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REVIEW

CARBON AND NITROGEN METABOLISM IN LEGUME ROOT NODULES

STEPHEN RAWSTHORNE*, FRANK R. MINCHIN*, RODNEY J. SUMMERFIELD*, CLAIRE COOKSON† and JAMES COOMBS†

* University of Reading, Department of Agriculture and Horticulture, Plant Environment Laboratory, Shinfield Grange, Cutbush Lane, Shinfield, Reading, RG2 9AD, U.K.; † Tate and Lyle Ltd., Group Research and Development, P.O. Box 68, Reading, RG6 2BX, U.K.

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Abstract—The literature concerning the metabolism of carbon compounds during the reduction, assimilation and translocation of nitrogen in root nodules of leguminous plants is reviewed. The reduction of dinitrogen requires an energy source (ATP) and a reductant which are both supplied by respiratory catabolism of carbohydrates produced by the host plant. Photosynthates are also required to generate the carbon skeletons for amino acid or ureide synthesis during the assimilation of ammonia produced by the bacteria within the nodule tissue. Competition for photosynthates occurs between the bacteroids, nodule tissue and the various vegetative and reproductive sinks in the host plant. The nature of carbon compounds involved in these processes, their routes of metabolism, the mechanisms of control and the partitioning of metabolites between the various sites of utilization are only poorly understood. It is apparent that dinitrogen is reduced to ammonia in the bacteroids. Both fast- and slow-growing strains of *Rhizobium* possess the Entner–Doudoroff pathway of glucose catabolism, and some, if not all, enzymes of the Emden–Meyerhof pathway. Some bacterial cultures also metabolize carbon through the ketogluconate pathway but only the fast-growing strains of cultured rhizobia possess the key enzyme of the pentose phosphate pathway (6-phosphogluconate dehydrogenase). The host cells are thought to contain the complete Emden–Meyerhof pathway and tricarboxylic acid cycle, which provides the carbon skeletons for assimilation of the ammonia, formed by the bacteroids, into α-amino acids. A pathway of anapleurotic carbon conservation, operative in the host cells, synthesizes oxaloacetic acid through β-carboxylation of phosphoenol pyruvate. This process could be important in the recapture and assimilation of respired CO_2 in the rhizosphere. The main route of assimilation of ammonia produced by the bacteroids would appear to be via the glutamine synthetase–glutamate synthase pathway in the host cells. However, glutamate dehydrogenase may also be involved in ammonia assimilation. These enzymes also occur in *in vitro* cultures of *Rhizobium* and in bacteroids where they presumably participate in the synthesis of amino acids for growth of the bacteria or bacteroids. Nitrogen assimilated into glutamine or glutamate is exported from the nodules in a variety of forms, which include asparagine, glutamine, aspartate, homoserine and allantoates, in proportions which depend on the legume species. Studies on regulation of the overall process have focussed on expression of bacteroid genes and on the control of enzyme activity, at the level of nitrogenase and enzymes of nitrogen assimilation in particular. However, due to the wide range of experimental techniques, environmental conditions and plant species which have been used, no clear conclusions can yet be drawn. The pathways of carbon flow in nitrogen metabolism, particularly in relation to the synthesis of ureides and the regulation of carbon metabolism, remain key areas for future research in symbiotic nitrogen fixation.

INTRODUCTION

The importance of grain legume crops is highlighted by the increasing world demand for food and fodder protein. Production costs of nitrogen fertilizer continue to escalate, problems associated with fertilizer application (e.g. leaching from agricultural soils, eutrophication of waterways and nitrates in drinking water) result in adverse reactions in developed countries, whilst population densities continue to increase in developing countries wherein the rural sector cannot usually afford fertilizer. Further knowledge on all aspects of leguminous crop plants is essential if the legume–*Rhizobium* symbiosis is to be exploited more effectively in human nutrition.

Detailed studies of the biochemical regulation of and/or limitations to carbon metabolism and nitrogen nutrition in nodules are essential if the symbiotic

Table 1. Host preference of *Rhizobium* species

Species	Preferred host genus
1. *Rhizobium leguminosarum*	*Pisum, Vicia, Lathyrus, Lens*
2. *R. trifolii*	*Trifolium*
3. *R. phaseoli*	*Phaseolus*
4. *R. meliloti*	*Medicago, Melilotus, Trigonella*
5. *R. lupini*	*Lupinus, Ornithopus*
6. *R. japonicum*	*Glycine max*
7. 'Cowpea type'	*Vigna, Macroptilium* and others

Species 1–4 are fast growers and 5–6 are generally slow growers. The 'cowpea rhizobia' group contains a diverse range of *Rhizobium* which cannot be accommodated in groups 1–6. They include both slow- and fast-growing strains, some of which may even infect non-leguminous angiosperms.

system is to be manipulated and improved. A potential for improvement undoubtedly exists. For example, CO_2 enrichment of the foliar environment causes a significant increase in the nitrogen fixation activity of nodulated soyabeans (*Glycine max*; [1]) which strongly suggests that in ambient conditions the potential of nodules to fix N_2 is limited by photosynthate supply.

Photosynthates supplied to nodules are used for (a) generation of bacteroid reducing power and ATP to supply the nitrogenase system [2], (b) maintenance of normal host-cell cytosol metabolism and (c) a supply of carbon skeletons, ATP and reducing power for the synthesis of nitrogenous compounds which are then exported selectively back to the growing parts of the host plant via the xylem. The precise details of the carbon sources involved and how they are utilized to support these activities in different legume species are unknown.

Previous uncertainties concerning the mechanism of ammonia assimilation in nodules have been largely resolved. However, the exact relationships between the activity of nitrogenase, the production of ammonia and the pathways of carbon metabolism are still unknown. The importance of ureides has only recently been recognized; the pathways and regulation of biosynthesis of these compounds are still uncertain. In particular, concentration of research on the mechanism of nitrogenase action and the expression of *nif* genes has diverted attention from the equally important aspects of carbon metabolism. That work which has been carried out is confused in part by the use of *Rhizobium* cultures *in vitro* for metabolic studies (under conditions in which their metabolism may well differ from that in the bacteroid/host-cell association), and in part by the variations which occur in the metabolism of various strains of *Rhizobium* or host legume species.

The purpose of this review is to highlight those areas in which knowledge is lacking or confused and to suggest that only by a broad treatment of both carbon and nitrogen metabolism in intact nodules will it be possible to fully resolve the mechanism of symbiotic nitrogen accumulation.

THE GENUS *RHIZOBIUM*

The genus *Rhizobium* contains those bacteria which are able to form morphologically distinct nodules on the roots of members of the Leguminosae. It also includes bacteria which no longer possess an invasive characteristic, but have an authentic history of origin from an invasive strain. Rhizobia are Gram-negative, rod-shaped bacteria ($0.5–0.9 \times 1.2–3.0\ \mu m$) which often contain prominent granules of poly-β-hydroxybutyrate. The genus is subdivided into two classes of species; (a) the fast growers which have a mean generation time of 2–4 hr and form relatively large (2–4 mm diameter) colonies on agar media within 3–5 days and (b) slow growers which have a mean generation time of 6–8 hr and form small colonies ($\leq$1 mm diameter) on agar media after 7–10 days. Strain differences exist amongst species and each strain is defined as a culture not known to have a common clonal history with any other culture.

Table 1 lists various *Rhizobium* species and the host genera which they preferentially invade. Within an infected nodule the rhizobia enlarge and change shape to form bacteroids. Depending on the host–strain combination, one or more bacteroids are found enclosed within a membranous envelope located within host cells of the inner region of the mature root nodule. Not all cells of this inner region are invaded and an outer cortex of uninvaded host-cells surrounds the bacteroid region [3].

NITROGEN FIXATION

In general, rhizobia only fix N_2 *in vivo* as bacteroids which contain the induced nitrogenase enzyme. However some slow-growing strains of rhizobia may be cultured which can fix N_2 *in vitro* [4–6a]. An overall reaction involved in the reduction of dinitrogen to ammonia in *Rhizobium* can be summarized as follows, although the nature of the reductant is not firmly established:

$$N_2 + n\,ATP + 6NADPH + 2H^+ \rightarrow 2NH_4^+ + n\,ADP + n\,P_i + 6NADP^+ + 6e^-$$

where $n = 6.0–6.9$ or 6.5 ATP/NH_4^+ depending on whether cell-free or cell mass balance figures, respectively, are used [7].

