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REVIEW

THE PATHWAY OF NITROGEN ASSIMILATION IN PLANTS

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Key Word Index—Nitrate reduction; nitrogen fixation; ammonia; amino acids; assimilation; regulation; glutamine synthetase; glutamate synthase; glutamate dehydrogenase.

Abstract—The major route of nitrogen assimilation has been considered for many years to occur via the reductive amination of α-oxoglutarate, catalysed by glutamate dehydrogenase. However, recent work has shown that in most bacteria an alternative route via glutamine synthetase and glutamine: 2-oxoglutarate aminotransferase (glutamate synthase) operates under conditions of ammonia limitation. Subsequently the presence of a ferredoxin-dependent glutamate synthase in green leaves and green and blue–green algae, and a NAD(P)H and ferredoxin-dependent enzyme in roots and other non-green plant tissues, has suggested that this route may also function in most members of the plant kingdom. The only exceptions are probably the majority of the fungi, where so far most organisms studied do not appear to contain glutamate synthase. Besides the presence of the necessary enzymes there is other evidence to support the contention that the assimilation of ammonia into amino acids occurs via glutamine synthetase and glutamate synthase, and that it is unlikely that glutamate dehydrogenase plays a major role in nitrogen assimilation in bacteria or higher plants except in circumstances of ammonia excess.

INTRODUCTION

For many years the main pathway of ammonia assimilation in living organisms has been considered to be by the reductive amination of α-oxoglutarate, catalysed by the enzyme glutamate dehydrogenase (GDH) (EC. 1.4.1.3. Reaction 1)

$$\alpha\text{-Oxoglutarate} + NH_3 + NAD(P)H + H^+ \rightleftharpoons \text{Glutamate} + NAD(P)^+ + H_2O$$

This enzyme has been found in virtually all organisms [1–4]. In higher plants it is mainly localised in the mitochondria [5–7]. The evidence in favour of its role in nitrogen assimilation has been (a) its ability to catalyse glutamate synthesis *in vitro* with a reaction equilibrium favouring such synthesis; (b) the kinetic labelling studies of Sims and Folkes [8–10] using *Candida utilis*, and of Bassham and Kirk [11] using *Chlorella*; and (c) the unsuitable nature of the other routes for ammonia assimilation.

The alternatives available are glutamine synthetase (GS) (EC. 6.3.1.2. Reaction 2)

$$\text{Glutamate} + NH_3 + ATP \rightarrow \text{Glutamine} + ADP + P_i$$

and certain dehydrogenases analogous to GDH. The action of GS results in the incorporation of ammonia-N into the amide-N of the molecule. Subsequently this nitrogen can be donated to carbamoyl phosphate and to purines, but it was only considered to contribute to the non-α-amino-N of tryptophan, arginine and histidine [12,13]. The reductive amination of pyruvate and oxaloacetate to give alanine and aspartate, respectively, has been claimed and in some cases substantiated [14–17]. However, only in a few tissues is the activity of alanine and/or aspartate dehydrogenase comparable to GDH and GS, and the ^{15}N kinetic data of Sims and Folkes [8–10] do not support a role for them in the primary assimilation of ammonia. Further evidence for their lack of involvement has come from a number of studies, as discussed by Brown *et al.* [18].

In general terms, therefore, the GDH pathway seemed well established in the central dogma of biochemistry. However in 1970, Tempest, Meers and Brown [19–21] reported experiments with ammonia-limited chemostat cultures of *Klebsiella aerogenes* that began to undermine the role of GDH in assimilation. Many subsequent experiments have continued this process and it now seems unlikely that GDH plays any major role in nitrogen assimilation in bacteria or higher plants, except in the relatively unusual circumstances of high external ammonia concentrations. The purpose of this review is to record this recent series of events, especially as it relates to higher plants.

STUDIES ON THE ENZYMES OF NITROGEN ASSIMILATION

In bacteria

The K_m for ammonia for most bacterial GDHs is *ca* 4 mM, indicating a low affinity of the enzyme for its substrate. Thus, in cases where ammonia is limiting and its intracellular concentration is of the order of 0.5 mM [19], it might be expected that ammonia could only be assimilated if the enzyme responsible was derepressed under these conditions. However, when the level of GDH was measured in ammonia-limited cultures of *Klebsiella*

aerogenes, it was found to have dropped to 3% of its level in glucose-limited cultures [20]. This behaviour is inconsistent with GDH being responsible for ammonia assimilation. Following a pulse of ammonia added to ammonia-limited cultures there was an initial rapid rise in glutamine, which then fell as glutamate continued to rise. This suggested that the primary assimilation of ammonia occurred into glutamine followed by a secondary formation of glutamate [20–22]. Although the first part of this reaction can be carried out by GS, there was no enzyme known to catalyse a net synthesis of glutamate from glutamine. However, Tempest, Meers and Brown [19–21] were able to demonstrate the existence of such an enzyme which catalysed the reductive transfer of the amide-amino group of glutamine to α-oxoglutarate to give two molecules of glutamate (Reaction 3)

$$\text{Glutamine} + \alpha\text{-Oxoglutarate} + \text{NAD(P)H} + \text{H}^+ \rightarrow 2\ \text{Glutamate} + \text{NAD(P)}^+$$

The amount of this enzyme increased when bacteria were grown on nitrate or with low levels of ammonia, and decreased at high levels. The reverse was found to be true for GDH [20]. The original name given to this enzyme was glutamine(amide):2-oxoglutarate aminotransferase (oxido reductase NADP), although it has been classified by the I.U.B. as EC. 2.6.1.53 glutamine:2-oxoglutarate aminotransferase (NADPH oxidising) [23].* This is less appropriate since the enzyme is clearly different in character from the usual aminotransferase catalysing the transfer of the α-amino group of amino acids, and allows confusion with EC. 2.6.1.15 which relates to the non-reductive transfer of the α-amino group of glutamine. The enzyme has been given the trivial name 'glutamate synthase' or the acronym 'GOGAT'. The term GOGAT will be used in this review to avoid confusion between glutamine synthetase and glutamate synthase.

Since its initial description [19], GOGAT has been found in a wide range of bacteria [18,24]. The enzyme has been purified from *Escherichia coli* by Miller and Stadtman [25–27]. It is a flavoprotein containing non-haem iron and labile sulphur. The data suggest that the enzyme consists of eight subunits, four each of two types, with molecular weights of 135000 and 53000 aggregated to give a final MW of 800000. Each subunit pair (dimer) is probably catalytically active and contains eight iron atoms, eight labile sulphur atoms, and two flavin residues. The electroparamagnetic resonance spectrum [26] of the protein does not suggest that the iron is present as a ferredoxin type group. The enzyme is highly specific for glutamine and α-oxoglutarate and the K_m for both of these substrates is low. In some organisms the enzyme is specific for NADPH (e.g. *K. aerogenes* and *E. coli* [19,25]) and in others NADH (e.g. *Chromatium* spp. and *Pseudomonas* spp. [24]).

In higher plants

Attempts in several laboratories, chiefly using leaf tissue, initially failed to demonstrate the presence in higher plants of a pyridine nucleotide-dependent GOGAT (source—personal experience and conference discussion). However in 1974, Dougall [28] reported the presence of such an enzyme system in extracts of tissue cultures, and Lea and Miflin [29] described the discovery of a ferredoxin-dependent GOGAT in leaf tissue which was inactive with pyridine nucleotides.

