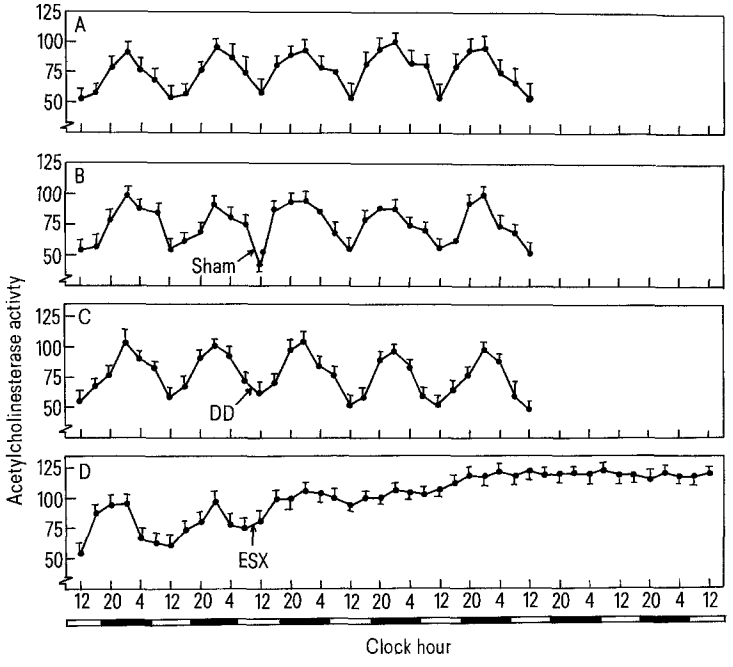

A Changes in AChE activity in the thoracic ganglionic mass of intact crab over a period of 5 days. *B* Changes in AChE activity in the thoracic ganglionic mass of crab before and after sham operation. *C* Changes in AChE activity in the thoracic ganglionic mass of crab before and after keeping it in complete darkness (DD). *D* Changes in AChE activity in the thoracic ganglionic mass of crab before and after bilateral eyestalk ablation (ESX). AChE activity was measured at 4-h time intervals and are expressed as μmoles of ACh hydrolyzed/mg protein/h. Each point is the mean of five individual observations. Vertical bars represent standard deviation. Operations were performed at the indicated times.

investigations of the sinus gland show that the number of vesicles is lower in the axon terminals between 19.00 and 20.00 h. Furthermore sinus gland homogenates, quantitatively analyzed by polyacrylamide-gradient-gel-electrophoresis in microgels, indicate a decline in hormone content at the transition from light to darkness with a minimum at 22.00 h. It is reasonable, therefore, to suggest that the abolition of rhythmic change of AChE activity by eyestalk ablation in the present study could have resulted from a disruption of an endogenous diurnal variation in hormonal and neuronal activity that affects AChE activity.

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Interaction of *Rhizobium loti* strain and host on the biosynthesis of unusual amino acids in leguminous plants

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Summary. The effect of different *Rhizobium loti* strains on the biosynthesis of 2,3-diamino-butanoic acid and 2,4-diamino-3-methyl-butanoic acid in root nodules of *Lotus tenuis, Anthyllis vulneraria* and *Lupinus densiflorus* has been investigated. Results suggest that biosynthesis is *Rhizobium* strain dependent, that the bacteroid is the site of synthesis of the compounds and that their biosynthesis is confined to the symbiosis.
Key words. 2,3-Diamino-butanoic acid; 2,4-diamino-3-methyl-butanoic acid; biosynthesis; *Rhizobium loti;* leguminous plants.

It has been known for some time that leguminous root nodules contain, in addition to the common amino acids, a number of ninhydrin positive compounds of unusual Rf values[1,2] as determined by paper chromatography. The synthesis of these unusual amino acids in *Lotus*[1] and a range of other leguminous species[2] was shown to be *Rhizobium* strain dependent. Recently, two of the unusual amino acids found in *L. tenuis* nodules have been identified as 2,4-diamino-3-methyl-butanoic acid[3] and 2,3-diamino-butanoic acid[4]. The former compound was found in large amounts in *L. tenuis* nodules containing *R. loti* strains NZP2227 or NZP2238 and the latter was abundant in *L. tenuis* inoculated with NZP2213. In order to confirm that the biosynthesis of these two unusual amino acids is *Rhizobium* strain dependent, we have examined the amino acid content of nodules from three different leguminous species inoculated with the different *R. loti* strains.

Materials and methods. Rhizobium strains and growth conditions.

The effect of different *R. loti* strains on the biosynthesis of 2,3-diaminobutanoic and 2,4-diamino-3-methyl-butanoic acids in root nodules of *L. tenuis*, *A. vulneraria* and *L. densiflorus*

	Rhizobium strain NZP2213			NZP2227			NZP2238		
Amino acid (μg/g fr wt)	Lotus tenuis	Anthyllis vulneraria	Lupinus densiflorus	Lotus tenuis	Anthyllis vulneraria	Lupinus densiflorus	Lotus tenuis	Anthyllis vulneraria	Lupinus densiflorus
2,3-DA-B	3.21	2.03	ND	ND	ND	ND	ND	ND	0.12
2,4-DA-3M-B	0.35	0.52	0.34	4.14	3.38	0.56	5.93	2.31	–

2,3-DA-B: 2,3-diamino-butanoic acid. 2,4-DA-3M-B: 2,4-diamino-3-methyl-butanoic acid. ND, Not detected (< 0.1 μg/g fr.wt).

