BBA 25715

PRIMARY PRODUCTS OF SYMBIOTIC NITROGEN FIXATION

II. PULSE-LABELLING OF SERRADELLA NODULES WITH $^{15}N_2$

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(Received April 27th, 1966)

SUMMARY

1. By supplying serradella nodules with a pulse of tracer $^{15}N_2$, and observing the rate of displacement of excess ^{15}N from certain nitrogen compounds, the role of these compounds in symbiotic nitrogen fixation has been examined.

2. The labelling patterns obtained support the primary position of ammonia as a stable intermediate, and indicate that glutamic acid *plus* glutamine are the primary amino compounds. Other amino acids appear to be of secondary origin.

INTRODUCTION

Results of short-term exposures of serradella nodules to $^{15}N_2$ were consistent with the primary role of ammonia in N_2 incorporation, and indicated that glutamic acid and glutamine are formed from it[1]. However, it remained possible that other amino compounds are also formed directly from ammonia, since several were labelled after only 45 sec exposure to $^{15}N_2$.

In this paper more definite evidence on precursor–product relationships in symbiotic N_2 fixation is presented. This was obtained by supplying detached serradella nodules with a pulse of tracer ^{15}N, by exposure to $^{15}N_2$ for several minutes, followed by their re-exposure to air. By halting the metabolism of the nodules at selected times after pulsing with $^{15}N_2$, it was possible to observe the rate of displacement of excess ^{15}N from ammonia and amino acids. The pattern of this displacement provides further evidence for deciding the sequence of formation of the primary compounds.

METHODS

Nodule material

Nodules were obtained from serradella (*Ornithopus sativus* Brot.) plants cultured in solution[1] for 5 weeks. Serradella seed was inoculated with cultures of *Rhizobium lupini* (D-25 strain, ref. 2) before germination. At 5 weeks of age, the nodules were a uniform pink-brown in the infected tissue.

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^{15}N analysis

Techniques for production of $^{15}N_2$ from $^{15}NH_4Cl$, preparation of gas mixtures with oxygen and argon, and mass spectrometric analysis of ^{15}N have been described[1]. Total-N was recovered as ammonia from nodule extracts after Kjeldahl digestion; ninhydrin-N was obtained by reaction of extracts with ninhydrin[3]; uncombined nodule ammonia was recovered by vacuum distillation[4]; amino-N of amino acids separated by paper chromatography in two dimensions was converted to ammonia by reaction with ninhydrin. These procedures are described at length in ref. 1.

Pulsing of nodules with $^{15}N_2$

Nodules were removed from the plants in the laboratory, thoroughly mixed, and 10.0-g samples were weighed into muslin bags. The nodules were exposed to a gas mixture containing 0.10 atm N_2 (69.8 atom per cent ^{15}N), 0.25 atm O_2 and 0.65 atm argon after a preliminary flushing with argon. After 8 min exposure at 23° to ^{15}N-enriched gas, an exposure flask containing several of the samples of nodules was evacuated, flushed with argon, re-evacuated and exposed to air. This operation was complete in 15 sec. Reaction was then terminated at intervals in the following 8 min by plunging the muslin bags into liquid N_2. Samples of nodules continuously exposed for 4, 8 and 16 min were also "killed" in this way. By freezing the nodules, it was possible to sample the pulsed series at intervals as low as 15 sec without difficulty. To extract nitrogen compounds from the frozen nodules they were immersed in boiling 80 % aqueous ethanol (5 ml per g nodules), boiled for 1 min, and crushed. After 1 h, the nodule material was filtered with suction and washed with 75 % ethanol. The extract *plus* washings was centrifuged at 20000 × *g* for 15 min, ethanol removed from the supernatant by vacuum evaporation at 30°, and known aliquots of the concentrate (pH approx. 5) taken for analysis.

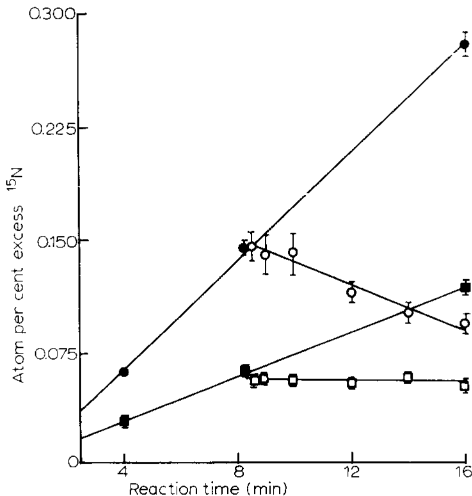

Fig. 1. Enrichment of total-N and of ninhydrin-N in nodules pulsed with $^{15}N_2$. The procedure for exposure is described in METHODS. Total-N was recovered by Kjeldahl digestion of the ethanol-soluble extracts, ninhydrin-N by reaction with ninhydrin. Total-N: ■—■, continuous; □—□, pulsed; ninhydrin-N: ●—●, continuous; ○—○, pulsed. The range of duplicate analyses is indicated.