The discovery of the ferredoxin-dependent enzyme arose out of a series of studies on the assimilation of nitrogen in leaves and isolated chloroplasts. Although the location of nitrate reductase is probably in the cytosol [30], the next enzyme of the nitrate reduction sequence–nitrite reductase, which leads to the production of ammonia, was found by non-aqueous and sucrose density gradient techniques to be associated with the leaf and root plastids [6,30,31].

Further studies by several groups showed that isolated intact chloroplasts were capable of the light-dependent reduction of nitrite to α-amino-N in the absence of any added cofactors [32–34]. If this assimilation to α-amino-N takes place via GDH, then the enzyme should be present in the chloroplasts. Previous studies by Leech and coworkers demonstrated the presence of low levels of GDH in chloroplast lamellae and the ability of intact chloroplasts to reduce α-oxoglutarate to glutamate [35,36]. Lea and Thurman [37] isolated chloroplast-GDH which preferentially used NADPH, and showed that it had a K_m for ammonia of 5.8×10^{-3} M, similar to mitochondrial-GDH. Considering that ammonia uncouples chloroplasts at 2 mM [38], it is unlikely that ammonia concentrations reach levels anywhere approaching the K_m of GDH. However, it is always possible that the *in vivo* K_m of chloroplastic-GDH is different from that of the isolated enzyme, or that the enzyme is in close physical proximity to nitrite reductase in a special compartment of the chloroplast.

Another reason for doubting the role of GDH in ammonia assimilation in chloroplasts was the finding by several groups that chloroplasts separated by differential centrifugation contained GS [39–41]. This was confirmed by Miflin [31] using density gradient techniques. GS has been found and characterised from several plant tissues [42–46]. Its level of activity is generally high, and it has a low K_m for ammonia: O'Neal and Joy have recently reported a value of 1.9×10^{-5} M for the pea leaf enzyme [46]. The activity in chloroplasts is several times that of GDH, and it is difficult to see how GDH could compete for ammonia. However, since nitrite is stoichiometrically reduced to α-amino-N [33,34], some way must exist for the transfer of the amide-N to the α-amino position if GS is the assimilatory enzyme. To test this possibility Lea and Miflin [29] repeated some of the experiments of Givan *et al.* [36] with intact chloroplasts. It was found that, whereas externally supplied ammonia had little effect on the synthesis of glutamate (confirming the original observation of Givan *et al.*), glutamine stimulated glutamate formation 16-fold. The stoichiometry of the reaction was such that two glutamates were formed for every glutamine used. As chloroplasts did not contain a NAD(P)-dependent GOGAT and since reduced ferredoxin is the electron donor for nitrite reductase, it seemed relevant to try this as a potential donor. The results showed that there was a ferredoxin-dependent GOGAT present in the chloroplast extracts, which could also accept electrons from viologen dyes but not from reduced pyridine nucleotides [29]. The enzyme is specific for glutamine and α-oxoglutarate. This enzyme has now been purified *ca.* 100-fold from *Vicia faba*, and some of its properties have been characterised (Wallsgrove, R. M., Lea, P. J. and Miflin, B. J. unpublished). The plant enzyme has a lower MW (145000) than the

* Ferredoxin-dependent glutamate synthase has now been given the number EC 1.4.7.1.

bacterial one (800000) and as yet its iron and sulphur content has not been determined. The K_m for glutamine (0.3 mM) is similar to the bacterial enzyme (0.25 mM) but the K_m for α-oxoglutarate (150 μM) is considerably higher (cf. to 7.3 μM).

The strict substrate specificity of the bacterial and leaf enzyme contrasts with that of the GOGAT isolated from tissue cultures, as described by Dougall [28] and Fowler *et al.* [47]. In both of these reports the enzyme system used asparagine as well as glutamine, and Fowler *et al.* [47] also reported that oxaloacetate could act as an alternative nitrogen acceptor to α-oxoglutarate. These observations were based on measurements of NAD(P)H oxidation in a spectrophotometer. Studies in our laboratory on the ferredoxin-dependent enzyme, using a chromatographic assay system, had failed to show these results when extracts of pea roots were used as the enzyme source. However, when the reaction was assayed in a spectrophotometer, glutamine and asparagine both stimulated the oxidation of NADH in the presence of α-oxoglutarate. The two reactions showed several differences. The stimulation of NADH oxidation caused by asparagine, unlike that caused by glutamine, was independent of the presence of the reductant, inhibited by transaminase inhibitors, not affected by reagents that blocked the glutamine-amide transfer site, and stable at 52° for 10 minutes. It was also additive to the glutamine reaction. The cause of these differences was eventually traced to contamination of commercial asparagine with aspartate, and the presence of aspartate transaminase and malate dehydrogenase in the extracts. Repurified asparagine caused no stimulation of NAD(P)H oxidation. GOGAT was also specific for α-oxoglutarate, the reaction was more active with NADH than NADPH, and ferredoxin would also act as an electron donor [48]. Since the pea root preparation used was relatively crude, the role of *trans*-hydrogenases cannot be eliminated, and the sequence of electron donation to the enzyme was not determined. The lack of substrate specificity observed by Dougall [28] and Fowler *et al.* [47] can probably be explained by the use of impure asparagine, and in the case of Fowler *et al.* by the use of crude extracts containing contaminating amino acids. In our opinion these make the assay of the enzyme from most tissues difficult unless they are removed by ammonium sulphate precipitation and gel filtration through Sephadex. In green tissues Sephadex G.75 should be used to remove ferredoxin from the enzyme [49].

In green algae

The algae are the one group of green plants which have a GDH with suitable characteristics for ammonia assimilation. For example, Morris and Syrett [50] stated that the level of activity of NADP-dependent GDH was three times that required for the nitrogen assimilation by *Chlorella vulgaris*. The K_m for ammonia for *Chlorella* GDH is also much lower than in other plants, and is in the range of $3–5 \times 10^{-4}$ M ([18] and Lea, P. J. and Miflin, B. J. unpublished results). The low K_m value should allow the rapid assimilation of ammonia before it rises to toxic levels. In other algae, such as the marine plankton diatom *Ditylum brightwellii* [51], the NADP-dependent GDH has a K_m value of 10 mM but the internal ammonia pool ranges from 5–10 mM, independent of the nitrogen source, suggesting that if the enzyme is involved in ammonia assimilation it is working below its optimum conditions. Other studies with marine phytoplankton suggest that GDH is not behaving in a manner consistent with its role as an assimilatory enzyme [18].

Initial attempts to demonstrate the presence of a ferredoxin-dependent GOGAT in *Chlorella* were hindered by the presence of a reductant-independent glutamine transaminase and difficulties in solubilizing GOGAT activity. The transaminase could, however, be inhibited by amino-oxyacetate, and the poor extraction of the enzyme overcome by utilising the frozen and thawed cell technique described by Syrett [52]. By these means high activities of GOGAT could be detected in intact cells. Subsequently the enzyme was liberated from the cells in a soluble form by ultrasonication [53]. The presence of high levels of activity of GS in *Chlorella* has also been reported ([54,55] and Lea, P. J. and Miflin, B. J. unpublished), and this enzyme coupled with GOGAT is probably responsible for ammonia assimilation in algae. Further evidence for this view is presented later.