Biological N_2 fixation (energy requirement 355 kJ/mol NH_4^+) is roughly twice as efficient as the industrial process which has an energy requirement of approximately 680 kJ/mol NH_3. The energy input in both biological and industrial systems is needed to

overcome the activation energy for the reduction of nitrogen [7] plus, in the industrial process, an energy requirement for the production of hydrogen.

The basic requirements for dinitrogen reduction are (a) the enzyme nitrogenase, (b) a strong reducing agent, (c) ATP and Mg^{2+} and (d) low oxygen tension. Only nitrogenase is unique to N_2-fixing organisms and this enzyme has thus attracted considerable attention; for review see [8]. Nitrogenase is a multi-subunit protein. In most organisms studied it has been found to consist of two major components with MW of *ca* 200 000 and 50 000 daltons, respectively. The larger component, molybdoferredoxin, has 4 subunits and contains molybdenum, non-haem iron and sulphide whereas the smaller component, azoferredoxin, has only 2 subunits and contains iron and sulphide [9]. Shortage of molybdenum, or iron, can restrict N_2-fixing capacity [10].

The systems which generate reducing power for N_2 fixation are quite varied and different forms of reductant may be used by different organisms, or even the same organism growing under different conditions [11]. However, although it has been suggested, on the basis of *in vitro* experiments, that in *Rhizobium* the reductant is NADPH [12] the evidence is not conclusive. It has also been suggested that other aerobic nitrogen-fixers (blue-green algae and *Azotobacter*) use NADPH as the reductant for nitrogen fixation [11].

Nitrogenase is very readily inactivated by O_2 [7]. In nodules, a particularly elaborate system has evolved which uses a special form of haemoglobin (leghaemoglobin), synthesized by the host plant [13–15] in order to regulate O_2 concentration at the site of nitrogen fixation. This red pigment, which accounts for the colouration characteristic of active nodules, is probably located inside the membrane surrounding each group of bacteroids [16] and has a high affinity for oxygen [17]. The bacteroids require relatively large amounts of O_2 for the respiratory production of reductant, and leghaemoglobin apparently not only facilitates O_2 diffusion but also maintains low levels of free O_2 within the bacteroid, so that nitrogenase activity remains unimpaired [17–19]. Although some of the ammonium produced by bacteroid nitrogenase may be used by the rhizobia for cell growth and maintenance in active nodules, a large proportion is exported to the host-cell cytosol [20].

CARBON METABOLISM

Ammonia produced by the activity of nitrogenase is incorporated into organic compounds before transportation to the shoot system of the plant. The initial assimilation of ammonia has been studied in detail and is discussed in the next section. Carbon metabolism has two main roles in the nodule. The first is to provide energy and reducing power to both bacteroids and host cell cytosol. Catabolic pathways have been investigated in free-living bacteria, but little is known of the major pathways in the symbiotic state. The second role of carbon metabolism is to provide carbon skeletons for the transport of fixed nitrogen.

Legumes can be divided into two groups on the basis of the major nitrogenous compound which is transported. One group transports mainly asparagine (e.g. *Pisum*, *Vicia* and *Lupinus* [21]), whereas the other group transports mainly the ureides, allantoin and allantoic acid (e.g. *Phaseolus*, *Glycine* and *Vigna* [22–24]). It is to be expected that these two groups will show differences in their major pathways of carbon metabolism since asparagine can be formed from oxaloacetate, a TCA cycle intermediate, whereas allantoin and allantoic acid are products of purine metabolism.

Primary carbon source

The importance of carbohydrate supply in symbiotic activity has long been recognized [25]. The dependence of bacteroid nitrogenase activity on a continued supply of current photosynthate to the nodule has been demonstrated by manipulating entire nodulated plants. When shoots are darkened, defoliated or decapitated, nitrogenase activity in soyabean nodules declines [26, 27]. Other experiments have shown a diurnal change in N_2-fixing activity, which is more rapid during daylight [28]. The difference in N_2-fixation abilities of equivalent masses of attached and detached nodules, as a function of time, is greatest during the photoperiod [29, 30]. Wong and Evans [26] interpret reductions in nitrogen fixation rate in the dark as a feedback inhibition on nitrogenase synthesis by ammonium ions that cannot be assimilated because carbon skeletons are unavailable. On the other hand, Ching *et al.* [2] attribute the decline in nitrogenase activity during darkness to a decline in both the total usable cellular energy in adenine nucleotides (energy state) and reductant concentration in the bacteroids, both of which are essential for maintenance of nitrogenase activity. The relative contributions of repression and/or energy supply to diurnal changes in nitrogenase activity have yet to be fully assessed.

Photosynthate is transported in the phloem as sucrose in many plant species [31]. Some $^{14}CO_2$ feeding studies [32, 33] and analyses of phloem-bleeding sap [34] have shown that sucrose can be translocated from host shoot to nodules in legumes. Indeed, sucrose may be the primary source of carbon for nodules, but further work is required to confirm this. In general, photosynthate is metabolized rapidly by nodules. For example, nodular accumulation of ^{14}C was maximal 90 min after foliar feeding of $^{14}CO_2$ to *Vicia faba* [35]. ^{14}C-Labelled sucrose was the main labelled sugar in the roots but it was not detected in the nodules where glucose and fructose were the main labelled sugars [35]. In contrast, Antoniw and Sprent [36] found sucrose more intensely labelled than glucose in *Phaseolus* nodules. The carbon requirements of nodules from peas (*Pisum sativum* [37]), lupin (*Lupinus albus* [38]) and cowpeas (*Vigna unguiculata* [39, 40]) have been studied in detail. Pea nodules consume 32% of the net photosynthetic carbon fixed in a 9-day vegetative period (between 21 and 30 days after sowing) whilst lupin and cowpea nodules consume 10–13% of the net shoot photosynthetic carbon during a growth period of 78–94 days, commencing two weeks after sowing. In all cases, carbon returned to the shoot as nitrogenous compounds accounted for *ca* 50% of these totals, respiration accounted for *ca* 40% and the remainder was used in nodule maintenance and growth [41].

A positive correlation between the concentration of

sucrose fed to excised nodulated cowpea roots and N_2-fixing activity also indicates that sucrose, or a product of its metabolism, may be the primary carbon source for the nodule [42]. Both sucrose and (+)-pinitol concentrations in soyabean nodules are positively correlated with nitrogenase activity [43], and incubation of nodule slices in glucose, fructose or sucrose stimulates nitrogen fixation[44]. Sucrose does not stimulate nitrogen fixation in isolated soyabean bacteroids [45]. However, the identification of a neutral invertase (EC 3.2.1.26) in the host-cell cytosol of lupin nodules [46] indicates that sucrose may be the primary carbon source for the nodule, whereas absence of invertase activity in the bacteroid [46] confirms its dependence upon the host for a respiratory substrate. The fact that sucrose is hydrolysed by a neutral invertase [46] implies immediate rather than a delayed usage following a period of storage. The latter could be indicated if the conversion was mediated via an acid invertase since this requires diffusion into and out of a vacuole. Both glucose and fructose can enter the bacteroid and host-cell cytosol hexose utilization pathways.

Organic acids (e.g. succinate and malate) prove the most effective substrates for stimulation of respiration of isolated bacteroid suspensions, although they are present in soyabean nodules in smaller concentrations than glucose [47–49]. Glucose can support metabolism in isolated soyabean bacteroids [50]. Kidby [51] explains the poor utilization of glucose by isolated lupin nodule bacteroids on the basis of damage to the sugar transport system in the bacteroids during the isolation procedure. The chloramphenicol-sensitive restoration of glucose utilization, after bacteroids have been incubated at room temperature for a few hours, strongly suggests the induction of a glucose permease [51].

Even though the importance of current photosynthate supply to symbiotic fixation rate and the metabolism of sucrose to hexoses in the nodule has been established, the carbon source for the bacteroids has yet to be fully identified. Investigations with $^{14}CO_2$ foliar feeding to *Phaseolus* [36] indicate that any one of a diverse range of carbon compounds (e.g. sucrose, glucose, fructose, an unidentified carbohydrate, malate for an unidentified organic acid) may be the carbon source for the bacteroids.