RESULTS

The pattern of enrichment in total-N and in ninhydrin-N, with time of exposure, is shown in Fig. 1. The enrichment in total-N was constant after pulsing. This is good evidence for the absence of transfer of excess ^{15}N to insoluble material during this time, confirming the result from ^{15}N analysis of the residue. With continuous exposure to $^{15}N_2$, the increase in enrichment of total-N in the soluble nitrogen was linear.

The rate of enrichment in ninhydrin-N with continuous exposure was also linear, but there was a marked decline in enrichment after pulsing. This indicates that excess ^{15}N was progressively lost to nitrogen compounds which are not degraded to ammonia by ninhydrin. Ninhydrin-N includes the nitrogen of uncombined ammonia, amino-N of amino acids and of amides, and other unstable nitrogen compounds.

The enrichment pattern for uncombined ammonia is shown in Fig. 2. Two types of curve for enrichment with time are given, the atom per cent excess ^{15}N, and μg excess ^{15}N in ammonia per g of fresh nodules; the μg of excess isotope was calculated from the total pool of nodule ammonia-N and its enrichment, with a correction for the greater mass of ^{15}N.

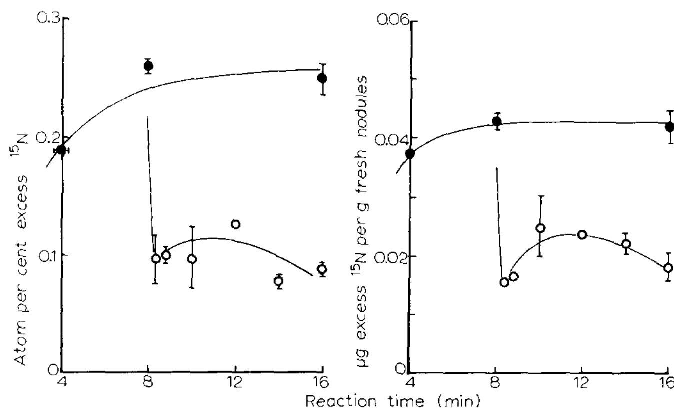

Fig. 2. Enrichment of ammonia in nodules pulsed with $^{15}N_2$. The protocol for exposure of nodules and extraction of ammonia is described in METHODS. Ammonia was recovered by vacuum distillation. Continuous exposure, ●—●; pulsed exposure, ○--○. The range of duplicate analyses is indicated.

As was shown in earlier exposures[1], the nodule ammonia active in early reactions of N_2 fixation was saturated with excess ^{15}N after several minutes of exposure. In the pulsed series, there was a rapid displacement of excess ^{15}N from ammonia once the $^{15}N_2$ was replaced by air. In 15 sec, the amount displaced was 0.027 μg excess ^{15}N per g of nodules from a saturated pool containing 0.043 μg excess ^{15}N per g. The previous rate of enrichment in the total-N of the nodule sample concerned was 0.06 μg excess ^{15}N per g per 15 sec. Thus about half the extra ^{14}N incorporated in the 15 sec of re-exposure to air was presumably located within the ammonia pool, replacing ^{15}N.

This is specific, direct evidence that ammonia is rapidly assimilated into other compounds. In addition, the labelling pattern supports the existence of a second ammonia pool in serradella nodules, also saturating with excess ^{15}N, but not on the direct pathway of N_2 incorporation into amino compounds, since the initial rapid

rate of displacement of excess ^{15}N from ammonia was followed by a period of several minutes in which a less rapid displacement occurred. This possibility for serradella nodules was postulated previously[1].

Enrichment patterns in several amino compounds are shown in Figs. 3–7. The enrichment in glutamic acid was much greater than in any other nitrogen compound.