In nitrogen-fixing organisms

Nagatini *et al.* [24] showed the presence of a pyridine nucleotide-dependent GOGAT in free living nitrogen fixing bacteria. Dharmawardene *et al.* [56] reported low levels of activity of the enzyme in blue–green algae, but subsequently the same group commented that the activity could have been due to the combined action of glutaminase and GDH [57]. Nielson and Doudoroff [58] were unable to find GOGAT in a number of blue-green algae, and considered that assimilation was via GDH and alanine dehydrogenase. However, the circumstantial evidence in favour of the GS/GOGAT system operating in blue–green algae appears substantial, since they have very low levels of GDH and high levels of GS [56,57]. This GS is well suited to being an assimilatory enzyme due to its relatively low K_m [59]. It is also present under nitrogen-fixing conditions at considerably higher levels than both GDH and alanine dehydrogenase. The failure to find an NAD(P)-dependent GOGAT in blue–green algae, and the presence of the ferredoxin enzyme in the chloroplasts, suggested to us that, on the basis of the endosymbiont hypothesis [60], blue–green algae should have a ferredoxin-dependent GOGAT. As with *Chlorella*, initial studies were largely inconclusive, due to the interference of glutamine transaminase. However, using amino-oxyacetate and the whole cell assay techniques that had proved successful with *Chlorella*, we were able to demonstrate the presence of the enzyme [61]. The enzyme was subsequently solubilised by ultrasonication and the levels of activity obtained in cell free extracts were of the same order as those previously reported for GS and nitrogenase [61].

The presence of the GS/GOGAT system in symbiotic nitrogen-fixing associations has likewise been subject to contradictory reports. Nagatini *et al.* [24], Sloger [62] Kennedy [63], Dunn and Klucas [64], and Ryan and Fottrell [65] have all reported the presence of both enzymes in legume root nodules, and have suggested that they had a role to play in the assimilation of fixed nitrogen. Further studies by Brown and Dilworth [66] showed that the enzymes were present in several species of *Rhizobium*, growing in chemostat cultures with ammonia or nitrate as the limiting nutrient. However, when these species formed bacteroids in legumes GS was found in every case, but GOGAT only in *Vicia faba*,

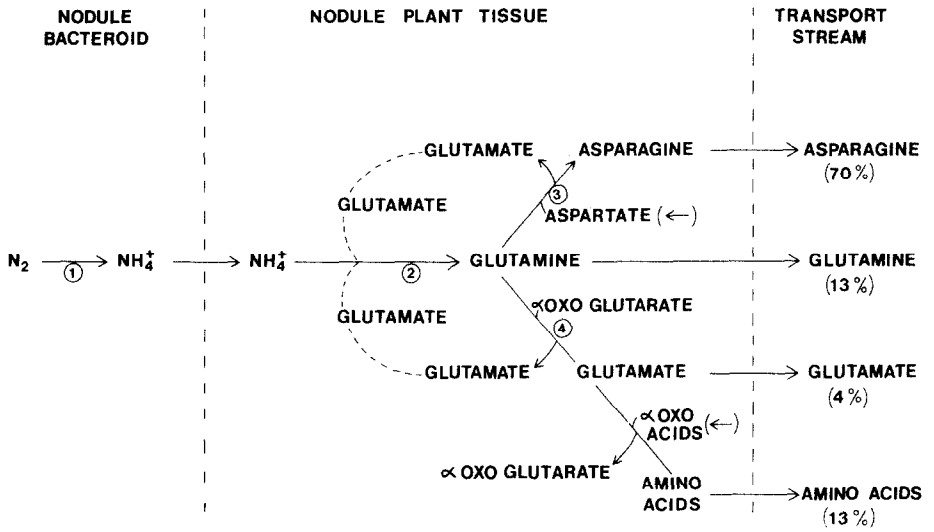


Fig. 1. Pathways of nitrogen assimilation and movement in nodules. (←) indicate net inputs required to balance the loss of compounds in the transport stream. The values in parenthesis indicate the relative composition of the transport stream based on the data in refs [67,69,70]. Enzymes ① Nitrogenase ② GS ③ Asparagine synthetase ④ GOGAT.

Pisum sativum, *Lupinus* spp. and *Phaseolus* spp. but not in *Medicago* spp. or *P. coccineus*. Even in those bacteroids containing GS and GOGAT, the levels of these enzymes were insufficient to support the levels of nitrogen fixation observed. In contrast to the low levels of GS in the bacteroid, the supernatant fractions of these nodules contained up to 1000-fold more GS, well in excess of that required to assimilate the products of nitrogen fixation. The presence of this excess of enzyme calls into question the values obtained for the bacteroids, since the isolation techniques used to separate the bacteroids involve a centrifugation which could also have led to the harvest of intact plastids (which contain GS- [31]) derived from the root tissue of the nodule.

Although GOGAT was found in the bacteroids, Brown and Dilworth [66] were unable to find it in the nodule supernatant (plant) tissue, which may have been due to the methods used. Recently, Robertson *et al.* [67] have found considerable levels of GOGAT in *Lupinus* root tissue in association with nodule formation. The levels of GS and GOGAT in the plant fraction of the nodule increase markedly during this formation, whereas GS and GOGAT of the bacteroid remained constant. The level of the bacteroid GS was extremely low, but that in the plant was more than twice that required to assimilate the products of nitrogen fixation. The final level of GOGAT was considerably less than that of GS, and probably only sufficient to deal with 10–20% of the nitrogen flux.

The levels of the assimilatory enzymes in the bacteroid [66,67] and the studies of $^{15}N_2$ fixation by isolated bacteroids [68] suggest that most of the nitrogen fixed may be excreted from the bacteroid as ammonia, and assimilated into glutamine via GS in the cytosol of the nodule. The great excess of GS in the nodule cytosol could be of key importance in the nitrogen-fixing symbiotic relationship since it would generate a steep concentration gradient of ammonia away from the bacteroid and allow continued fixation to occur. The formation of asparagine and its transport away from the nodule would complete the removal of the products of fixation from the nitrogen-fixing area. Further research is required to determine this point. The glutamine so formed can be metabolised in three ways (see Fig. 1). First, it can be exported and the results of Pate *et al.* [69] suggest that it accounts for 8–9% of the nitrogen in the bleeding sap of *Vicia* nodules. Secondly, it can serve as a substrate for GOGAT; this reaction should occur at a rate sufficient to replenish the glutamate lost by transport of glutamine and glutamate out of the nodule. This glutamate is required to accept the ammonia. The level of GOGAT should also be sufficient to provide the small amount of amino acids necessary to maintain the growth of the nodule. Thirdly, it can serve as a substrate for asparagine synthetase. Since 65–70% of the N_2 fixed appears to leave the nodules as asparagine [67,69,70] this is probably the major fate of the glutamine produced. Asparagine synthetase has been found in germinating legumes and shown to be a glutamine and ATP-dependent enzyme (EC. 6.3.5.4. Reaction 4) [71–76].

