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THE AMINO ACIDS OF NON-LEGUME ROOT NODULES

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Abstract—The free amino acids in the nitrogen-fixing root nodules of nine further species (in seven genera) of non-legumes have been determined. In the species of *Myrica* now studied and also in *Hippophaë, Elaeagnus, Ceanothus* and *Casuarina*, the pattern of amino acids resembled that previously reported in the nodules of *Myrica gale*, asparagine being the predominant amino acid in terms of nitrogen content, with substantial amounts of glutamine often present also. In *Alnus inokumai*, citrulline was prominent, as previously shown for the nodules of *A. glutinosa*, though now amides were also present in substantial quantities. In nodules of *Coriaria myrtifolia* glutamic acid, glutamine and arginine were the chief amino acids.

THE IDENTITY and relative abundance of the free amino acids in nitrogen-fixing root nodules are of interest in connexion with the chemical mechanism for the acceptance and conveyance to the rest of the plant of the ammonia which may be presumed to be produced in the fixation. In respect of non-legume root nodules, previous quantitative and semi-quantitative studies of amino acid content appear to have been confined to *Alnus glutinosa*[1-3] and *Myrica gale*.[4] These indicate that in the nodules of the former species citrulline is the dominant amino acid, with substantial amounts of glutamic acid, γ-aminoisobutyric acid and arginine also present. In *M. gale* nodules asparagine replaces citrulline as the major amino acid and is accompanied by substantial amounts of aspartic acid, glutamic acid and glutamine.

It was of interest to determine whether these respective patterns of amino acids are also shown in the nodules of other species of the above genera and to learn the situation in the nodules of other non-legume genera quite unrelated to *Alnus* or *Myrica*. Accordingly the nodules of the species listed in Table 1 have now been examined, those of *A. glutinosa* and *M. gale* being included to provide fully comparable data. All these species eventually attain shrub or tree size, but in the present study the nodule material was taken from younger plants in culture in a greenhouse at this Department, the plants of *A. inokumai, M. gale* and *Hippophaë* being in their first year of growth, those of *Ceanothus* and *Coriaria* in their third year and the remaining species in their second year. All plants were growing in rooting media (solution culture or Peralite) free of combined nitrogen and were associated with their normal nodule endophytes except that *A. inokumai* had been inoculated from *A. glutinosa* nodules. The plants of all species were growing satisfactorily, indicating that nitrogen fixation was proceeding normally in the nodules over the general period of use. Nodules for analysis were collected during the period 13.00 to 16.00 hr B.S.T. on fine days during the summers of 1967 and 1968. One collection was made for each species.

[1] J. K. MIETTINEN and A. I. VIRTANEN, *Physiol. Plantarum* **5**, 540 (1952).
[2] G. LEAF, I. C. GARDNER and G. BOND, *J. Expt. Botany* **9**, 320 (1958).
[3] K. B. ASEEVA, Z. G. EVSTIGNEEVA and V. L. KRETOVICH, *Dokl. Akad. Nauk SSR* **169**, 463 (1966).
[4] G. LEAF, I. C. GARDNER and G. BOND, *Biochem. J.* **72**, 662 (1959).

TABLE 1. FREE AMINO ACIDS IN NON-LEGUME NODULES IN TERMS OF μmole NITROGEN per g fresh wt.

Species	Aspartic acid	Glutamic acid	Citrulline	Asparagine	Glutamine	Other amino acids*	Total amino acid N
Alnus glutinosa (L.) Gaertn.	0·25	0·78	1·25	Not detected	0·30	0·60	3·18
A. inokumai Murai and Kusaka	0·08	0·96	0·89	0·99	1·02	0·66	3·60
Myrica gale L.	0·07	0·38	Not detected	1·81	0·59	0·53	3·38
M. cerifera L.	1·07	1·30	Not detected	7·60	1·75	1·29	13·01
M. pilulifera Rendle	0·67	0·71	Not detected	1·18	0·07	0·43	3·06
M. cordifolia L.	0·31	1·11	Not detected	13·04	2·25	2·19	18·90
Hippophaë rhamnoides L.	0·81	1·50	Not detected	7·04	0·36	1·70	11·41
Elaeagnus angustifolia L.	0·67	0·48	Not detected	3·22	0·60	0·68	5·65
Ceanothus velutinus Dougl. var. *laevigatus* Torr. and Gray	0·46	0·47	Not detected	21·10	0·84	2·56	25·43
Casuarina cunninghamiana Miq.	1·20	0·81	Not detected	27·08	6·45	0·92	36·46
Coriaria myrtifolia L.	0·20	1·06	Not detected	0·18	1·10	2·08	4·52

* Comprising, where detected, threonine, serine, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, γ-aminoisobutyric acid, ornithine, lysine, histidine and arginine.

Preliminary inspection of the analytical results showed that aspartic acid, glutamic acid, citrulline, asparagine and glutamine were, in terms of nitrogen content, the predominant amino acids and in Table 1 separate data are given for these constituents only. Except in *Coriaria*, these amino acids account for 80 per cent or more of the TAAN (total amino acid nitrogen), although, of them, citrulline was found only in the *Alnus* species, while one of the latter lacked asparagine. In *Coriaria* the acids named in the table accounted for only 55 per cent of the TAAN; among the "other" amino acids in this species arginine was most prominent, contributing 32 per cent of the TAAN. The presence of citrulline in the xylem sap of the stem of *Coriaria ruscifolia* has been reported;[5] that it also occurs in plants of *C. myrtifolia* is made somewhat doubtful by the present authors' failure to find it in the nodules.