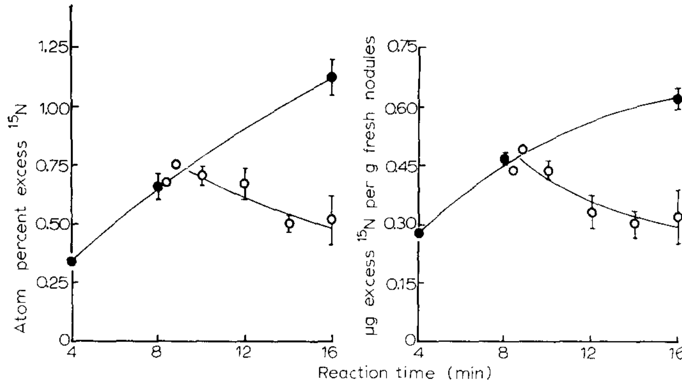

Fig. 3. Enrichment of glutamic acid in nodules pulsed with $^{15}N_2$. Glutamic acid was recovered by paper chromatography in two dimensions (ref. 1) and its amino-N released with ninhydrin. Continuous exposure, ●—●; pulsed exposure, ○--○. The range of duplicate analyses is indicated.

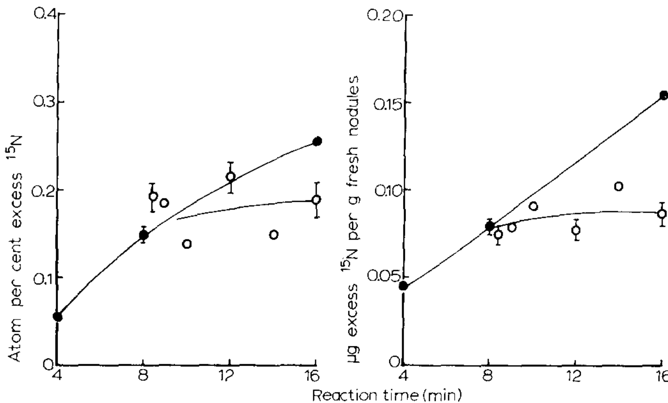

Fig. 4. Enrichment of aspartic acid in nodules pulsed with $^{15}N_2$. Aspartic acid was recovered by paper chromatography and its amino-N released with ninhydrin. Continuous exposure, ●—●; pulsed exposure, ○--○. The range of duplicate analyses is indicated.

This can also be seen in Table I, where representative values for enrichment of all forms of nitrogen examined are given. In the pulsed series, there was a marked displacement of excess ^{15}N from the nodule glutamic acid. The rate of transfer from glutamate shown in Fig. 3 approximates a first-order kinetic process, characteristic of a primary labelled compound[5]. This is good evidence that, as a nett process, glutamic acid is a precursor for other soluble nitrogen compounds.

The labelling in aspartic acid (Fig. 4) gives no indication that it was a precursor for other nitrogen compounds. The pulsed curve is characteristic of an end-product, or of an intermediate secondary to glutamate since the excess ^{15}N remained approxi-

mately constant. The curve for enrichment of alanine after pulsing (Fig. 5) is less conclusive. Insufficient data were obtained to definitely exclude alanine as a primary amino acid, but the low enrichment of alanine by comparison with that in glutamate, and the near constancy of its enrichment after pulsing suggest it was not.

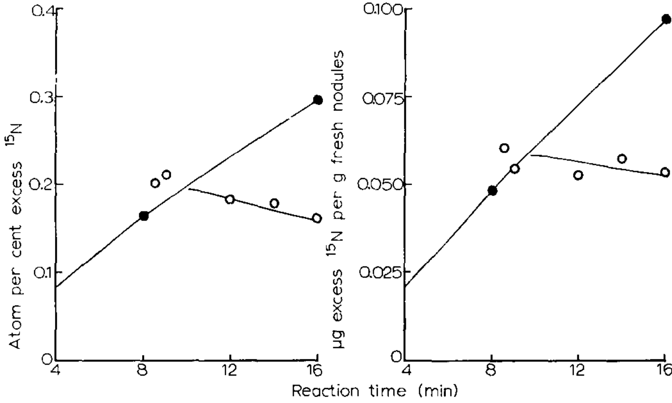

Fig. 5. Enrichment of alanine in nodules pulsed with ^{15}N. Alanine was recovered by paper chromatography and its amino-N released with ninhydrin. Continuous exposure, ●—●; pulsed exposure, ○—○.