L-aspartate + L-glutamine + ATP →
L-asparagine + L-glutamate + PP_i + AMP

However, it has not yet been shown to be actually present in nodules. The origin of the aspartate for this reaction is not known. If only carbohydrates are transported into the nodule then the amino group of aspartate is probably derived by transamination from glutamate. This would place a further demand on GOGAT, and the levels reported so far would be insufficient to sustain nitrogen flow. This may be due to difficulties of isolating and assaying the enzyme and there is also a ferredoxin-dependent GOGAT present (Lea P. J. and Miflin B. J. unpublished).

In yeasts and fungi

Brown *et al.* showed that several species of yeasts, including *Saccharomyces cerevisiae* and *Candida utilis* do

not contain GOGAT [22]. Subsequently, Roon *et al.* [77] have claimed that GOGAT is present in *S. cerevisiae*, but the levels were very low and the significance of this finding is uncertain. The authors appear to have ruled out the possibility that their low levels were due to GDH activity, although Blumenthal and Smith [78] have shown that GDH from *Neurospora* can apparently use glutamine as a substitute donor for ammonia at very low comparative rates. Subsequent studies by Brown *et al.* [79,80] showed that GOGAT was present in some, but not all, species of *Schizosaccharomyces*. Brown [81] has also found very low levels of GOGAT generally 1% or less of the NADP-dependent GDH in several strains of *S. cerevisiae*, but considers it unlikely that this enzyme plays any role in ammonia assimilation in this organism.

The absence, or extremely low levels, of GOGAT in a wide range of yeasts appears to be genuine, and the conclusion that these organisms normally assimilate ammonia via GDH is compatible with the ^{15}N incorporation experiments by Sims and Folkes [8–10] and Jones *et al.* [82]. Further support for this conclusion comes from genetic work by Fincham *et al.* [83,84] in which they have shown that strains of *N. crassa*, unable to grow in ammonia, have a defective NADP-dependent GDH.

OTHER EVIDENCE FOR THE GS/GOGAT PATHWAY IN PLANTS

The presence of GS and GOGAT does not prove that they are used *in vivo* as the major route of nitrogen assimilation. Favourable circumstantial evidence is that the system has a low K_m for the initial substrate and is irreversible, properties it shares with a number of other biosynthetic systems. More direct evidence is available from studies with labelled nitrogen and from the use of specific inhibitors. From the known characteristics of the GDH and GS/GOGAT pathways, which are shown in schematic form in Fig. 2, certain predictions can be made.

Prediction 1

Where GDH is the only route to α-amino-N and the amide-N of glutamine is transferred only to a limited range of compounds formed in the absence of GOGAT, then $^{15}NO_3^-$ and ^{15}N-glutamate should donate their label in the same manner to all amino acids, but ^{15}N-amide glutamine should donate its label only to tryptophan, arginine and histidine. Where, however, the GS/GOGAT assimilatory pathway is operating, $^{15}NO_3^-$, ^{15}N-amide glutamine, and ^{15}N-amino glutamate are all in series and should all donate their label to amino acids in approximately the same proportions. The results of such an experiment in pea leaves carried out by Lewis and Pate [85] are shown in Table 1. The relative incorporation of ^{15}N from all three substrates is virtually identical. The results strongly support the operation of the GS/GOGAT assimilatory pathway.

Prediction 2

In the GDH pathway, labelled ammonia should be incorporated primarily into *both* the amino group of glutamate *and* the amide group of glutamine. On transfer back to $^{14}NH_3$, the amino group of glutamate and the amide group of glutamine should both immediately begin to lose their label. In the GS/GOGAT route, labelled ammonia should be incorporated into the amide group of glutamine first, and secondly into the amino

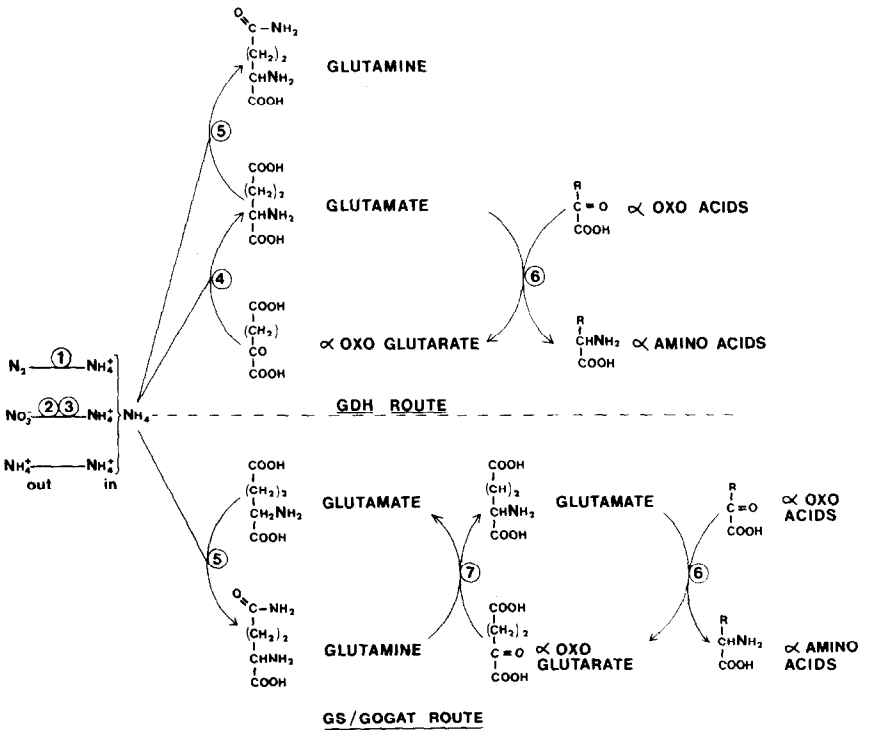


Fig. 2. Route of nitrogen assimilation into amino acids via the GDH and GS/GOGAT pathways. Enzymes ① Nitrogenase ② Nitrate reductase ③ Nitrite reductase ④ GDH ⑤ GS ⑥ Transaminase ⑦ GOGAT.

Table 1. The relative incorporation of ^{15}N, 10 hr after feeding labelled substrates, into the protein amino acids of pea leaves (calculated from Lewis and Pate [85])*

Leaf amino acid residue	Labelled substrates $^{15}NO_3$	^{15}N-glutamic acid	^{15}N(amide)-glutamine
Glutamyl-†	5.2	3.5	4.2
Serine	3.2	2.3	2.1
Aspartyl-†	3.1	2.7	3.4
Glycine	2.1	2.5	2.3
Alanine	2.0	2.4	2.1
Threonine	1.7	1.5	1.4
Isoleucine	1.6	1.2	1.2
Phenylalanine	1.1	1.5	1.2
Arginine	1.0	1.5	1.6
Valine	1.0	1.0	1.0
Leucine	1.3	1.1	1.0
Tyrosine	1.3	1.1	1.2
Lysine	0.9	1.0	1.2
Histidine	0.9	0.7	0.9

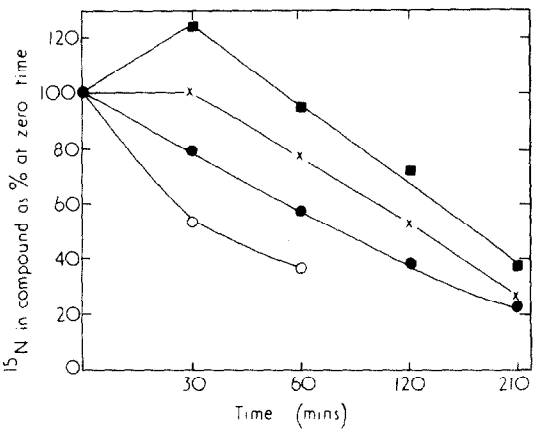
* The data of Lewis and Pate have been adjusted so that the incorporation into valine is set at 1.0 for each substrate. This allows for differences in the rate of uptake and transport of the different substrates to be partially eliminated.