Glucose metabolism

Bacteroid and bacterial metabolism. Several enzymes of the Emden–Meyerhof (EM) pathway have been identified in rhizobia [51–56]. However, the almost total absence of a key enzyme, fructose-1,6-diphosphate aldolase (EC 4.1.2.13), precludes the operation of this pathway in most slow-growing species of *Rhizobium* [55]. The slow-growing *R. japonicum* and *R. lupini* have negligible aldolase activity and 'cowpea rhizobia' have very limited activity in culture [55]. The fast growers, *R. meliloti*, *R. trifolii*, *R. leguminosarum* and *R. phaseoli* have slightly greater specific activities of aldolase when cultured and could therefore catabolize glucose by the EM pathway, but at a slower rate than that through the Entner–Doudoroff (ED) pathway.

The activity of the ED pathway, measured by the rate of formation of pyruvate from 6-phosphogluconate, even without correction for metabolism of pyruvate formed during the assay period, is double that of the EM pathway in fast growers [55]. The ED pathway is the only known major oxidative pathway demonstratable in slow growers which apparently have a deficiency of enzymes of both the EM and PP pathways [54, 55]. In fast growers, measured activity of the ED pathway is less than that of the EM pathway in some experiments [55]. However, when the results are corrected for the further metabolism of pyruvate, the ED pathway accounts for the major proportion of the glucose catabolism in both slow- and fast-growing species of *Rhizobium* in culture [53–55].

Using radiorespirometric techniques to monitor release of $^{14}CO_2$ from specifically labelled C atoms in the glucose molecule, a pattern of catabolism consistent with the operation of the ED pathway alone has been shown in cultured *R. japonicum* [53]. Similar experiments on *R. japonicum* cultures with gluconate as a carbon substrate also indicate operation of the ED pathway, accompanied by a C-1 decarboxylation. This decarboxylation pattern does not correspond to operation of the pentose phosphate (PP) pathway, which is unlikely to occur in slow-growing *R. japonicum* since they apparently lack 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44), a key enzyme of the PP pathway [53]. In the *R. japonicum* strain examined it is possible that gluconate is metabolized through the ketogluconate pathway [54] as shown in *Acetobacter* [57]. This is indicated by the presence of 2-ketogluconate and 2,5-diketogluconate, two of the intermediates, and gluconate dehydrogenase (EC 1.1.99.3), the initial enzyme of the pathway [54]. Since the product of this pathway, α-ketoglutarate, can enter the TCA cycle it is possible that the ketogluconate pathway serves an anapleurotic function, i.e. a non-oxidative preservation of carbon atoms. The value of this in the apparently 'static' bacteroids where ribosomes are absent and protein synthesis presumably cannot occur [58] seems to be questionable. Indeed, unless C-4 acids or α-ketoglutarate are transported out of the bacteroid, operation of the ketogluconate pathway could lead to an accumulation of bacteroid TCA cycle intermediates. However, recent work by Stovall and Cole [59] demonstrates that isolated bacteroids have the ability to perform some synthetic reactions, when incubated aerobically, using carbon gained anapleurotically, and thus the ketogluconate pathway combined with CO_2 fixation enzymes may serve some function in bacteroids.

In contrast to the data described above, Mulongoy and Elkan [56] suggest that both the EM and ED pathways operate simultaneously in isolated derivatives of *R. japonicum* strain 3I1b110. Radiorespirometric measurements indicate that two isolates which differ in symbiotic nitrogen-fixing efficiency [60] operate the two pathways at different relative rates. The more efficient isolate, I-110, shows larger rates of operation of the EM pathway whereas the ED pathway predominates in LI-110, the less efficient strain [56].

The occurrence of the pentose phosphate (PP) pathway has been examined in detail in rhizobia [55]. Only fast-growing strains of cultured rhizobia contain extractable amounts of the key enzyme for operation of this pathway, 6-phosphogluconate dehydrogenase. This enzyme has not been detected in slow-growing

strains of *R. japonicum* [53]. The initial enzyme of the PP pathway, glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), has been detected in cultures of both slow- and fast-growing strains, although the specific activity of this enzyme is several times greater in cell-free extracts of the fast-growing strains [55]. The PP pathway is therefore more likely to operate in fast-growing strains and may be the major source of reducing power in the form of NADPH for nitrogenase activity. The presence of transketolase (EC 2.2.1.1) in *R. japonicum* indicates that pentose phosphate, which is essential for nucleic acid synthesis, could be synthesized from fructose-6-phosphate by the transketolase–transaldolase (EC 2.2.1.2) reactions in slow-growing species [53, 54, 56].

Although *R. japonicum* does not possesss a decarboxylating $NADP^+$-dependent 6PGDH, a constitutive, non-decarboxylating, NAD^+-dependent 6PGDH is present in isolated derivates of *R. japonicum* strain 311b110 [56]. This enzyme may represent the initiation point of some hitherto unknown hexose catabolic pathway in *Rhizobium*. The product of the NAD^+–6PGDH reaction has been identified as a phosphorylated keto or diketohexonic compound [56]. Operation of gluconeogenesis is suggested by the presence of hexose diphosphatase (EC 3.1.3.11) and radiorespirometry, although compartmentation of this synthetic pathway from catabolic processes would be necessary [56].

Host-cell cytosol metabolism. No detailed studies of host-cell cytosol metabolism have been reported, though Peterson and Evans [61] assumed that respiration proceeds via a classical EM/TCA cycle pathway. These authors examined the regulatory properties of pyruvate kinase (EC 2.7.1.40) in soyabean nodule cytosol and report a possible inhibition of the enzyme by increasing ammonium concentration at certain phosphoenol pyruvate (PEP) and potassium concentrations. They propose that such an inhibition is likely to reduce competition of pyruvate kinase for PEP and so allow greater production of oxaloacetate, by PEP carboxylase (EC 4.1.1.31), to provide more carbon skeletons for the assimilation of increasing numbers of ammonium ions. However, they admit that because of some ambiguity further studies which involve purer enzyme preparations are desirable. The possible interrelationships between glycolysis and ammonium assimilation in angiosperm tissues have recently been summarized [62].

Metabolism of other sugars

Although sucrose is considered to be the primary carbon source for nodule growth, respiration, maintenance and N_2-fixation, various other sugars occur in nodules of soyabean [43] and cowpeas [63], many in significant quantities. Streeter and Bosler [43] report hexoses and disaccharides which include sucrose, glucose, pinitol and inositol, a hexose probably derived from glucose for carbohydrate storage. Halliday [63] identified a range of sugars, but of particular interest is the large concentration of arabinose recorded during the time of maximum nodule efficiency. This pentose supported the growth of 35 out of 36 strains of 'cowpea rhizobia' in pure culture and was the most successful of the carbon sources tested [64]. Similarly, arabinose and fructose were equally the best substrates for growth of *R. lupini*, *R. japonicum* and fast-growing *Rhizobium* species [64]. Since all these species are likely to possess transketolase–transaldolase enzyme systems, they could utilize arabinose and fructose by established pathways. Conversion of arabinose to fructose and thence to 6-phosphogluconate for metabolism via the ED pathway seems likely.

The fact that approximately 25% of the pentose-utilizing cowpea, soyabean and lupin rhizobia cannot utilize glucose [64, 65] in culture can best be explained by a deficiency or lack of glucose permease or glucokinase (EC 2.7.1.2) and hexokinase (EC 2.7.1.1) activities, both of which are required for formation of glucose-6-phosphate within the bacterium. These are the only two deficiencies which might offer an explanation for the observed results since metabolism of arabinose via the known pathways must culminate in the formation of glucose-6-phosphate for entry into the ED pathway (as for fructose), which eliminates any enzyme deficiency after glucose-6-phosphate in the reaction sequence. However, as Vincent [66] points out, metabolism of pentose by some hitherto unknown direct pathway could explain its relative superiority for growth support of cultured rhizobia. Direct oxidative metabolism of arabinose, in cultured *R. japonicum*, has been reported by Pedrosa and Zancan [67]. Arabinose is metabolized to pyruvate and glycoaldehyde, the latter being a source of glyoxylate which could feed into the partial glyoxylate cycle reported in bacteroids from *R. japonicum* [59].

