FREE AND BOUND AMINO ACIDS IN LEGUME ROOT NODULES: BOUND γ-AMINOBUTYRIC ACID IN THE GENUS *TRIFOLIUM*

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Summary

"Free" and "bound" amino acids in root nodules from 10 legume species have been studied. In general, the free amino acids comprise a small proportion (25 per cent. or less) of the total nitrogenous compounds soluble in 80 per cent. (v/v) ethanol.

Bound amino acids were present in substantial amounts in the nodules from all 10 legumes.

In particular, "bound γ-aminobutyric acid" was present in large amounts in nodules of *Trifolium repens* and in smaller amounts in *T. pratense* and *T. medium*.

In *T. repens*, bound γ-aminobutyric acid was present only in effective nodules, being absent from leaf, stem, and root tissues, from ineffective nodules, and from *Rhizobia* in liquid culture.

Some properties of bound γ-aminobutyric acid are described.

I. Introduction

Steward and Thompson (1950) have suggested that the composition of the non-protein-nitrogen fraction of nodules should be further elucidated. This paper deals with the amino acids which can be found in "free" and "bound" form in protein-free extracts from legume nodules. In the particular case of nodules from white clover (*Trifolium repens*), the presence of considerable amounts of bound γ-aminobutyric acid is reported. A preliminary report of part of this work has been given elsewhere (Butler and Bathurst 1957).

II. Methods

(*a*) *Sampling and Extraction*

Roots, together with nodules, were removed from plants growing freely in the field in association with other species and immediately freeze-dried, after which the nodules, consisting of specimens of all ages and sizes, were dissected out and cleaned by sieving followed by dusting with a camel-hair brush. Solubility in 80 per cent. (v/v) ethanol was used as the criterion for the presence of nitrogenous substances of low molecular weight. Extracts were made by homogenizing nodule tissue (100 mg dry weight) with 25 ml 80 per cent. ethanol at room temperature, followed by centrifugation for 5 min at 2000 *g*. The supernatant was concentrated to dryness by means of a rotary vacuum drier, taken up in 1 ml water, and clarified by centrifugation at 5000 *g*.

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TABLE 1

FREE AND BOUND AMINO ACIDS IN ETHANOL EXTRACTS OF NODULES OF LEGUMINOUS PLANTS

Amino acids (expressed as mg amino nitrogen per 100 g dry wt.) in ethanol-soluble fraction before and after hydrolysis with 6N HCl for 16 hr

Amino Acid	*T. repens*		*T. pratense*		*T. medium*		*Lotus uliginosus*		*Lotus corniculatus*		*Pisum sativum*		*Cytisus scoparius*		*Lupinis angustifolium*		*Galega officinalis*		*Medicago lupulina*	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Aspartic acid	6	101	4	136	16	108	Trace	42	Trace	25	7	56	32	236	39	149	5	175	7	165
Asparagine	72	—	104	—	82	—	12	—	Trace	—	55	—	116	—	14	—	158	—	173	—
Glutamic acid	4	37	12	22	28	28	17	29	6	13	6	18	8	68	21	143	32	46	4	25
Glutamine	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—	—		—	—
Glycine	Trace	Trace	Trace	3	Trace	Trace	Trace	Trace	—	Trace	Trace	20	Trace	28	Trace	Trace	—	Trace	Trace	Trace
Alanine	13	31	24	25	Trace	Trace	Trace	18	Trace	Trace	2	30	4	68	28	38	6	11	5	Trace
Valine	Trace	Trace	Trace	9	—	—	Trace	14	Trace	Trace	1	6	—	Trace	Trace	12	Trace	11	Trace	Trace
Isoleucine	Trace	Trace	Trace	18	—	—	Trace	Trace	Trace	Trace	2	5	—	Trace	Trace	15	Trace	19	Trace	Trace
Serine	6	6	4	11	Trace	Trace	Trace	21	Trace	13	2	6	3	8	*	11	3	7	Trace	76
Threonine	Trace	Trace	4	8	—	—	—	—	—	—	—	—	—	18	—	12	4	13	—	Trace
γ-Aminobutyric acid	2	1006	12	120	—	104	—	29	—	Trace	12	18	8	12	11	12	13	6	Trace	Trace
Others	Trace	—	16	28	—	—	Trace	15	Trace	35	32	53	70	100	46	69	108	127	20	45
Total amino nitrogen	103	1181	180	380	126	240	29	167	6	86	119	212	241	538	162	461	329	415	209	311
Total ethanol-soluble nitrogen (mg % dry wt.)	1390		736		740		358		358		619		1840		1075		1350		989	

* Included with glutamic acid.