† Includes the α-amino-N but not the amide-N of the corresponding amide.

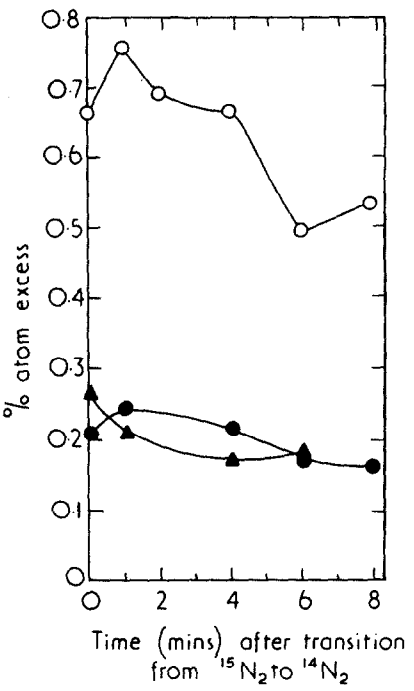
group of glutamate. On transfer to $^{14}NH_3$, the label should immediately decrease in the amide group of glutamine, but there should be a lag before the label in the α-amino group of glutamine and glutamate decreases. Because of difficulty in interpreting the initial labelling rates with $^{15}NH_3$, it is often more informative to look at the changes occurring after the transfer back to $^{14}NH_3$. In Fig. 3 two experiments have been chosen, one from rice roots grown on *ca* 3 mM $(NH_4)_2SO_4$ [86], and the other from nodules fixing $^{15}N_2$ [87]. The results have been redrawn to emphasise the changes occurring after the transition back to ^{14}N substrates. Although in the work with rice roots (Fig. 3a) no discrimination is made between the amide and amino-N of glutamine, it is clear that the ^{15}N is lost from glutamine before glutamate. The unchanged level of incorporation into glutamate for 30 minutes after the transition is entirely consistent with glutamate being formed from glutamine. These results also confirm previous studies showing a major incorporation of $^{15}NH_3$ fed to roots into the amide-N of glutamine [15,88]. In root nodules the results (Fig. 3b) allow discrimination between the two nitrogens of glutamine, and their different behaviour can be demonstrated. After transition there is a rapid initial loss of label from the amide group, but the α-amino group continues to incorporate ^{15}N, as does the α-amino group of glutamate. These results are consistent with the operation of the GS/GOGAT pathway in which glutamate acts as the ammonia acceptor. The higher specific activity of ^{15}N in glutamate than in glutamine probably reflects the size of the internal unlabelled pools rather than the sequence of nitrogen assimilation. It is known that glutamine is present in large amounts in nodule tissues and is one of the compounds transported away from the nodule [69]. The levelling off of the incorporation pattern of glutamine, three minutes after the transition, is probably due to the transport of glutamine away from the site of the GOGAT activity.

Recent work by Thomas *et al.* [89] using ^{13}N has confirmed that the flow of N_2 into amino acids in blue-green algae is via GS/GOGAT. Using very short incorporation times of 15–120 sec, they have shown that the label is found first in the amide-N of glutamine and only secondarily in the α-amino group of glutamate.

The nitrogen incorporation data of Bassham and Kirk [11] has generally been considered compatible with the GDH pathway. However, there are certain aspects of these experiments which limit the generality of the conclusions that can be drawn. Work in bacteria, referred



(a)



(b)

Fig. 3(a). The loss of ^{15}N from ammonia (○——○), glutamate (×——×), glutamine (●——●) and alanine (■——■) after transfer of rice roots to $^{14}(NH_4)_2SO_4$ after 2 hr in $^{15}(NH_4)_2SO_4$. Redrawn from the data of Yoneyama and Kumazawa [86]; 3(b). The loss of ^{15}N from glutamate (○——○) and from the amide (▲——▲) and α-amino (●——●) N of glutamine after transfer of *Seradella* nodules from $^{15}N_2$ to $^{14}N_2$. Redrawn from Kennedy [87].

to later under Section IV, suggests that at high levels of ammonia GDH may become the predominant route of incorporation. Although the method of culture of the *Chlorella* used by these workers probably leads to a variable level of ammonia in the medium, it is likely that this was considerable and thus the results may not be relevant to the situation of nitrate nutrition, more commonly found in nature. The results also do not give much information about incorporation into glutamine, and the authors state, "The results do not preclude the possibility that there was a small actively-turning-over pool of glutamine which saturates very quickly and which accounts for substantial amounts of ammonia incorporation". Part of the reason for the lack of information regarding glutamine may be due to the way in which the cells were harvested. In this and in many other studies [e.g. 86,90] tissues were extracted under conditions (particularly by boiling) which may lead to the loss of glutamine, due to the formation of pyrrolidone carboxylic acid [91,92]. The suspicion that such a rapidly turning-over pool of glutamine did exist in the *Chlorella* used by Bassham and Kirk [11] is supported by the shape of the incorporation curve of ^{15}N into glutamate (Fig. 2 of ref. [9]). This curve shows a sigmoid shape unlike the hyperbolic shape reported by Folkes and Sims [8–10] for *Candida utilis*, but resembles the incorporation curves into secondary products found by the latter authors. The exact ^{15}N atom excess in the *Chlorella* medium at the onset of incorporation is not known thus making calculations of the sort carried out by Folkes and Sims [10] questionable; however, using their plot of $\log_e (*A/*A - *B)$ a line is obtained that does not pass through the origin as expected for primary assimilation products.

Baker and Thompson [55] had previously investigated $^{15}NH_3$ incorporation into nitrate-grown *Chlorella*. They found that after one minute's incorporation the amide group of glutamine contained twice as much label as either the amino group of glutamine or glutamate, whilst after 5 min the amino groups had increased relatively more than the amide group. Great care was taken in these experiments to prevent degradation of glutamine.

The above experiments using labelled compounds are open to criticism on the grounds that compartmentation and small, actively turning-over pools can confuse their interpretation. Thus they cannot provide an unequivocable answer. However, in each case the most straight forward explanation is that the GS/GOGAT pathway is operating. Despite the fact that GDH has been known for many years, there is no labelling data that substantiates its role in ammonia assimilation in green plants, comparable with that provided by the studies of Sims and Folkes [8–10] for fungi.

Prediction 3

Methionine sulphoximine (MSO) is an analogue of the γ-glutamyl phosphate enzyme complex of GS [93] and is a potent inhibitor of the enzyme. It does not inhibit GDH but has some small effect on GOGAT [94,48].