The formation of arabinose in significant quantities by 'cowpea rhizobia' [63] may not be due to action of the PP pathway since many 'cowpea rhizobia' are slow growing and hence are unlikely to possess an active 6PGDH, the key enzyme of this pathway. These results indicate a need for more detailed examination of carbohydrate metabolism in host-cells and bacteroids in order to determine the significance and metabolism of some of these less common sugars.

The citrate and glyoxylate cycles

When glucose is metabolized by either the EM or the ED pathways the end product is two molecules of pyruvate which are converted to acetyl CoA by pyruvate dehydrogenase (EC 1.2.4.1). The metabolism of acetyl CoA can follow either of two pathways which have several enzymes in common. The first, and most important, is the citrate or tricarboxylic acid (TCA) cycle. Determinations of enzyme activity and respirometric measurements indicate operation of this cycle in cultured rhizobia [53–56], in isolated bacteroids of *R. japonicum* and in several fast-growing species of *Rhizobium* [45, 55, 68, 69]. This pathway may generate reducing power either in the form of NADH or NADPH. The former is generated by most mitochondrial dehydrogenases. Reports of a $NADP^+$-specific isocitrate dehydrogenase (EC 1.1.1.42) in bacteroids of pea nodules [69] and $NADP^+$-specific malate dehydrogenase (EC 1.1.1.82) in roots of *Phaseolus vulgaris* [70] may be of particular significance since NADPH is thought to be the electron donor for nitrogenase [12]. The coincidence of rapid isocitrate dehydrogenase activity in pea nodule bacteroids with the peak of nitrogen fixation in entire

plants is further presumptive evidence that isocitrate dehydrogenase provides NADPH for fixation [69].

The alternative metabolic fate of acetyl CoA is one of carbon conservation by the anapleurotic glyoxylate cycle. It has been suggested that this pathway does not occur in bacteroids of several species of *Rhizobium* (e.g. *R. meliloti* and *R. leguminosarum*) because isocitrate lyase (EC 4.1.3.1) could not be detected [71]. However, isocitrate lyase has been demonstrated in other species of *Rhizobium* in culture where conditions provide increased concentrations of acetyl CoA in the cell, e.g. fat catabolism, induced by addition of oleate to the culture medium [71]. Malate synthetase (EC 4.1.3.2) has been reported in extracts of bacteroids from nodules of several legume species (e.g. *Vigna*, *Lupinus* and *Glycine*) but is not found in others (e.g. *Medicago*, *Trifolium* and *Pisum* [71]). Acetyl CoA could be utilized directly by this route providing glyoxylate is available. A source of glyoxylate is as yet unidentified although various possibilities exist (e.g. glycolate, glycine or oxalate by known metabolic pathways).

Utilization of approximately 50% of total isotopic acetate fed, by a partial glyoxylate cycle, was demonstrated in *R. japonicum* bacteroids by Stovall and Cole [59] although they offered no suggestion as to the source of glyoxylate. Malate synthesized by this route could leave the bacteroid to supply a C-4 intermediate for ammonium assimilation in the host-cell although these authors report incorporation of ^{14}C-label into all cellular components of the bacteroids, which indicates that anapleurotic synthesis of malate may be necessary to replace organic acid pools depleted by other synthetic reactions. This incorporation may, however, be artefactual since the suspensions of bacteroids were incubated aerobically which could lead to an inactivation of nitrogenase, releasing energy for other purposes such as synthesis of cellular components [59].

Wong and Evans [26] observed induction of isocitrate lyase in soyabean bacteroids but only after a significant decrease in the polyhydroxybutyrate content of the bacteroids (which were presumed to be dependent on this carbon source alone) had occurred as the plants aged. By this time, certain irreversible changes (e.g. cellular disorganization and the onset of senescence) may have commenced in the nodule. That this may have occurred is suggested by the lack of restoration of nitrogenase activity when sugar was supplied exogenously to detached nodules in which the bacteroids had shown the same delayed induction of isocitrate lyase as the whole plant [26]. Unfortunately, these workers compared enzyme activities in an *in vitro*, aerobic environment, where nitrogenase may be inactive, with nitrogenase activity measured in intact nodules. Since these are very different biochemical situations, conclusions from these data can only be tentative. Johnson *et al.* [71] detected little or no activity of isocitrate lyase or malate synthetase in the nodule cytosol of several legume species. No other experiments on the operation of the glyoxalate pathway in the host cell cytosol have yet been reported.

Carbon dioxide fixation by legume root nodules

Carbon dioxide fixation by legume roots was reported by Jackson and Coleman [72] who demonstrated the fixation of CO_2 by PEP carboxylase into citrate cycle intermediates in *Phaseolus vulgaris*. Mülder and Van Veen [73] showed that in red clover growth of the host plant and nitrogen fixation are both increased if the CO_2 concentration of the root environment is increased, an observation which could be explained by the activity of PEP carboxylase in the nodules, as in *Vicia faba* where activity is 50 times faster than that in the root [74]. In lupin nodules, both *in vivo* CO_2-fixation rate and *in vitro* PEP carboxylase activity in the host-cell cytosol are correlated with the onset of nitrogen-fixation activity [75]. Christeller *et al.* [75] suggest that the amount of CO_2 fixed by this system would provide sufficient C-4 and other organic acid skeletons for assimilation of the ammonia produced by N_2-fixation in the bacteroids.

Lowe and Evans [76] reported that *Rhizobium* cultures have a growth requirement for CO_2 in the medium and that fixation of CO_2 into PEP and propionyl CoA occurs via carboxylase activity. They did not suggest a source of propionyl CoA, but metabolism of methylmalonyl CoA, the fixation product of propionyl CoA carboxylase (EC 6.4.1.3), via the TCA cycle after conversion to succinate, would ultimately lead to oxaloacetate, which is also the product of PEP carboxylase.

Another nodular CO_2-fixing enzyme is malic enzyme (EC 1.1.1.40) which Lawrie and Wheeler [74] found in *Vicia faba* nodule cytosol, although only at activities equivalent to 10% of PEP carboxylase activity. Since malic enzyme catalyses a readily reversible reaction and requires NADPH for carboxylation, the direction of its activity will be regulated by the reductive state of the host-cell cytosol. Hence, malic enzyme may function preferentially in the reverse reaction, i.e. decarboxylation of malate, and thus generate NADPH in the host cytosol which could be used for example in the synthesis of lipids, which can represent approximately 20% of the fresh weight of nodules in 7-week-old soyabeans [71].

PEP carboxylase requires bicarbonate ions rather that CO_2 [77, 78] suggesting a role for carbonic anhydrase (EC 4.2.1.1 which converts CO_2 to HCO_3^-) which has been reported to occur in nodules from a number of legumes (e.g. *Glycine max*, *Vicia faba*, *Phaseolus* and *Pisum* sp. [79]. This enzyme might also facilitate the excretion of respired CO_2 from the nodule centre to the external environment [79, 80].

Carbohydrate and other carbon storage compounds

The importance of glycogen as a storage carbohydrate in rhizobia was noted by Bergersen [81]. The metabolism and associated regulation, formation and breakdown of this compound have been well documented for mammalian systems [82] but not for rhizobia. Another major and more thoroughly investigated storage compound of bacteroids is poly-β-hydroxybutyric acid (PHB) [83] which can represent as much as 50% of the dry weight of soyabean nodules [84]. This polymer may act as an insoluble carbon store [66] which may be utilized to support dinitrogen fixation during periods of darkness when photosynthate is less readily available [85], although Wong and Evans [26] concluded that PHB is neither likely to act as a source of energy or of carbon skeletons for

maintenance of nitrogenase activity. However, their failure to detect decreases in PHB concentration when nitrogenase activity was reduced by the removal of other carbon sources could be attributed both to small N_2-fixation rates (2 μ mol C_2H_4 g/nodule/hr) [86] and the analytical method used to measure small decreases in a relatively large mass of polymer [87].