(*b*) *Chemical Determinations*

(i) *Total Nitrogen.*—This was determined by the micro-Kjeldahl procedure. The extracts were first spot-tested with a 0·1 per cent. solution of diphenylamine in conc. H_2SO_4 for the presence of nitrate. Where nitrate was present, a preliminary reduction step, using 20 mg reduced iron in 3 ml 30 per cent. H_2SO_4, was carried out prior to the micro-Kjeldahl digestion.

(ii) *Free Amino Acids.*—These were determined by two-dimensional paper chromatography. For each extract, suitable aliquots were applied to Whatman No. 1 papers and chromatograms run at 20°C, using butanol–acetic acid–water (25 : 6 : 25 by volume) in one direction followed by water-saturated phenol in the other. The papers were dried in a current of slightly warmed air and sprayed with a solution of 1 per cent. ninhydrin in 95 per cent. (v/v) ethanol containing 1 per cent. collidine. They were placed in the dark for 24–48 hr at 40 per cent. relative humidity and 20°C for full development of the colours. The intensities of the coloured spots were measured photometrically according to Wellington's modification (1952, 1953) of the method of Thompson, Zacharias, and Steward (1951) and Thompson and Steward (1951). Standard curves were prepared for each amino acid so that the amount of each could be read off. For unknown amino acids a curve giving average amino nitrogen values was used.

(iii) *Bound Amino Acids.*—To determine the amino acids released by hydrolysis of ethanol extracts, suitable aliquots were refluxed in 6N HCl for 16 hr. Excess HCl was removed by evaporating the extract down to dryness two to three times from distilled water, the pH being finally brought to 6–7. The residue was taken up in 80 per cent. ethanol, the ethanol removed *in vacuo*, and the aqueous solution finally made to the same volume as the original aliquot. Amino acids were then estimated by paper chromatography, as above.

III. Results

(*a*) *Free and Bound Amino Acid Content of Nodules*

In Table 1 are presented the results of analyses of the ethanol-soluble fraction of nodules from 10 legumes for free amino acids and for amino acids present after hydrolysis with 6N HCl for 18 hr. The data for each plant refer to a single harvesting of nodules. Since the harvests were made from plants growing under a variety of field conditions at differing times of the year, detailed quantitative comparisons of the data for different plants would be invalid. The following conclusions can, however, be drawn:

(1) The free amino acids, in general, comprised a small proportion (up to 25 per cent.) of the total ethanol-soluble nitrogen. Asparagine was present in comparatively large amounts in the *Trifolium* species examined, in *Medicago lupulina*, and in *Galega officinalis*. In the latter species a particularly high proportion of the ninhydrin-positive nitrogen was unidentified; it was mainly present in two spots whose R_F values in phenol–water and butanol–acetic acid–water respectively were 0·18, 0 and 0·33, 0.

(2) In all species, the occurrence of bound amino acids in the ethanol-soluble fraction could be demonstrated by hydrolysis with 6N HCl. In most cases there was still a considerable gap between the sum of free and bound amino acids and the total ethanol-soluble nitrogen. This is partly accounted for by ammonia nitrogen liberated from asparagine by hydrolysis. In white clover nodules, however, γ-aminobutyric acid was formed in large quantities on acid hydrolysis and accounted for the major portion (about 70 per cent.), of the ethanol-soluble nitrogen. Bound γ-aminobutyric acid was also present in comparatively large amounts in *T. pratense* and *T. medium*; it was not present in significant amounts in the nodules of other species examined.

Of the unidentified ninhydrin spots on chromatograms of acid hydrolysates, most were probably basic amino acids.