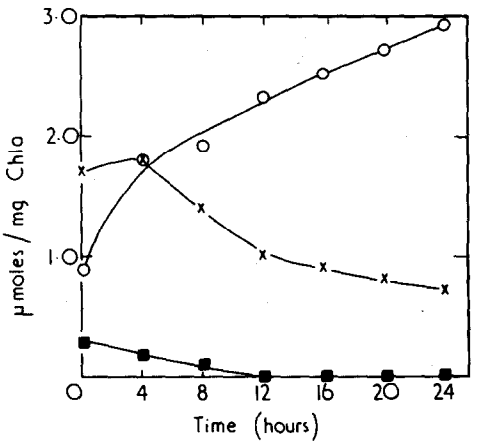
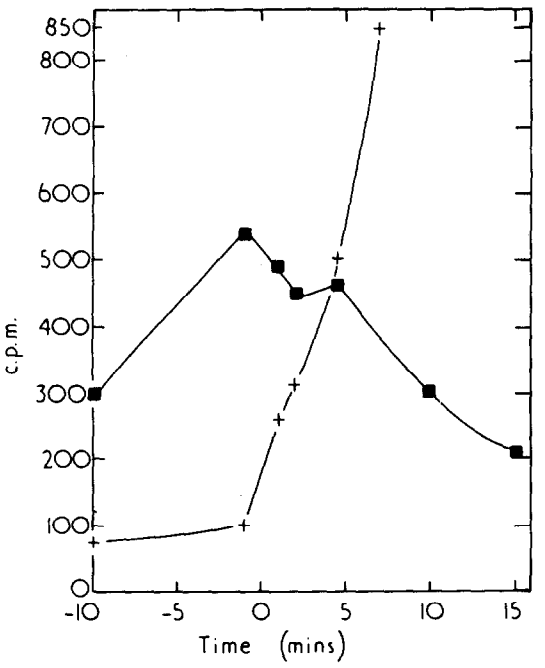


Fig. 4. The effect of methionine sulphoximine on the intracellular pools of N_2-fixing *Anabaena*. Redrawn from Stewart and Rowell [95]. o——o Ammonia; ×——× glutamate; ■——■ glutamine.



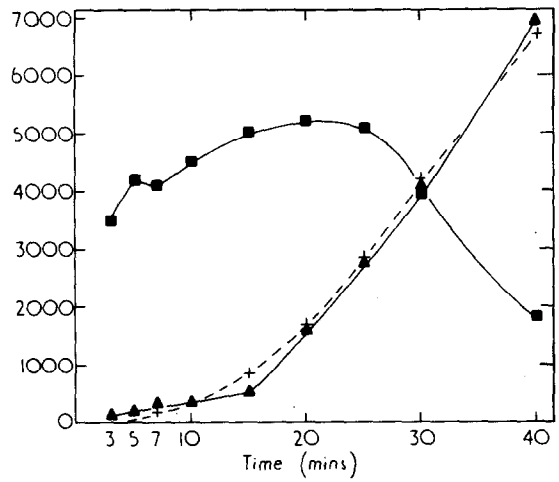


Fig. 5(a). The effect of azaserine on the $^{14}CO_2$ incorporation in *Scenedesmus*; 5(b) The effect of DON on $^{14}CO_2$ incorporation in *Chlorella*. ■——■ Glutamate; +——+ glutamine; ▲——▲ α-oxoglutarate. Redrawn from van der Meulen and Bassham [99].

Consequently, it should inhibit nitrogen assimilation via the GS/GOGAT pathway, but have no effect on that via GDH. Recently Stewart and Rowell [95] have tested its effect on nitrogen-fixing blue–green algae. The results of their experiments (Fig. 4) clearly show that the fixed nitrogen is assimilated by GS because the glutamine level drops and ammonia rises upon addition of the analogue. Thomas *et al.* [89] have obtained the same result using $^{13}N_2$.

Prediction 4

Azaserine and 6-diazo-5-oxo-L-norvaline (DON) are analogues of glutamine [96,97] and inhibit all glutamine amide transfer reactions, including GOGAT [25,48,98]. They do not affect GDH [48]. Thus, on addition to a system in which the GS/GOGAT pathway is operating, they should cause a rapid increase in the levels of glutamine and α-oxoglutarate and a decrease in glutamate. Unfortunately this experiment does not seem to have been done with labelled nitrogen, but a study of $^{14}CO_2$ labelling in *Chlorella* and *Scenedesmus* was reported by van der Meulen and Bassham in 1959 [99], and their redrawn results are shown in Fig. 5. The rise in ^{14}C incorporation into α-oxoglutarate and glutamine, and the decrease in glutamate, are consistent with the synthesis of glutamate via the GS/GOGAT pathway and cannot be explained by its synthesis via GDH. The conclusion is not limited to the route of newly assimilated $^{14}CO_2$ into glutamate since the total levels also fall markedly upon the addition of DON. Subsequent results from Bassham's laboratory are also consistent with the GS/GOGAT pathway. Thus Smith, Bassham and Kirk [90] found that the kinetics of ^{14}C incorporation into glutamate and glutamine were identical. They considered this inconsistent with the major pool of glutamate being the precursor of glutamine and suggested that these two compounds came from a common precursor. However, if glutamine and glutamate are formed as in the GS/GOGAT scheme (Fig. 2), each is the precursor of the other, and thus the results obtained by Smith *et al.* would be expected. Later work by Kanazawa *et al.* [100] showed that when ammonia is added to *Chlorella* the transient changes in the levels of ^{14}C and ^{32}P in various intermediates are consistent with the assimilation of ammonia into glutamine at the expense of ATP, according to Reaction 2.

CONTROL OF NITROGEN ASSIMILATION

Allosteric feedback control mechanisms

The regulation of bacterial GS had been the subject of hundreds of papers and reviews [12,13,101] before the discovery of its central role in nitrogen assimilation, and will only be summarized.

The *Escherichia coli* enzyme can exist in two states: (i) the unadenylated form, which is biosynthetically active and not subject to feedback control; and (ii) the adenylated form, which is less active and subject to feedback regulation. The difference between the two forms lies in the enzymic addition of adenylic acid to the enzyme. The adenylation reaction is stimulated by glutamine and several products of glutamine metabolism, and inhibited by ATP and α-oxoglutarate. The reverse reaction is stimulated by ATP and α-oxoglutarate, and inhibited by glutamine and AMP. In some other bacteria (e.g. *Bacillus subtilis* [102]), in blue–green algae [103], and in higher plants [104], this reversible adenylation does not appear to occur. The feedback control of the adenylated form of the *E. coli* enzyme and the *B. subtilis* enzyme is by the cumulative effect of several amino acids and other end products of glutamine metabolism. The possible physiological significance of this multi-valent effect of amino acids was clear after the central role of GS in nitrogen metabolism became known [105].