Kretovich *et al.* [88] have suggested that PHB is an energy source for dark N_2-fixation in lupin nodules, and they demonstrated an inverse correlation of PHB concentration with energy demands in the nodule. They also suggest that PHB might be a possible source of carbon skeletons for ammonia assimilation. A weak electron-donating system from NADH to nitrogenase (including artificial electron carriers) in which β-hydroxybutyrate served as a NADH-generating system has been demonstrated in bacteroids [84, 89]. All of the enzymes which are required for the conversion of PHB into acetyl CoA have been identified in soyabean bacteroids [26, 84]. Metabolism of acetyl units via the citrate or partial glyoxylate cycles has been discussed previously. Glycolytic intermediates required for CO_2-fixation would be reduced in the absence of photosynthate supply unless another source of sugars was available, and so assimilation of ammonia would deplete both bacteroid and cytosol citrate cycles unless the entire glyoxylate pathway was operational.

Starch is stored in amyloplasts of uninfected interstitial cells of the nodule [85] and in combination with glycogen could well sustain the glycolytic processes and citrate cycles in both bacteroid and cytosol during darkness; a proposal supported by a night time depletion in extractable starch from nodule tissue of peas [37] and cowpeas [63].

AMMONIA METABOLISM

Initial fixation of atmospheric nitrogen

Studies in which $^{15}N_2$ was fed to detached nodules have demonstrated that ammonia is the first stable product of fixation by nitrogenase [90]. The bacteroid fraction of soyabean nodules is without doubt the site of nitrogen fixation [45]. Many of the early experiments carried out on the mechanism of ammonium assimilation were based on a fundamental assumption which has more recently been challenged viz. that ammonia was assimilated in the bacteroid and then excreted into the host-cell cytosol as amino acids. The original theory was supported by demonstrating the presence of both nitrogenase and ammonium assimilatory enzymes in the bacteroid. However, it has now been suggested that ammonia is excreted from the bacteroid and assimilated in the host-cell cytosol [91]. This view is supported by the observations that bacteroids excrete ammonia *in vitro* [92] as do N_2-fixing *R. japonicum* in culture [20]. The two major ammonium assimilatory enzyme systems are those catalysed by (a) glutamate dehydrogenase (GDH; EC 1.4.1.3) and (b) glutamine synthetase–glutamate synthase (GS; EC 6.3.1.2./GOGAT; EC 2.6.1.53; see Fig. 2).

Assimilation of ammonium via glutamate dehydrogenase. Incorporation of ammonia into glutamate has been noted in nodules of soyabean [93] and seradella (*Ornithopus sativus* [90]). Furthermore, Kennedy [90] identified isocitrate and glutamate dehydrogenase activities in seradella nodules, GDH being the assumed assimilatory enzyme at this time. Kennedy *et al.* [94] showed that isocitrate and glutamate dehydrogenase are active in both the host cytosol and the bacteroids of *Ornithopus*, as did Grimes and Fottrell [68] for the host cytosol of *Pisum*, *Trifolium* and *Medicago* nodules. The former authors also noted the presence of intensely labelled glutamate in the nodule following short term labelling experiments with $^{15}N_2$. However, pulse labelling experiments [95] demonstrated that amido groups ($CO\text{-}NH_2$) become labelled with ^{15}N sooner, and more intensely, than amino groups ($CH\text{-}NH_2$). Despite this evidence, Mooney and Fottrell [96] suggested that isocitrate and glutamate dehydrogenase were the enzymes responsible for glutamate synthesis in the bacteroid.

Assimilation of ammonium via the GS/GOGAT pathway. An additional pathway for assimilation of ammonium ions during N_2-fixation, the GS/GOGAT pathway, has now been established [91, 97, 98]. This pathway was shown to occur in cultures of *R. japonicum* by Nagatani *et al.* [99] and was subsequently demonstrated in bacteroids of the same species [100–102]. Dunn and Klucas [101] proposed this to be the main assimilatory pathway and they suggested, from their enzyme activity measurements, that GS/GOGAT could assimilate all the ammonia synthesized by nitrogenase in soyabean bacteroids. However, their figures for nitrogenase activity were obtained from experiments performed at a different time and in a different laboratory [103] and yet the bacteroids in both cases were assumed to be comparable. Kurz *et al.* [104] also measured GDH and GS/GOGAT activities in *R. leguminosarum* bacteroids and concluded that neither of these pathways could account for the ammonium assimilated by pea plants during a 9 week period.

Brown and Dilworth [105] demonstrated the presence of GS and GOGAT in several species of *Rhizobium* when grown in chemostat culture with ammonia or nitrate as the limiting nutrient. However, when these species formed bacteroids, GS was found in every case but GOGAT only in *R. leguminosarum*, *R. japonicum*, *R. lupini* and *R. phaseoli*. Substantial GOGAT activities were detected only in *R. japonicum* and *R. lupini* bacteroids, perhaps notable as they are both slow-growing species, whereas the other species are fast growers. Bacteroid GS activities were generally small and much greater activities were recorded for the host-cell cytosol. GOGAT activity was not detected in the cytosol of any of the nodules investigated and because the activity of bacteroid GS/GOGAT enzymes was insufficient to cope with NH_4^+ fixed via nitrogenase (assayed concurrently) it was concluded that GS/GOGAT did not operate as a major assimilatory pathway in legume root nodules. They suggested that the function of these enzymes in the bacteroid could be to maintain adequate amino acid supplies for bacteroid protein synthesis or that their presence may merely be constitutive.

Brown and Dilworth [105] were unable to detect GOGAT activity in the cytosol of nodules of various legumes (e.g. *Vicia*, *Lupinus* and *Phaseolus*) but Robertson *et al.* [106] detected considerable GOGAT activity in lupin nodule cytosol. The failure of previous

workers to detect GOGAT activity in the host plant cytosol may be explained by inhibition of the enzyme by glutamate and glycine which was overcome in the later experiments [106] when the amino acids were removed by gel filtration. The activities of GS and GOGAT in the cytosol increased in parallel with increases in leghaemoglobin content and nitrogenase activity of nodules, an observation which strongly indicates that GS/GOGAT could be the major assimilatory pathway [106, 107]. Boland *et al.* [98] measured host cytosol enzyme activities and K_m values for GS, GOGAT and GDH in a large range of legume species and also concluded that the major route of ammonia assimilation is via the GS/GOGAT pathway. Excretion of NH_4^+ from the bacteroid is more likely if a diffusion gradient is maintained by the presence of a large excess of GS in the cytosol [105, 106, 108]. Robertson *et al.* [106] estimated GS to be capable of assimilating more than twice the amount of ammonia produced by the bacteroids in 18-day-old lupin nodules.

More recent work using $^{15}N_2$ provides yet further evidence that GS/GOGAT is the primary assimilation route in nitrogen-fixing soyabean nodules [109]. The initial labelled organic product was glutamine, though glutamate became more radioactive than glutamine within one minute. After 15 min, most of the ^{15}N-labelling was found in glutamate and alanine and little glutamine-[^{15}N] was detected. The labelling in alanine is thought to have occurred, via transamination, from glutamate and this is consistent with the time course of labelling. Only a small proportion of ^{15}N-label was present in asparagine though as much as 30% of the total ^{15}N extracted from attached nodules was present in an unidentified compound. Furthermore, this unknown compound (which may be a ureide or a precursor) was only labelled in attached nodules which suggests that a cofactor, or substrate, supply from the host plant is involved [109].

Alternative routes of NH_4^+ *assimilation and further metabolism.* Various other combinations of enzymes have been implicated as assimilatory systems for ammonium ions. For example, bacteroid alanine dehydrogenase (EC 1.4.1.1; [101]) and a combination of alanine aminotransferase (EC 2.6.1.2) and GDH in the host-cell cytosol [102] could contribute to ammonium assimilation in soyabean nodules. Although initial ammonia assimilation is into glutamine or glutamate the nitrogenous compounds exported to the host plant have been identified as asparagine, glutamine, aspartate, homoserine and allantoates in proportions which depend on the host legume species. Asparagine was found in large concentrations in soyabeans and peas [110, 111]. The discovery of aspartate aminotransferase (EC 2.6.1.1) activity in the bacteroid and host cytosol (see Fig. 2) and measurements of enzyme K_m values indicated that aspartate could be synthesized in the bacteroid and transported into the cytosol [112, 113]. Indeed, aspartate aminotransferase activity in the cytosol increases during development of lupin nodules [102, 113]. Asparagine synthesis from aspartate transported into the cytosol could be effected by glutamine-dependent asparagine synthetase (EC 6.3.5.4; see Fig. 2; [112, 113]).