(*b*) *Bound γ-Aminobutyric Acid in Clover Nodules*

(i) *Location of Bound γ-Aminobutyric Acid on Paper Chromatograms.*—When two-dimensional chromatograms were prepared, two to three small spots, which gave a very weak reaction with ninhydrin, were observed at $R_F = 0$–$0 \cdot 05$ in butanol–acetic acid–water and $R_F = 0 \cdot 80$–$1 \cdot 00$ in phenol–water. One-dimensional chromatograms of a white clover nodule extract applied to 1-in. bands were therefore run and sectors were eluted and analysed for total nitrogen. Blank spaces on each chromatogram were also eluted to give paper blanks for each sector. The results of such an experiment are shown in Table 2

The recovery of the applied nitrogen from sectors of the phenol chromatograms totalled 93 per cent., of which 73 per cent. was present at $R_F = 0 \cdot 70$–$0 \cdot 90$. Recoveries of only 60 per cent. could be obtained from the butanol–acetic acid chromatograms, indicating incomplete elution of the bound γ-aminobutyric acid after chromatography, possibly due to chemical modification during running in the acidic solvent. Of the 60 per cent. of nitrogen recovered, two-thirds was present at $R_F = 0$–$0 \cdot 10$.

In order to obtain bound γ-aminobutyric acid relatively free from amino acids, 1 g dry weight white clover nodules was extracted by the method described above and made to a final volume of 5 ml. The extract was applied in 16-in. bands to four sheets of Whatman No. 1 paper and run in phenol–water. There was good separation with no overloading. Three fluorescent bands were observed under ultraviolet light and strips were cut from each paper comprising the zone $R_F = 0 \cdot 80$–$0 \cdot 90$, this being just below the lowest fluorescent band. The strips were extracted with three changes of water at room temperature, each extraction taking 10 min. The combined extract was reduced in volume by freeze-drying, and the preparation used in the tests detailed below.

(ii) *Reaction of Bound γ-Aminobutyric Acid to Spray Reagents.*—The following spray reagents and tests were used on two-dimensional chromatograms in phenol–water and butanol–acetic acid–water and on one-dimensional chromatograms in phenol–water:

Ninhydrin.—Different preparations of bound γ-aminobutyric acid gave a negative reaction to ninhydrin after running in phenol–water. When run in butanol–acetic acid followed by phenol–water, some preparations gave a faint reaction to ninhydrin, the spot tailing from $R_F = 0{\cdot}90$–$0{\cdot}70$ in phenol–water and $R_F = 0{\cdot}05$ in butanol–water.

Tests for Guanido Derivatives.—The extended ninhydrin (Steward, Zacharias, and Pollard 1955) and Sakaguchi (Roche, Thoai, and Hatt 1954) tests were both negative.

Ureide Test.—On spraying with dimethylaminobenzaldehyde reagent (Fink *et al.* 1956), a faint yellow spot was observed at $R_F = 0{\cdot}75$ in phenol–water and $R_F = 0{\cdot}57$ in butanol–acetic acid–water. This was ascribed to urea, which was present to the extent of 13·2 mg per cent. nitrogen in the nodules, as assayed by the method of Conway (1957).

TABLE 2

DISTRIBUTION OF THE 80 PER CENT. ETHANOL-SOLUBLE NITROGEN FRACTION OF WHITE CLOVER NODULES ON ONE-DIMENSIONAL PAPER CHROMATOGRAMS

Results expressed as a percentage of the nitrogen applied

R_F	Solvent Systems	
	Phenol–Water (saturated)	Butanol–Acetic Acid–Water (25 : 6 : 25 v/v)
0 –0·10	0	46·2
0·10–0·20	1·5	8·4
0·20–0·30	1·5	1·5
0·30–0·40	4·3	1·2
0·40–0·50	7·0	0
0·50–0·60	0	0
0·60–0·70	3·0	0
0·70–0·80	33·4	1·0
0·80–0·90	39·2	0
0·90–1·00	2·9	2·0
Nitrogen recovered (%)	92·8	60·3

(iii) *Chromatographic Identification of γ-Aminobutyric Acid.*—Suitable aliquots of bound γ-aminobutyric acid were hydrolysed with 6N HCl for 18 hr as described earlier. Superposition tests of equivalent amounts of authentic γ-aminobutyric acid and the test material on one- and two-dimensional chromatograms in butanol–acetic acid–water and phenol–water indicated their chromatographic identity. The test material did not complex with copper carbonate impregnated on the paper chromatograms in the path of the compounds during the first solvent movement, whereas α-amino acids characteristically do so (Crumpler and Dent 1949). Also tests with α-alanine, β-alanine, ethanolamine, and α-aminobutyric acid showed that these compounds moved to positions clearly distinct from the test material.