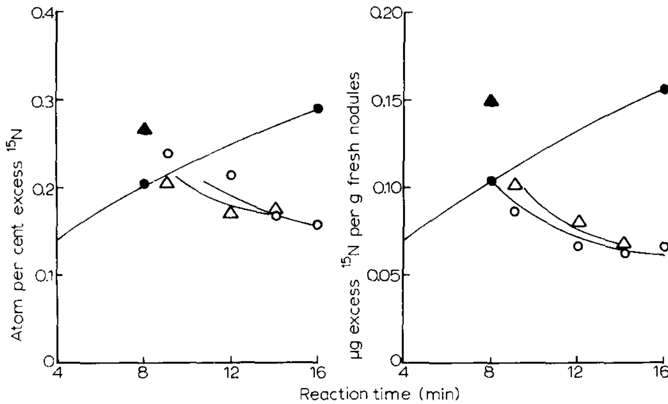

Fig. 6. Enrichment of glutamine in nodules pulsed with $^{15}N_2$. Glutamine was separated by paper chromatography and its amino-N released with ninhydrin. The residue from reaction with ninhydrin, after removal of the ammonia, was Kjeldahl-digested to determine the enrichment in the amide-N. Continuous exposure: ●—●, amino-N; ▲—▲, amide-N; pulsed exposure: ○—○, amino-N; △—△, amide-N.

The primary role of glutamine in nodule metabolism is indicated in Fig. 6. The µg of excess ^{15}N data for glutamine were more variable than for glutamic acid, due to incomplete recovery of glutamine, particularly of the nitrogen referred to as amide-N. This was obtained by Kjeldahl digestion of the residue from reaction of separated glutamine with ninhydrin. Since the enrichment in the nitrogen released from glutamine by reaction with ninhydrin was similar to that in the residual nitrogen, there is no problem in assigning it to amino-N and amide-N — a possible result of the incomplete specificity of the method for release of amino-N with ninhydrin[3]. Clearly,

both the amino-group and the amide-group of glutamine act as precursors for nitrogen in other compounds.

Though the atom per cent excess ^{15}N in nitrogen groups of asparagine was low, the total excess ^{15}N in this compound was comparable to that in glutamine, since

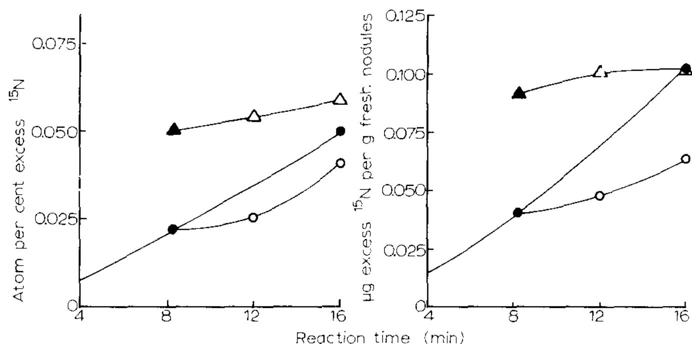

Fig. 7. Enrichment of asparagine in nodules pulsed with $^{15}N_2$. Asparagine was separated by paper chromatography and its amino-N released with ninhydrin. The enrichment in amide-N was determined with Kjeldahl digestion of the residual nitrogen from reaction with ninhydrin. Continuous exposure: ●—●, amino-N; ▲—▲, amide-N; pulsed exposure: ○—○, amino-N; △—△, amide-N.

asparagine is quantitatively the major amino compound in serradella nodules. Its pattern of enrichment after pulsing was unique among the compounds examined. The amount of excess ^{15}N in both nitrogen groups of asparagine increased in the 8 min following pulsing, indicating it is an end-product of the primary reactions of N_2 incorporation in serradella nodules.

All ninhydrin-positive compounds recovered from the ethanol extracts were examined for enrichment. None of the remainder (Table I) appeared to be significant in the early reactions examined here, though they may be actively involved in nitrogen metabolism in regions remote from the site of fixation.

DISCUSSION

The results of this study confirm and extend conclusions which were reached from short-term exposures of serradella nodules to $^{15}N_2$ (ref. 1). They agree with the hypothesis that ammonia is the first stable product of symbiotic N_2 fixation, and that glutamic acid and glutamine are primary organic products.

There is a fundamental distinction between the data obtained by short-term labelling, and those obtained by pulse-labelling. The former should give a more accurate indication of the reactions immediately following activation and fixation of the N_2 molecule. But the lability of the nitrogen atom in its compounds constitutes a problem in deciding the pathway of nitrogen flow. Results obtained with pulse-labelling reflect a metabolic pattern possibly more remote from these initial reactions, depending on the extent to which pools of nitrogen compounds distant to the site of fixation have become labelled, and their turnover rate. However, with pulsing for a short time only, till the ammonia pool was just saturated, the reactions observed here should represent primary events in N_2 fixation.