Other pathways for the biosynthesis of aspartate, glutamate and alanine in cultured *R. japonicum* have been identified by Lillich and Elkan [114]. They confirmed that oxaloacetate, either from CO_2-fixation or from the TCA cycle, contributed to aspartate synthesis and that aspartate contributed to alanine synthesis by either direct decarboxylation of C-4 or decarboxylation of oxaloacetate or malate to pyruvate which was then transaminated to alanine. Aspartic acid-[4-^{13}C] was metabolized via the TCA cycle to α-ketoglutarate and glutamate, as was aspartate-[1-^{13}C]. The authors suggest that this could not occur via the TCA cycle since C_1 is lost in decarboxylation of isocitrate to α-ketoglutarate. However, if oxaloacetate is shunted through the TCA cycle in the reverse direction to fumarate, the labelled C atom would equilbrate between the C_1 and the C_4 position. This is supported by a less intense specific labelling of glutamate in the $^{13}C_1$ compared with the $^{13}C_4$ experiment which could well indicate that a proportion of the oxaloacetate is being inverted via fumarate.

Glutamine-dependent asparagine synthesis occurs in a range of germinating legume seedlings [115–119] and asparagine synthetase activity in the nodule cytosol was first reported in lupins [120]. This activity is glutamine and ATP-dependent and the properties of the enzyme are in good agreement with those previously reported in germinating lupins [118]. Ammonia can replace glutamine as the N source but the reaction rate is reduced and the K_m values greatly favour glutamine (K_m 0.26 mM) compared with ammonia (K_m 3–5 mM; see [116, 117]). The asparagine synthetase could only be detected when a sulphydryl-protecting agent (e.g. dithiothreitol) was present during extraction [120]. Furthermore, no ammonium or glutamine-dependent asparagine synthetase activity was detectable in the bacteroid fraction of the nodules or in cell-free extracts of cultured *R. lupini* [120]. Radyukina *et al.* [121] detected greater asparagine synthetase and GS activities in the cytosol than in bacteroids of lupin nodules, whereas they found the reverse was true for GOGAT. Of particular interest is the observation of 'switching on and off' of enzymes involved with asparagine metabolism: asparagine synthetase was detected only after 13 days from inoculation of lupins following cessation of asparaginase (EC 3.5.1.1) activity [120]. This was interpreted as a switch from dependence on nitrogen supplied from the cotyledons as asparagine to nitrogen produced by fixation in the nodule. In contrast, Streeter [122] detected asparaginase activity in soyabean nodules from between 20 and 60 days after initiation. He concluded that this activity may have been derived from the leakage of asparaginase from bacteroids damaged during isolation and separation procedures. However, the differences between these two species are understandable in terms of the major export compounds: lupin nodules export asparagine [22] while soyabean nodules are known to export allantoin and require aspartate for its synthesis. However, Meeks *et al.* [109] were unable to detect breakdown of asparagine-[^{13}C] in their soyabean nodule homogenates during extraction procedures.

Unfortunately, Streeter did not make comparisons between the cytosol and bacteroid enzymes although a K_m of 4.9 μM for asparagine was reported for asparaginase from 'nodules'. This value is similar to the small K_m reported for lupin nodule bacteroids

(6.6 μM) rather than the larger value for cytosolic asparaginase (K_m 7 mM). The smaller K_m of the bacteroid asparaginase is similar to that from several species of bacteria [123, 124].

Clearly, specific activities of enzymes vary even in the same *Rhizobium* species and strain variation has been reported for GDH and GOGAT in *R. japonicum* [125]. When strain differences are combined with different extraction procedures, and reports of enzyme activities from plants nodulated with unspecified strains or commercial inocula, combinations of data from different groups performing similar or interrelated experiments must be interpreted and extrapolated with caution.

Regulation of ammonium assimilation and nitrogenase

Many investigations on the regulation of ammonium assimilation have focussed on the ways in which both bacteroid gene expression and enzyme activity are controlled. Regulation of the assimilatory pathways causes production of ammonia (by nitrogenase) beyond the demands of the bacteroid system and results in excretion of ammonia into the host plant cell.

Control of nitrogenase activity. The first major control in assimilation is the nitrogenase enzyme complex which requires ATP as an energy source [126]. Nitrogenase activity has a linear response to increasing ATP/ADP ratios and has a small energy threshold below which it is inactive [127]. Ching [128] concludes that ATP (produced aerobically) stimulates nitrogenase activity *in vivo* as does an increase in the energy charge. (The term 'energy charge' used here is that of Atkinson [129, 130]. It is defined as the ratio of the concentrations of (ATP + 0.5 ADP/ATP + ADP + AMP) in the presence of adenylate kinase (EC 2.7.4.3) with a presumed equilibrium constant of 0.8. The charge is equal to unity when only ATP is present and is equal to zero when only AMP is present.) These results [128] also suggest participation of adenylate kinase in the production of ATP for maintenance of basal metabolism and nitrogenase activity during times of restricted energy supply (e.g. a shortage of respiratory substrate). As adenylate kinase uses 2 molecules of ADP (an inhibitor of nitrogenase) to produce 1 molecule of ATP, it is more efficient at relieving inhibition of nitrogenase than a system which simply converts ADP into ATP *pro rata*. Organic and inorganic products of nitrogenase activity (i.e. amino acids and NH_4^+) show no inhibitory effect on the nitrogenase enzyme complex *in vitro* [131].

An indirect control of nitrogenase activity may arise from the partitioning of electrons between N_2 reduction and ATP-dependent H_2 production. A consistent feature of many experiments in which hydrogen evolution from nodules is measured is that, as photosynthate supply to the nodule increases, either by manipulation of the environment (e.g. light intensity) or the plant (e.g. depodding), the rate of hydrogen evolution increases relative to the rate of N_2-fixation [132–134]. This may represent an increase in ATP-dependent H_2 evolution, possibly removing excess reducing power, or it may be a manifestation of smaller hydrogenase activity which when active recaptures reducing power lost in H_2 production [135].

Control of nitrogenase biosynthesis. A possible case of regulatory control is that of glutamine synthetase activation of nitrogenase synthesis as in *Klebsiella* [136]. GS activity is controlled by adenylylation of the subunits of the enzyme. The presence of large concentrations of glutamine (which would arise through the assimilation of NH_4^+ into glutamine by GS) activates the adenylylation enzyme system, converting GS to its inactive form, which presumably fails to stimulate nitrogenase synthesis. Thus, the inhibition of nitrogenase synthesis by NH_4^+ in *Klebsiella* is mediated via glutamine increasing the adenylylation of GS. Adenylylated GS has been implicated as a positive control element of the *nif* operon (genes coding for the nitrogenase enzyme), and GS may be directly involved in transcription of *nif* DNA [136, 137]. However, the regulation of *nif* expression in *Klebsiella* is also likely to involve an ammonium-sensitive repressor which has been reported in mutant strains which have *nif* transcription independent of GS activation [137].

Bishop *et al.* [138] demonstrated repression of GS, accompanied by increased adenylylation, in the presence of NH_4^+ in free-living *R. japonicum*, although Scowcroft *et al.* [139] failed to demonstrate this type of control in cultured N_2-fixing 'cowpea rhizobia'. Bergersen and Turner [140] and Ludwig and Signer [141] have reported regulatory effects of GS on nitrogenase activity in 'cowpea rhizobia'. A mutant of 'cowpea rhizobia' strain 32H1, which has no GS activity, also lacks nitrogenase activity [141]. Bergersen and Turner [140] noted that changes in the adenylylation state of GS occur prior to changes in nitrogenase activity in cultured N_2-fixing 'cowpea rhizobia', especially when an imposed state of repression of nitrogenase is progressively alleviated. Repression of GS by ammonia has been reported in cultures of N_2-fixing *Rhizobium* spp. [20, 141, 142], though not in bacteroids. The activity and extent of adenylylation of bacteroid GS was unaltered by ammonium treatment of either bacteroid suspensions or of intact nodulated soyabean plant, even though nitrogenase activity was reduced markedly in the latter case [138].