Some studies of the regulation of GS in higher plants have been done without very clear conclusions being reached. Webster and Varner [44] showed that the pea seed enzyme is inhibited by ADP and P_i, and Kingdon [104] has shown that AMP, histidine, lysine and alanine also inhibit the enzyme from this source. Similar inhibitory effects of these amino acids have been reported for the enzyme from pea leaves [106], carrots [107], soyabean nodule cytosol [108] and *Lemna* [109], but not from rice roots [110]. These effects are much more marked in the presence of Mn^{2+} than Mg^{2+}, and appear to be cumulative [106], but the levels of amino acids used are high (*ca* 10–13 mM) and the physiological relevance is somewhat doubtful. Probably more relevant is the inhibition of the enzyme by AMP, ADP and P_i which, in contrast to the amino acids, is more effective in the presence of Mg^{2+}.

Aspartate and methionine inhibit *E. coli*-GOGAT by more than 50% at concentrations less than 10 mM, which may be physiologically significant [25]. Little other information is available from studies of GOGAT in other organisms.

As mentioned earlier, in most but not *all* bacteria and fungi, NADP-dependent GDH is considered to be a biosynthetic enzyme (see review [4]). Except for the results of Jones [111] on the *C. utilis* enzyme quoted by Folkes and Sims [10], there is little to suggest that GDH is subject to allosteric feedback inhibition by amino acids. However the enzyme from several sources is affected by nucleotides [112,113]. Evidence for allosteric controls of higher plant-GDH are also inconclusive [114–117], and the results obtained do not suggest a role for GDH in nitrogen assimilation.

Changes in enzyme levels

Tempest *et al.* [22] showed that when ammonia is limiting the growth of *K. aerogenes*, the levels of GS and GOGAT rise and GDH falls (Table 2). In contrast, at higher levels of ammonia and under conditions of carbon limitation, GDH rises and GS and GOGAT fall. This is consistent with nitrogen assimilation occurring via GS/GOGAT under conditions of limiting nitrogen and sufficient energy, and via GDH in excess ammonia and low energy. Analyses of the intracellular pools of ammonia and glutamine in bacteria suggest that it is the level of ammonia itself that regulates the synthesis of GS [22].

The GS/GOGAT pathway is probably also the major assimilatory route in *K. aerogenes* and *Pseudomonas aeruginosa* when the nitrogen source is nitrate, even when present in excess (Table 2). Work from Brown's group with a number of species of bacteria shows that although the variation in levels of the enzymes between ammonia and carbon limitation are not as clear cut as in *K. aerogenes*, they follow a general trend ([18,81] and Table 2). One contrary situation appears to be in *E. coli* W, based on the results of Senior [118] although, despite

Table 2. The effects of nitrogen and carbon limitation on the levels of enzymes of nitrogen assimilation in various organisms

Organism	Nitrogen source	Limitation	Rates (nmol/min/mg protein) GS	GOGAT	GDH(NADP)	GDH(NAD)
Klebsiella aerogenes	ammonia	nitrogen	91	39	<1	—
	ammonia	carbon	<1	<1	671	—
	nitrate	carbon	43	47	73	—
Pseudomonas aeruginosa	ammonia	nitrogen	171	30	<1	3
	ammonia	carbon	<1	15	37	11
	nitrate	carbon	164	23	3	12
Rhodospirillum rubrum	ammonia + N_2	nitrogen	122	131	—	39
	ammonia + N_2	carbon	11	11	—	44
Escherichia coli W.	ammonia[a]	nitrogen	710	100	100	—
	ammonia[b]	nitrogen	690	120	590	—
	ammonia[c]	carbon	180	50	400	—
Saccharomyces ludwigii	ammonia	nitrogen	16	17	28	—
	ammonia	carbon	13	17	46	—
Saccharomyces cerevisae						
(NCYC 622)	ammonia	nitrogen	17	<1	710	<1
(NCYC 712)	ammonia	nitrogen	8	7	496	10
Schizosaccharomyces pombe	ammonia	nitrogen	11	22	29	—
	ammonia	carbon	ND	15	27	—

The results of all organisms except *E. coli* W. are from the work of Brown and colleagues [18,22,81]. Those for *E. coli* W. were derived from graphs in a paper by Senior [118]. The different growth rates of the cultures were a, 0.062; b, 0.046; and c, 0.044 mass doublings/hr.

his claims to the contrary, it is by no means certain that this organism uses GDH for assimilation under conditions of ammonia limitation.

In yeasts there appear to be different responses, since Burn *et al.* [119] have shown that for eight different yeasts the level of GDH is much greater at low, rather than high, concentrations of ammonia in the growth medium. However, the same group [79–81] have also shown that this does not hold for some species of *Schizosaccharomyces*, and that GOGAT and GS are present under conditions of ammonia limitation (Table 2).

Ferguson and Sims [120] have shown that increases in the ammonia supply, or a decrease in the glucose supply, to *C. utilis* cause a rapid fall in the level of GS. This fall is due to rapid inactivation of the enzyme which appears to involve the dissociation of its polymeric structure [121,122]. The level of the internal pool of glutamine is implicated in the control of the enzyme in that glutamine appears to act as a co-repressor of GS synthesis; however, the control mechanisms are obviously complex [122,123]. There is little *in vivo* evidence in this organism for allosteric feedback control of GS by the cumulative effects of amino acids as postulated for the *E. coli* enzyme [124].

The changes of GDH in algae have been studied by Kretovich *et al.* [125] using *Chlorella pyrenoidosa*. The NAD-dependent GDH shows little variation with nitrogen source, but the NADP-dependent GDH increases rapidly upon adding ammonia and is repressed in the presence of nitrate. The authors suggested that GDH was responsible for nitrogen assimilation, but did not explain why this 'biosynthetic' enzyme remained at a low level when nitrate was added to nitrogen-starved cells (which then produced large amounts of glutamate). Further, glutamine, which the authors proposed was deamidated to glutamate and ammonia, also fails to stimulate NADP-dependent GDH, which would have been expected if the ammonia were released and accumulated [125]. Similar increases in the levels of NADP-dependent GDH in thermophilic strains of *Chlorella* have been shown by Talley *et al.* [126]. Studies using marine algae have shown that NADP-dependent GDH is present in cultures grown on nitrate, nitrite or ammonia and remains high following depletion of nitrogen [51], showing some similarity to the results obtained in fungi. However, other work with marine phytoplankton shows that there is little correlation between GDH and ammonia assimilation [18]. A full picture of the control of enzyme levels in the algae awaits more detailed studies involving the measurement of GS and GOGAT levels as well as GDH.

