. Samples were chromatographed, and glutamate and glutamine concentrations determined as described in the Methods section. NADH oxidation was corrected for that occurring in the absence of glutamine, and glutamate appearance and glutamine disappearance were corrected for that occurring in the absence of NADH. Values in parentheses are the ratios to NADH oxidized.

NADH oxidized (nmol)	Glutamine lost (nmol)	Glutamate formed (nmol)
180	220 (1.2)	329 (2.18)

that the glutamine-dependent nicotinamide nucleotide oxidation could be due in any way to the operation of glutamate dehydrogenase.

Certain characteristics of the NADH-dependent glutamate synthase system, as measured in the crude Sephadex G-75 eluate, were determined. The K_m values for glutamine and α-oxoglutarate were found to be about 1.0 and 0.5mM respectively. For NADH the value was 7μM. The stoicheiometry of the reaction was investigated and found to be in reasonable accordance with that predicted from reaction 2 (Table 7). The enzyme activity was inhibited by glutamine analogues, as shown in Table 2, and the concentration dependence of this inhibition was investigated. The enzyme is most sensitive to azaserine, followed by albizziine and methionine sulphoximine, the concentrations required in the presence of 5mM-glutamine for 50% inhibition of activity being about 0.2, 2 and 4mM respectively.

Discussion

The stimulation by asparagine of the oxidation of nicotinamide nucleotides, in the presence of α-oxoglutarate, that we have observed in pea root extracts is almost certainly due to the contamination of commercially available asparagine with aspartate. The reaction was shown to be independent of added reductant and to be inhibited by transaminase inhibitors. It was not observed with purified asparagine. Our results do not necessarily mean that a reductant-dependent asparagine amide nitrogen transferase does not exist in plants, or that the previously reported (Dougall, 1974; Fowler *et al.*, 1974) asparagine-dependent nicotinamide nucleotide oxidation was necessarily due to the same cause. However, they emphasize the extreme care which must be taken in interpreting nicotinamide nucleotide oxidation rates in crude extracts which contain a wide range of enzymes and various possible coupled reactions. These difficulties are increased if residual substrates present in the cells are not removed from the extracts (Fowler *et al.*, 1974). Our results also emphasize that in experiments involving asparagine, care should be taken to see that the substrate used is aspartate-free.

Because the asparagine reaction is an artifact, it is advisable to be extremely sceptical of the glutamine-dependent NADH oxidation. However, there are a number of reasons which suggest that it is a genuine glutamate synthase reaction. First, the absence of glutaminase from the preparations (Table 1), the ability to separate glutamate dehydrogenase from glutamate synthase activity, and the complete dependence on reductant (Fig. 2) all argue against the results being due to the combined operation of glutaminase and glutamate dehydrogenase. Secondly, the reaction shows the expected stoicheiometry, and can be assayed by both chromatographic and NADH-oxidation techniques.

The presence of glutamate synthase in roots means that they, as well as leaves, have an alternative route for assimilating NH_3. The importance of glutamate synthase in N assimilation in root and other tissue, despite the inference in the results of Dougall (1974), and Fowler *et al.* (1974) does not depend on its abundance relative to glutamate dehydrogenase. Glutamate dehydrogenase is present in large quantities in tissues, particularly roots. Although it is possible to choose extraction methods (e.g. Table 6) so that the relative activity of glutamate synthase is favoured, the only fair comparison (if it were relevant and possible) would be under optimal extraction and assay conditions for both enzymes. Joy (1971, 1973) and Lea & Thurman (1972) have demonstrated some of the difficulties and pitfalls that occur in measuring glutamate dehydrogenase activity.

The importance of glutamate synthase is that, in conjunction with glutamate synthetase, it forms a system capable of incorporating NH_3, present in low concentrations, into α-amino N, owing to the favourable K_m of glutamine synthetase. The location in the plastid of at least part of the activity of three of the enzymes in the scheme, namely nitrite reductase, glutamine synthetase and glutamate synthase (Miflin, 1974; B. J. Miflin, unpublished work) suggests that this route may be the major one for the assimilation of nitrate, which in most soils is the chief form of N available. Further, as both glutamine synthetase and glutamate synthase are present in the bacteroids (Dunn & Klucas, 1973) and the roots of legumes, they may also be expected to play an important role in the assimilation of the products of N fixation. However, under conditions of high NH_3 concentration, glutamate dehydrogenase could be an important enzyme in assimilation. In any event, further experiments are required before the true relative importance of the two routes can be assessed.

Comparison of the pea leaf and root glutamate synthase systems show that both can utilize reduced ferredoxin and Methyl Viologen as electron donors. Additionally, the root enzyme can use reduced nicotinamide nucleotides. More work is required to show whether or not coupled diaphorase systems are involved. The dual specificity of the nucleotides could also be due to the activities of other enzymes (Wells & Hageman, 1974). In our studies we have generally observed more activity with NADH than with NADPH, but this varies with the preparations. In contrast with variabilities in the specificity for electron donors, both leaf and root enzymes appear to be specific for glutamine as the N donor and α-oxoglutarate as the N acceptor.

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