If NH_4^+ does regulate GS adenylation and hence nitrogenase synthesis in bacteroids, very precise control must exist. Since nitrogenase produces ammonium ions, which may repress GS *in vivo*, the NH_4^+ and active GS concentrations in the bacteroid would have to be closely maintained. Active GS would be necessary for the continued synthesis of nitrogenase and hence ammonia production, but assimilation of the ammonia in the bacteroid would increase the glutamine concentration and hence reduce the concentration of active GS. However, Upchurch and Elkan [143] have described derivatives of *R. japonicum* which can simultaneously derepress nitrogenase whilst repressing synthesis of ammonium assimilatory enzymes.

Control of bacteroid ammonium assimilation. O'Gara and Shanmugam [20] have demonstrated that as much as 94% of $^{15}N_2$ fixed by *R. japonicum* in culture is excreted into the medium as $^{15}NH_4^+$ and they adopt the opinion that rhizobial assimilatory enzymes may be 'switched off' during symbiotic nitrogen fixation. Several assimilatory enzymes are present with small specific activities in bacteroids of various species of *Rhizobium* [20]. Further

biochemical studies indicate that GOGAT activity fluctuates markedly as a function of nitrogen source and stage of growth of cultured *R. japonicum* [142]. Mutants of *R. trifolii* which are able to assimilate NH_4^+ in the absence of organic nitrogen (unlike the parent strain) lack repression of GOGAT by glutamate, a feature noted for the parent strain [142]. It is possible that repression of GOGAT by glutamate may be important in the symbiotic state. Repression of GOGAT by glutamate and cyclic 3',5'-adenosine monophosphate (cAMP) has also been reported in cultured *R. japonicum* [143]. Glutamate repression of GS was also noted for *R. japonicum* in contrast to the findings of Ludwig and Signer [141] for 'cowpea rhizobia'.

Control of cytosol ammonium assimilation. Investigations of the control of cytosolic enzymes have centred on GS and GOGAT. NADH-dependent GOGAT from lupin nodules is inhibited by glutamate (and, less severely so, by oxaloacetate, aspartate and asparagine) competitively with respect to α-ketoglutarate, and by NAD^+ competitively with respect to NADH [144]. Both of these effects are examples of product feed-back inhibition, the most direct and rapid method for controlling enzymatic activity. McParland *et al.* [145] report feed-back inhibition of cytosol GS of soyabean nodules by glutamine and an apparent regulation by energy charge as judged by inhibition by AMP or ADP additions to the purified enzyme in solution.

Studies on the control of asparagine synthetase from lupin cotyledons show inhibition by AMP at a concentration of 2.5 mM and strong inhibition by ATP concentrations greater than 5 mM [119]. The physiological significance of the latter was not discussed but AMP could act as a feed-back inhibitor since ATP is cleaved to AMP and PPi (pyrophosphate) by asparagine synthetase.

Clearly, considerably more research is required on regulation of assimilatory enzymes in both the cytosol and the bacteroids, especially in the former since the assimilatory pathways are located here. Further studies may help to resolve contradictory reports of control mechanisms in different species of *Rhizobium*, both in cultured and bacteroid forms.

Ureides and legume nodules

In spite of evidence that ureides are present in legumes and that they are the major compounds exported from nodules of various species (*Phaseolus* [22], soyabean [23, 146]) their synthesis has long been ignored in studies on ammonium assimilation in nodules where it has long been assumed that N export occurs solely as amino acids and amides [91, 97, 120]. Recent work has demonstrated that allantoin is exported from the root nodules of soyabean [147] and cowpea [24, 40] in appreciable quantities, and in larger amounts than are exported from the roots of nitrate-dependent plants. Allantoin production and export to the shoot and fruits are both reduced when inorganic nitrogen is supplied to the host roots [148], while amino-N concentrations are either not affected or are increased slightly in all tissues [149]. The advantage to the nodule of exporting ureides can be seen by considering the carbon balances [24]. Ureides have a C:N ratio of unity, compared with 2 for asparagine, a commonly exported amide, and hence, less C is required to transport equivalent amounts of N. The allantoin content of soyabean plants is maximal during the early reproductive stage which correlates with the time of maximal N_2-fixation rates [150].

Allantoin synthesis in nodules has been confirmed by feeding $^{15}N_2$ and $^{15}NH_4^+$ to intact nodulated soyabean and cowpea plants [151–153]. Matsumoto *et al.* [151] concluded from ^{15}N-labelling patterns that allantoin was not synthesized by a direct pathway but

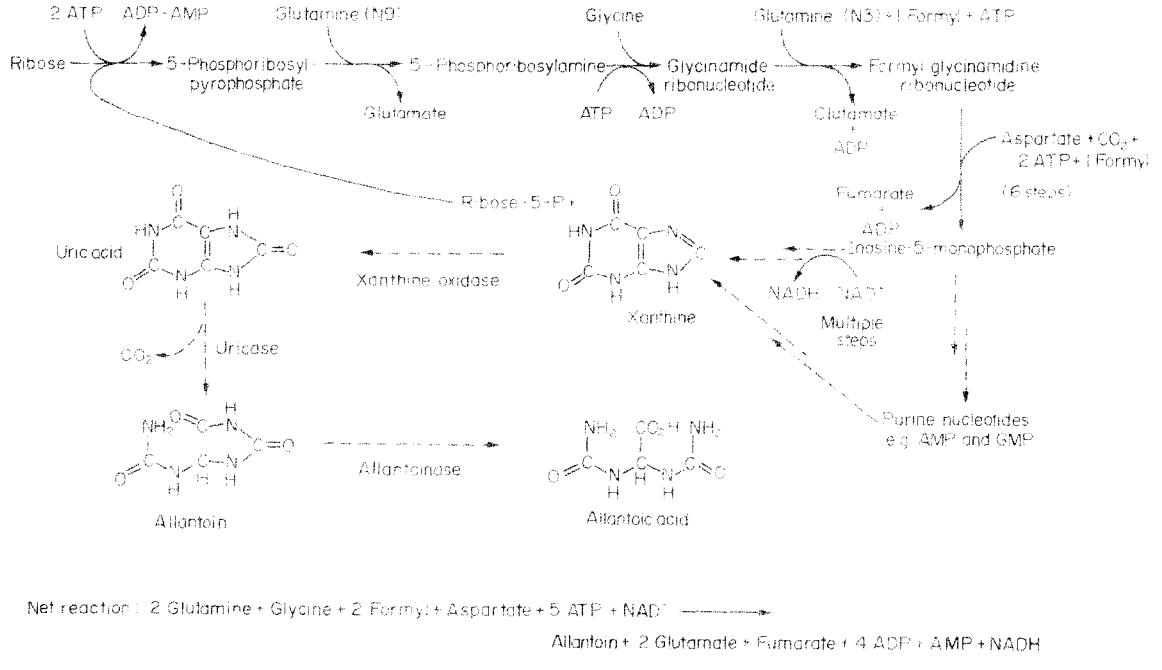

Fig. 1. Purine synthesis and degradation (after Larher [154]).

resulted from degradation of purines ([154]; Fig. 1). The labelling pattern indicated formation of allantoic acid from allantoin in the nodule. Ureide synthesis via purine degradation was also suggested by Fujihara and Yamaguchi [152] who detected small concentrations of glutamine in the nodule despite a very active GS, and therefore suggested that glutamine may be an N-precursor for allantoin. Glutamine contributes amide N to N_3 and N_9 of the purine ring in classical purine nucleotide biosynthesis (Fig. 1). An elegant experiment by Fujihara and Yamaguchi [150] has confirmed that allantoin is formed by purine degradation in nodules and seedlings of soyabeans. They inhibited xanthine oxidase (EC 1.2.3.2; Fig. 1; the critical enzyme in purine degradation) in the nodule by application of a purine analogue and this caused a large accumulation of xanthine in nodules and a reduction of allantoin in the stems of treated plants. These workers also treated soyabean cotyledons with azaserine, an inhibitor of purine synthesis, but could not demonstrate a significant difference in the allantoin and allantoate contents of treated plants. This indicates that purines which are degraded to form allantoates are not currently synthesized but originate from a stored form such as nucleic acids.