β-aminobutyric and β-amino*iso*butyric acids showed almost the same R_F values as the test material, but reacted more slowly with ninhydrin. The colours of spots developed from the test material and γ-aminobutyric acid were identical using isatin and alloxan spray reagents (Seifer and Oreskes 1956). On paper chromatographic evidence the test material could therefore be identified as γ-aminobutyric acid.

(iv) *Efficiency of Extraction of Bound γ-Aminobutyric Acid.*—In Table 3, the amounts of free and bound amino acids extracted from freeze-dried white clover nodules by 80 per cent. ethanol and by distilled water respectively at room temperature are presented. Nodules (0·2 g dry weight) were extracted with 25 ml solvent in each case.

TABLE 3

FREE AND BOUND AMINO ACIDS EXTRACTED BY 80 PER CENT. ETHANOL AND WATER RESPECTIVELY FROM WHITE CLOVER NODULES

Results expressed as mg per cent. nitrogen

Amino Acid	Ethanol		Water	
	Unhydrolysed	Hydrolysed	Unhydrolysed	Hydrolysed
Aspartic acid	6	70	14	172
Asparagine	99	—	105	—
Glutamic acid	8	18	24	122
Glycine	—	43	—	215
Alanine	9	11	19	74
Valine	Trace	Trace	7	96
Isoleucine	Trace	Trace	8	59
Serine	4	11	—	67
Threonine	Trace	6	20	58
γ-Aminobutyric acid	20	337	23	1130
Unidentified	26	42	159	153
Totals	172	538	379	2146
Total soluble nitrogen		787		3062

Of the free amino acids, most were extracted by 80 per cent. ethanol; in the water extract a large ninhydrin-positive spot at $R_F = 0{\cdot}01$ in butanol–acetic acid and 0·45 in phenol–water accounted for two-thirds of the unidentified ninhydrin-positive nitrogen. After hydrolysis, γ-aminobutyric acid was predominant in both extracts, more being present in the water extract than in the ethanol. Bound amino acids generally were present at higher levels in the water extract. When extractions of the same sample of freeze-dried nodules were made on separate occasions, it was found that bound γ-aminobutyric acid was extracted in varying amounts by 80 per cent. ethanol at room temperature, showing that it was incompletely soluble in 80 per cent. ethanol. Other bound amino acids were much less soluble in 80 per cent. ethanol.

(v) *Stability of Bound γ-Aminobutyric Acid in Acid.*—The stability to acid hydrolysis was investigated by refluxing aliquots of bound γ-aminobutyric acid extract with:

(1) 0·1M borate buffer at pH 6·5 for 2 hr.

(2) N HCl for 2 hr.

(3) 6N HCl for 16 and 24 hr.

Two-dimensional paper chromatograms were run using the hydrolysates. Negligible amounts of γ-aminobutyric acid were liberated at pH 6·5 and 100°C for 2 hr. 10–16 per cent. of the bound γ-aminobutyric acid was hydrolysed to γ-aminobutyric acid by N HCl at 100°C for 2 hr. Complete hydrolysis was achieved in 6N HCl at 100°C for 16 and 24 hr, as assessed by the amount of γ-aminobutyric acid formed.

TABLE 4

DISTRIBUTION OF BOUND γ-AMINOBUTYRIC ACID IN TISSUES OF WHITE CLOVER PLANTS AND IN RHIZOBIA

Unless otherwise stated, data are expressed as mg nitrogen per 100 g dry wt.