In higher plants much less work has been done on the levels of the enzymes of nitrogen assimilation. Some work in root tissues by Weissman [127] has shown that the levels of NAD-dependent GDH in soyabean roots is higher under ammonium nutrition than under nitrate, while the reverse is true for NADP-dependent GDH and GS. Similar effects were observed for GDH from sunflower roots: GS was highest in ammonium-grown roots. Analogous stimulatory effects of ammonia on GDH levels have been found in rice roots [128]. Few effects of ammonium feeding of roots on GDH in the leaves have been reported, but this is probably because ammonia is not normally translocated to the leaves. In aquatic plants, such as *Lemna*, possibly the external medium is more able to influence the levels of the assimilatory enzymes in the leaf tissues (fronds); this has been reflected in the results obtained. Several authors [129–132] have studied the effects of ammonia in the medium on the level of GDH. Although Joy [130] has concluded that the changes in the levels in GDH that he observed [129] were due to EDTA, Shepard and Thurman [131] have suggested that there is a real increase in NAD-dependent GDH when *Lemna gibba* is transferred from glutamate to ammonia, apparently involving *de novo* synthesis of GDH. Unfortunately, under

the conditions used, ammonia is toxic to the plants and induces chlorosis and inhibits respiration. Rhodes *et al.* [132] made a detailed study on GDH, GS and GOGAT in *Lemna*, in relation to growth medium and internal pool size. Their results suggest that the level of GS is dependent on the level of the internal pool of glutamine. The activity of GS and GDH varies in inverse relationship to one another. Thus, as the glutamine concentration rises, so the level of GDH goes up and GS falls. Some changes in the level of GOGAT occur independently of GS and GDH, and it is difficult to relate these to the internal concentration of any amino acid. Although the changes in levels are two- to three-fold, the absolute levels rarely approach zero and appreciable levels of activity of GS, GDH and GOGAT are present under all conditions.

One final aspect of control worthy of note is the relationship of GS to the control of nitrogenase. It has long been known that the presence of ammonia in the medium represses nitrogenase [133]. Recent studies using MSO, which combines with GS and blocks ammonia incorporation, have shown that in its presence ammonia repression does not occur in nitrogen-fixing bacteria and blue–green algae [133,134,95], suggesting that ammonia is not the true repressor molecule. An exception to the relief, by MSO, of nitrogenase repression by ammonia has been found in studies with free-living *Rhizobia* spp and N_2-fixing soyabean root nodules (Brill, W. J. personal communication).

As a result of studies on the control of various enzymes of amino acid metabolism, Magasanik's group have proposed that the deadenylated form of GS can act as a regulatory protein activating the *hut* (histidine utilisation) operon [135]. Streicher *et al.* [136] and Tubb [137] have extended this proposal to suggest that this form of GS in also responsible for the de-repression of nitrogenase in *Klebsiella*. However, many organisms, including blue–green algae [103], *B. subtilis* [102], and peas [104], do not have the adenylating control mechanism of GS and there are no changes in the ratio of adenylated to deadenylated GS upon ammonia repression of nitrogenase in soyabean root nodules [138]. An alternative hypothesis is that one or several eventual products of glutamine metabolism are the repressors; the repression of nitrogenase in blue–green algae [103] and *Clostridium* [139] by carbamoyl phosphate supports this idea. The use of azaserine or DON to block the further metabolism of the amide of glutamine apparently has not been tried. If these inhibitors are ineffective in preventing ammonia repression of nitrogenase, then it would suggest that GS or glutamine was the repressor. Alternatively, if DON does relieve ammonia repression it will not be consistent with a role for GS in nitrogenase control.

THE SIGNIFICANCE OF THE GS/GOGAT PATHWAY IN PLANTS

The evidence available on the basis of (a) the presence of the necessary enzymes, (b) the incorporation pattern of labelled nitrogen, and (c) the use of specific inhibitors, suggests that for plants obtaining their nitrogen from either nitrate or low levels of ammonia, or from N_2, the incorporation of ammonia proceeds via the GS/GOGAT pathway. Work with bacteria in which the level of GS approaches zero when ammonia is in excess and carbon is limited suggests that, under these conditions, assimilation is via GDH. Similar studies with eucaryotic plants, including aquatic plants such as *Chlorella* and *Lemna*, show much less clearly defined and dramatic changes in enzyme levels. Such results suggest that in higher plants a switch from the GS/GOGAT to the GDH pathway could occur at high ammonia and low energy levels. However, there is no direct evidence that this switch occurs; if it does there is then a need to determine the relative rates of nitrogen flux through the two pathways. Much more work with labelled nitrogen and specific inhibitors is required to define the conditions, if any, in which GDH acts as the *major* assimilatory pathway in higher plants.

In higher plants the steps of nitrogen assimilation to the level of amino acids are often separated in space. Thus, ammonia present in the roots, whether from uptake, nitrate reduction or nitrogen fixation, is incorporated chiefly into amides which are then transported to the rest of the plant [15]. Similarly, it has been found that glutamine is the predominant transport compound derived from $^{15}NO_3$ reduced in the leaf [140]. Consistent with this is the recent finding of Mitchell and Stocking [141] and Givan [142] that isolated intact chloroplasts catalyse a light-dependent glutamine synthesis. Under these conditions the level of GOGAT in the assimilatory tissue needs to be adequate to regenerate sufficient glutamate to act as an ammonia acceptor. However, in the tissue receiving nitrogen from the transport stream in the form of amides, mechanisms must exist for the further transformation of this amide-N to α-amino acids. The glutamine present can be converted to α-amino acids via GOGAT; consistent with this NAD-dependent GOGAT has been found in developing endosperms of barley, and ferredoxin-dependent GOGAT in developing cotyledons of lupins, ([76]; Lea, P. J., Wallsgrove, R. M. and Miflin, B. J. unpublished results). Mechanisms for the utilisation of asparagine are less defined. Although there is little evidence for the metabolism of carbon and amide-N labelled asparagine in the soluble pools of amino acids, both the carbon and amide-N are found in a range of amino acids in the protein of developing lupin seed [143–145]. This suggests that asparagine metabolism occurs immediately prior to protein synthesis. Atkins *et al.* [145] showed that this may be via asparaginase (EC. 3.5.1.1) which is present in crude extracts of *Lupinus albus* embryos. The enzyme is also extremely active in the developing cotyledons of L. *polyphyllus* from which it has been purified 200-fold to a specific activity of 520 nmoles/min/mg protein [146]. Asparaginase results in the formation of ammonia with the subsequent need for its reassimilation. Superficially this does not appear to be a satisfactory way for the plant to utilise asparagine amide-N. However, an amide-amino transfer reaction analogous to GOGAT has not so far been described (see Section II), and the only alternative is an asparagine transaminase leading to the production to α-oxosuccinamic acid [147,148]; the fate of this compound is unknown, but it probably breaks down to release the amide-N again as ammonia. Further research is required on the utilisation of amide-N, particularly asparagine, by developing seeds and other tissues which are not involved in the primary assimilation via GS.

It is perhaps worthwhile considering why it has taken so long to establish the importance of the GS/GOGAT route, since several of the vital clues were available some years ago, particularly in the early papers of van der Meulen and Bassham [99] and Baker and Thompson [55]. Although, in their discussion, the former authors mentioned that the accepted route for the entry of nitrogen was via GDH, they also wrote, 'on inhibition of some transamidation reactions glutamine will accumulate and glutamic acid (a reaction product of the transamidations in which glutamine donates its amide group) will no longer be regenerated'. This is a clear statement of the mechanism of action of GOGAT. However, for the most significant and elegant experiments using ^{15}N, the experimenters chose to use *Candida utilis* [8–10], one of the few organisms which genuinely appears to lack GOGAT. The generalisations that were drawn by others from these experiments served to cement the central role of GDH as the major entry point of nitrogen until, by another quirk of fate, Tempest, Meers and Brown [19–21] chose *K. aerogenes* for their studies of the effect of ammonia limitation. This is one of the few organisms in which the level of GDH declines so dramatically under these conditions (Table 2), that the authors were persuaded to look for alternative assimilatory mechanisms.

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