Rhizobium loti[5] strains NZP2213, NZP2227 and NZP2238 (syn. NZP2238/1) were obtained from the Applied Biochemistry Division, DSIR culture collection and were maintained on S10 defined medium[6]. Bacteria were grown at 28 °C in S10 medium and harvested by centrifugation at 6000 g for 5 min. Cells were washed twice in S10 salts solutions by resuspension and centrifugation and then stored at −60 °C until analyzed for amino acids.

Growth of plants. Surface sterilized seeds[6] of *Lotus tenuis* Waldst et Kit, *Lupinus densiflorus* and *Anthyllis vulneraria* were germinated on water agar at 22 °C and then planted in sterile pumice in stainless steel troughs and inoculated with a suspension of the appropriate strain of *R. loti*. The pumice was then covered with a layer of sterile polypropylene beads and after shoot emergence the plants were transferred to the green house where they were grown with a day temperature of 20-28 °C and a night temperature of 14–18 °C. Plants were supplied with a nitrogen free nutrient solution[7]. Nodules were harvested after one month and stored at −60 °C until analyzed for amino acids.

Preparation of bacteroids from Lotus tenuis nodules. Nodules were harvested from 4-week-old plants and crushed at 4 °C in a mortar and pestle with 10 volumes of a homogenizing medium consisting of 25 mM TES (adjusted to pH 7.4 with KOH), 2 mM $MgSO_4 \cdot 7H_2O$, 5 mM dithiothreitol, 0.5 M sucrose and 4% (w/v) polyvinylpyrrolidone (Type NP-K30, Chemical Products NZ Ltd). The homogenate was filtered through two layers of Miracloth and separated into a bacteroid and plant fraction using the differential centrifugation method described by Robertson et al.[7]. Samples of the crude homogenate, plant and bacteroid fractions were then prepared for amino acid analysis.

Isolation and quantitation of amino acids. The following procedure is primarily that of Kaiser et al.[8] with some modification to permit extraction of whole root nodules, nodule fractions, plant roots and bacteria.

Samples (100–500 mg fr.wt) were macerated in the presence of 0.1 mg transexamic acid (internal standard) and then extracted with hot 80% EtOH (3 × 20 ml) for 2 min before filtering. Combined filtrates were taken to dryness, redissolved in 2 ml distilled water and clarified by centrifugation at 4000 rpm for 3 min. The supernatant was hydrolyzed for 12 h at 100 °C in 6 N HCl and then taken to dryness. The residue was redissolved in 5 ml 0.1 N HCl and quantitatively transferred to an Amberlite IR-120[H^+] ion exchange column. After washing with water, the amino acid fraction was displaced from the column with 6 N NH_4OH.

The basic fraction was taken to dryness and derivatized as described by Kaiser et al.[8], giving a mixture of N-trifluoroacetyl n-butyl esters which was analyzed by GC and GC-MS[3,4].

For the quantitation of 2,4-diamino-3-methyl-butanoic acid and 2,3-diamino-butanoic acid both compounds were given a GC molar response factor as found for asparatic acid.

Results and discussion. High levels of 2,3-diamino-butanoic acid were found in nodules of *L. tenuis* and *A. vulneraria* with strain NZP2213 but this amino acid could not be detected (< 0.1 μg/g fr.wt) in nodules from other plant-*R. loti* strain combinations with the exception of a trace in *L. densiflorus* with NZP2238. Synthesis of 2,4-diamino-3-methyl-butanoic acid was found in all nodules except those from *L. densiflorus* containing NZP2238. The levels of this compound synthesized in nodules containing NZP2213 were in general lower than in nodules formed with strains NZP2227 and NZP2238, and this may reflect the fact that 2,3-diamino-butanoic acid is the more abundant unusual amino acid in these nodules. In fact, 2,3-diamino-butanoic acid is one of the most abundant amino acids in hydrolyzed extracts of nodules containing NZP2213 as is 2,4-diamino-3-methyl-butanoic acid in hydrolyzed extracts of nodules formed with strains NZP2227 and NZP2238. Neither amino acid could be detected in cells of the three *R. loti* strains grown in S10 medium, or in root tissue from the three different plants nodulated with NZP2213, demonstrating that the synthesis of these amino acids is confined to the symbiosis.

Furthermore, *L. tenuis* nodules formed with *R. loti* strains NZP2037, NZP2203 and NZP2205 do not contain these amino acids suggesting that their formation is strain dependent. The synthesis of each of these unusual amino acids in legumes from the widely different tribes Loteae (*L. tenuis* and *A. vulneraria*) and Genisteae (*L. densiflorus*) is further evidence that their synthesis is *Rhizobium* strain dependent. However, there is obviously a plant interaction as well, as *L. densiflorus*-NZP2213 nodules did not contain 2,3-diamino-butanoic acid and in *L. densiflorus*-NZP2238 nodules 2,3-diamino-butanoic acid was only just detectable. In addition, the levels of each amino acid differed between plants but this would also be dependent on the time of nodule harvest, the effectiveness of the symbiosis and the rate of metabolism of the compound by the *Rhizobium* strain. All *R. loti* strains were only partially effective on *L. densiflorus*.

In an attempt to localize the site of synthesis of both amino acids *L. tenuis* nodules formed with strain NZP2227 were fractionated into plant and bacteroid fractions[7]. The level of 2,4-diamino-3-methyl-butanoic acid in the plant and bacteroid fraction as a percentage of that in the original crude extract were 25% and 75% respectively. This result would suggest that the bacteroid is the site of synthesis of this compound.

The role of these unusual amino acids in leguminous root nodules is not known at present. One possibility is that these amino acids may serve as *Rhizobium* strain-specific growth compounds providing a chemical environment favorable for growth as has been proposed for opines in crown-gall tumors induced by *Agrobacterium tumefaciens*[9].

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