Allantoin synthesis has recently been investigated extensively and enzymes concerned with allantoin (and related ureide) synthesis have been found in nodules. Uricase (EC 1.7.3.3; Fig. 1) is far more active in the nodule than the roots and leaves of soyabean seedlings [155]. The nodule uricase was found in the 'mitochondrial fraction' (30 min × 12 000 g pellet from the initial supernatant of homogenized nodules) and did not depend on the presence of a cofactor absolutely required by uricase isolated from roots [156]. The pH optima of the two enzymes also differ, which indicates that they are chemically distinct. Changes in uricase activity during root development were attributed to altered concentrations of a cofactor since the apoprotein was present throughout the period of growth studied [156].

Activities of xanthine oxidase and allantoinase (EC 3.5.2.5; Fig. 1) were maximal in the nuclear and supernatant fractions of the nodule, respectively [155]. The term 'nuclear fraction' is used to describe the pellet obtained after centrifugation at 100 g for 10 min of ground, filtered nodule tissue. The location of the bacteroid fraction is not defined, and it is difficult to draw firm conclusions on enzyme distribution from this work as both 'nuclear' and 'mitochondrial' fractions are likely to be heterogeneous.

The cytosolic allantoinase had a larger specific activity than the other allantoin-producing enzymes in any of the nodule fractions. This implies that all allantoin should be converted to allantoic acid, but no data are yet available which describe the ratio of allantoin to allantoic acid in soyabean root bleeding sap although they are present in approximately equal quantities in stems and petioles of cowpea [24]. The allantoinase has an acidic pH optimum (pH 5.0) and this might indicate compartmentation, as the pH of the cytosol is thought to be neutral or even slightly alkaline, as judged by the pH optima of other cytosolic enzymes. The pH optimum of the allantoinase is different from that reported for mung bean (*Vigna radiata*) seedlings (pH 7.5–8.3 [157]), although both these results may represent the two different states of allantoinase in *Dolichos biflorus* [158]. This species has allantoinase

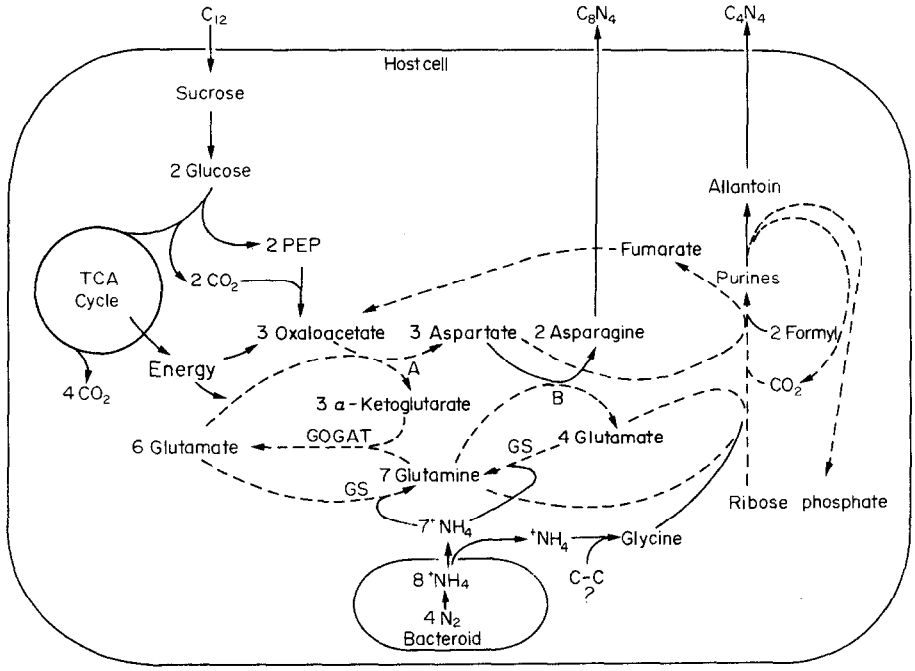

Fig. 2. A schematic representation of nodular symbiotic nitrogen assimilation (after Scott *et al.* [120]). ----, Cyclic pathways; ——, assimilatory pathways. A and B represent aspartate aminotransferase and asparagine synthetase, respectively.

activity with two different pH optima (pH 4.0 and 7.5) depending on whether the disulphide bridges in the enzyme are oxidized or reduced.

Allantoinase activity has been detected in leaves and stems of soyabean seedlings [155] and others have found large concentrations of allantoin-N in roots, stems, and especially, pods of effectively nodulated soyabean plants during the pod-greening period [148, 159]. The concentration of ethanol-soluble allantoin-N markedly decreases during the seed development stage which indicates use of allantoin in seed protein production. However, allantoinase activity has not yet been reported in soyabean pods and seeds although urease (EC 3.5.1.5) is known to be present in various leguminous seeds. The activity of allantoinase in cowpea seeds is maximal when greatest rates of storage protein accumulation are recorded (110 days after sowing) whilst activity in pod wall extracts declines with fruit age [24].

Work on ureide enzyme distribution by Tajima *et al.* [149] has suggested that allantoin and/or allantoic acid is formed in the nodules and then utilized by all other parts of soyabean plants. Uricase activity in soyabean nodules was associated with bacteroids separated by sucrose density gradient centrifugation. Herridge *et al.* [24] have recently found substantial uricase and allantoinase activities in the host-cell cytosol of cowpea, but negligible uricase activity in the bacteroids. These results contrast with those of Tajima *et al.* [149] but they are more logical with respect to purine synthesis, since the amino acid precursors required are also synthesized in the nodule cytosol. The relative amounts of allantoin and allantoic acid in root bleeding sap of nodulated cowpea plants reflect the relative activities of uricase (allantoin producing) and allantoinase (allantoic acid producing) in the nodule cytosol throughout growth [24].

Synthesis of allantoin via xanthine oxidase would generate hydrogen peroxide and this must be removed or damage to cellular components would be likely to occur. The activity of catalase (EC 1.11.1.6) in soyabean bacteroids has been correlated with rhizobial strain effectiveness [160] but the significance of the presence of purine degradation enzymes and catalase in the bacteroids requires further investigation since assimilation of NH_4^+ into the amino acid precursors of ureides occurs in the host-cell cytosol.

A diagrammatic representation of ammonium assimilation including both amino acids and ureides is presented in Fig. 2.

CONCLUSIONS

This review of current literature indicates that considerable gaps exist in the present understanding of carbon metabolism in relation to nitrogen reduction and assimilation in nodules. In part, results are complicated by the number of studies carried out on cultured rhizobia rather than bacteroids in the symbiotic host-cell association. In particular, further information would appear to be needed concerning the following points: (a) the nature of carbon compounds which are transported between host cytosol and the bacteroids, (b) the pathways of carbon assimilation in bacteroids and their interrelationship with carbon metabolism in the nodule cytosol; (c) the fixation of CO_2 by various enzyme-catalysed reactions and their respective locations and implications for assimilation of ammonium and the carbon economy of the nodule; (d) the significance of other anapleurotic pathways in both bacteroid and host cells; (e) the significance of the occurrence of various uncommon sugars in the nodule; (f) the metabolism of various storage compounds, and their energy, reductant and carbon-skeleton producing potential in relation to nitrogenase activity during periods of darkness; (g) the control and interrelationships of bacteroid and host-cell ammonium assimilatory processes; (h) the metabolic pathways linking glutamate and glutamine to the final production of exportable amino acids, amides and ureides, and the control mechanisms which regulate these pathways; (i) the nature and regulation of transport through the bacteroid membrane. Many processes in the nodule may involve movement of compounds from the cytosol to the bacteroid and vice versa. Since the bacteroid and cytosol perform different functions (fixation and assimilation, respectively) and reductant states may well differ, control over the movement of solutes and reducing power must somehow be exercised by the bacteroid cell wall. Research into this area has so far been almost non-existent. Finally (j) the nature of the regulatory mechanisms which reduce nodule activity when the intact plants, nodules or bacteroids are fed with exogenously supplied nitrate or ammonia have yet to be established.

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