Of the other amino acids analysed, γ-aminoisobutyric acid was generally the most prominent, contributing between 0·05 μmole nitrogen (in *Coriaria*) and 0·57 μmole (in *Hippophaë*). Arginine was a prominent constituent of some nodules, particularly (as noted above) those of *Coriaria* (1·46 μmoles), *Ceanothus* (1·6 μmoles) and *M. cordifolia* (1·0 μmole), all in terms of nitrogen. Proline was not detected in the *Alnus* or *Myrica* species, with the exception of *M. cordifolia*, while ornithine was generally present in trace amounts only and was not detected at all in *M. cerifera*, *Ceanothus* and *Coriaria*.

It is clear that apart from the *Alnus* species and *Coriaria* the predominant amino acid in terms of nitrogen content in the nodules of the species examined is asparagine, with substantial amounts of glutamine present also. These two substances together accounted for 92 per cent of the TAAN in *Casuarina* nodules, 86 per cent and 81 per cent respectively in *Ceanothus* and *M. cordifolia*, and somewhat smaller but still substantial proportions in the remaining species, except for a low value of 41 per cent in *M. pilulifera*. In *Coriaria* the corresponding value was only 28 per cent. It is notable that in *A. inokumai* citrulline was not so dominant as

[5] E. G. BOLLARD, *Australian J. Biol. Sci.* **10**, 292 (1957).

in *A. glutinosa*, since both the above amides were present and together accounted for more than twice as much nitrogen as did the citrulline.

It will be seen in Table 1 that the absolute amounts of these major constituents and also of the TAAN vary considerably between species. While there may be true specific differences in these respects, it is more certain that the differences are to a considerable extent fortuitous and are due to environmental conditions being less favourable to fixation for some species than for others. Thus, for reasons connected with other uses that were being made of them, the plants of *A. glutinosa* and *M. gale* were rooted in Peralite and experience has shown that in these particular species such plants fix nitrogen more slowly than those growing in solution culture. Further, in all species weather conditions on the days preceding nodule collection are likely to have affected the level of amino acids found. Also there is a marked diurnal fluctuation in fixation in these plants[6] and in some species the time of day of maximum fixation may not have coincided with the time of nodule collection.

Thus no new pattern of nodule amino acids has been detected in the plants examined. There are still just the two patterns established by previous work, namely those shown by the nodules of *A. glutinosa* and of *M. gale* respectively and it is the latter pattern which reappears in most of the plants now examined, despite the complete lack of taxonomic affinity between *Ceanothus*, *Hippophaë* or *Elaeagnus* on the one hand and *Myrica* on the other. *Coriaria*, where the most prominent acids are glutamic, glutamine and arginine, possibly has a distinct pattern and merits further study. It may be noted that the free amino acid composition of many legume nodules somewhat resembles that of *M. gale* nodules, with the exception that glutamine is often not detectable.[7]

The relative abundance of the amino acids named in Table 1 suggests that they must be the main repositories of the ammonia formed in fixation and the consideration that most of them can gain part or all of their nitrogen directly from ammonia is consistent with this belief. Previous work[2,4] showed, in fact, that after nodules of *A. glutinosa* had been exposed to gaseous nitrogen enriched with ^{15}N, the most highly-labelled constituents of the nodules were citrulline, glutamic and aspartic acids, while in similar tests with *M. gale* nodules the corresponding substances were asparagine, glutamine and glutamic acid. It is probably in the form of these same constituents that the transport of fixed nitrogen occurs from the nodules to other parts of the plant, an aspect which is now being studied.

EXPERIMENTAL

Nodulation in the species studied had been induced by the application to the roots of the endophytes in the form of crushed-nodule suspensions or habitat-soil suspensions, or by germinating the seed in habitat soil.

In most cases nodules were drawn from at least five plants of each species and the bulked nodules (fresh wt. usually 6–12 g) were washed, then frozen in a dry ice–alcohol mixture and stored at −20° until required for analysis. For the analyses a sample of 5 g fresh wt. drawn from the bulked nodules of each species was ground up and repeatedly extracted with hot 80% ethanol, followed by two extractions with distilled water. The extracts were combined and left to stand overnight at 2°, after which they were centrifuged to remove the slight precipitate which tended to form. The extracts were then cleared for analysis of free amino acids by the method of Plaisted.[8]

Quantitative estimations of free amino acids in the extracts were performed with a Beckman "Unichrom" amino acid analyser by the procedure recommended in the makers' instruction manual. Amides were estimated by hydrolysing a portion of the extract in 1 N HCl in a sealed, evacuated glass tube maintained at 110° for 3 hr and determining the additional aspartic and glutamic acid formed. Extracts were also chromatographed after hydrolysis *in vacuo* in 6 N HCl for 24 hr at 110° to ensure that none of the amino acid peaks identified

[6] C. T. Wheeler, *New Phytol.* **68**, 675 (1969).
[7] G. W. Butler and N. O. Bathurst, *Australian J. Biol. Sci.* **11**, 529 (1958).
[8] P. H. Plaisted, *Contrib. Boyce Thompson Inst.* **19**, 231 (1958).

was due to peptide material. Finally the composition of the extract suggested by the elution patterns obtained on the analyser was checked by qualitative examinations using TLC.[9,10]

Reproducibility of the Beckman analyser was within ±5% for each of the amino acids analysed. Suitable tests indicated that the loss of any particular amino acid in the clearing process indicated above did not exceed 13 per cent. Small amounts of other ninhydrin-positive compounds were found in the nodules of some species, but these were not studied further because of their very small contribution to the TAAN.

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[9] N. A. TURNER and R. J. REDGWELL, *J. Chromatog.* **21**, 129 (1966).
[10] H. H. WHITE, *Clin. Chim. Acta* **21**, 297 (1968).