Tissue	γ-Aminobutyric Acid		Ninhydrin-positive Nitrogen	Total Soluble Nitrogen
	Free	Bound		
Effective nodules	1·6	1004	1227	1390
Ineffective nodules	1·8	6·9	159	—
Rhizobia in culture	0·1*	0·0	9·0*	—
Roots	3·0	0·0	41	123
Stems and petioles	2·2	0·0	32	134
Leaves	2·7	5·3	79	249

* Expressed as mg nitrogen per 100 g fresh wt.

Measurements of the ammonia present after hydrolysis with 6N HCl by the method of Conway (1957) showed that ammonia nitrogen was formed in small quantities (*c.* 5 per cent.) relative to the γ-aminobutyric acid nitrogen.

(vi) *Distribution of Bound γ-Aminobutyric Acid in White Clover Plants.*—In Table 4 are summarized the results of analyses for free and bound γ-aminobutyric acid in ethanol extracts of leaves, stems, petioles, roots, and nodules of white clover plants. The plants sampled were growing vigorously in a ryegrass–white clover pasture in a soil of low nitrogen status. The clover roots were heavily nodulated and root tissue contiguous to nodules was chosen for analysis. It will be seen that the amount of bound γ-aminobutyric acid in effective root nodules of white clover is greatly in excess of the negligible amount present in other tissues of the plant.

In order to ascertain whether bound γ-aminobutyric acid was present in ineffective nodules of white clover, plants were grown in sterile pumice sand and inoculated with an ineffective *Rhizobium* strain. The amounts of free and bound

γ-aminobutyric acid present in the nodules are shown in Table 4; bound γ-aminobutyric acid was present in quantities less than 1 per cent. of that present in effective nodules.

A strain of *Rhizobium* effective for white clover was grown in a medium containing 10 g mannitol, 4 g yeast extract, 0·5 g KH_2PO_4, 0·2 g $MgSO_4.7H_2O$, 0·1 g NaCl, and 3·0 g $CaCO_3$ per litre. The bacteria were washed once in water by centrifugation and extracted by homogenizing in 80 per cent. (v/v) ethanol at room temperature. Analyses given in Table 4 indicate that bound γ-aminobutyric acid was not present in this material.

IV. Discussion

Of the free amino acids present in the various nodule extracts, glutamine was observed only in lupin nodules, whereas asparagine was present, usually in relatively large amounts, in nodules from all species examined. Bathurst (1954) using the non-specific hydrolytic method (Vickery *et al.* 1935) for estimating glutamine, reported higher values for glutamine in lupin nodules. Whereas Hunt (1951) and Virtanen and Miettinen (1953) reported the presence of glutamine in various legume nodules, Sen and Burma (1953) did not report it in a study of the amino acids in nodules of four legume species.

Compared with other plant tissues, free amino acids in nodules of the 10 species examined comprise a small proportion of the total nitrogenous compounds soluble in 80 per cent. ethanol. It is clear that elucidation of the nature of the other nitrogenous compounds present may well be of significance in studies of symbiotic nitrogen fixation.

The presence of bound γ-aminobutyric acid in such large amounts in white clover nodules and in smaller amounts in nodules of *T. pratense* and *T. medium* is therefore of particular interest. It would appear from Table 1 that the occurrence of bound γ-aminobutyric acid in root nodules is likely to be confined to the genus *Trifolium*. The amounts of bound γ-aminobutyric acid observed in white clover nodules represent about 20 per cent. of the total nitrogen content. The smaller amounts present in nodules of *T. pratense* and *T. medium* are possibly related to the slower growth of these two species at the time of sampling. From sampling of white clover nodules at various times during the year from plants growing under field conditions, it appeared that the content of bound γ-aminobutyric acid was directly related to the rate of nitrogen fixation, as assessed by the vigour and rate of growth of the plants. The small amount of bound γ-aminobutyric acid observed in ineffective nodules of white clover is in accord with this observation.

In the white clover plant, bound γ-aminobutyric acid appears to be virtually absent from tissues other than those involved in active fixation of nitrogen—it may therefore be involved in nitrogen fixation processes in this species.

Chemically bound β-alanine and γ-aminobutyric acid were found by Virtanen and Miettinen (1953) in pea plants; they suggested that compounds of amino acids with sugars might be involved. The state of combination of γ-aminobutyric acid in our material remains a matter for further investigation.

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