The close coincidence of the labelling pattern of the amino-N of glutamine and glutamic acid is probably due to their rapid equilibration. It is interesting to speculate that glutamine may represent the more easily transportable nitrogen compound, by reason of differing membrane permeabilities. In a situation where carbon skeletons for nitrogen incorporation may be limited, glutamine would be more economical for nitrogen transport from the region of the primary reactions.

TABLE I

ENRICHMENT OF NITROGEN COMPOUNDS IN SERRADELLA NODULES PULSED WITH $^{15}N_2$

Amino acids were separated by paper chromatography in two dimensions[1], first in phenol–water (80 %, w/v), with elution from the chromatograms in the groups indicated, followed by re-chromatography in either butanol–pyridine–water (1:1:1, v/v) (aspartic acid and asparagine groups), or in butanol–acetic acid–water (60:15:25, v/v) (glutamic acid, alanine and leucine groups). Only compounds for which definite identification was made have been named. Other ninhydrin-positive compounds are indicated in their relative position on the chromatograms by letters. Amino-N of the separated amino acids was released by reaction with ninhydrin.

Compounds	*Atom per cent excess* ^{15}N				
	Continuous exposure (min)		*Pulsed exposure (min) — pulse terminated at 8 min*		
	8¼	*16*	*12*	*14*	*16*
Total-N	0.058	0.118	0.053	0.053	0.051
Ninhydrin-N	0.145	0.277	0.116	0.101	0.095
Ammonia	0.265	0.247	0.135	0.078	0.092
Aspartate group					
Aspartic acid	0.154	0.265	0.237	0.156	0.202
Glutamate group					
Glutamic acid	0.730	1.124	0.678	0.490	0.501
Serine	0.045	—	0.183	—	—
Asparagine group					
Threonine	0.041	—	—	0.053	0.022
B	0.019	—	—	0.036	0.020
Glycine	0.056	0.073	—	0.044	0.044
Asparagine (amino-N)	0.023	0.052	0.026	—	0.042
Asparagine (amide-N)	0.051	—	0.056	—	0.062
Alanine group					
Alanine	0.139	0.298	0.181	0.187	0.155
Glutamine (amino-N)	0.213	0.320	0.226	0.163	0.155
Glutamine (amide-N)	0.280	—	0.163	0.171	
Leucine group					
Leucines	0.031	0.050	—	0.019	0.025
Phenylalanine	0.006	0.055	—	0.028	0.017
C	0.018	0.020	—	0.007	0.026
D	0.039	—	—	0.038	0.041
γ-Aminobutyrate	0.028	0.040	—	0.022	0.037
α-Aminobutyrate	0.012	0.021	—	—	—
G	0.026	—	—	0.014	0.017
H	0.026	0.028	—	0.011	0.012
Arginine	—	0.012	—	0.047	0.026
K	0.063	—	—	0.019	0.016
L	0.033	—	—	0.012	0.026

The slow but continuous rate of asparagine enrichment, even in the pulsed series, indicates its formation at a region removed from the primary reactions. Its synthesis may occur by transamination and transamidation with glutamine. The labelling pattern in asparagine indicates it is a sink or end-point for nitrogen in nodule compounds. Presumably asparagine is then transported to the vascular tissue and flows to aerial portions of the plant. This amide is commonly found as the major amino acid component of the xylem stream in legumes[6,7].

Nearly all the amino compounds recovered were enriched to some extent after 8 min exposure. In most cases, the enrichments were too low to detect definite patterns. In serradella nodules, γ-aminobutyric acid is second to asparagine in concentration, but the enrichment was low, increasing slightly in the 8 min following pulsing (Table I). This amino acid is therefore probably of tertiary origin in serradella nodules, and not a precursor for glutamic acid as was indicated in studies with ^{14}C-labelling on carrot tissues[8].

Thus the pathway of nitrogen from ammonia to glutamic acid and glutamine indicated for serradella nodules is similar to that shown in other organisms[9–11]. There are no major differences in the sequence of formation of amino acids, except that, as in *Candida utilis* assimilating $^{15}NH_4^+$ (ref. 11), the primary position of glutamine has been emphasized. Glutamic acid is favoured as the primary amino acid synthesized from ammonia because of the large number of glutamate-based transaminations which occur[12]. Enzymatic mechanisms for reductive synthesis of glutamic acid from α-ketoglutarate and ammonia are widespread in plants and bacteria (discussed by McKee[13] and Mortenson[14]). Glutamate dehydrogenase has now been shown in extracts of bacteroids from lupin and serradella nodules[16]. Thus this enzyme (with glutamine synthetase) is the logical mechanism for ammonia incorporation. The route of ammonia incorporation is dependent, in addition, on an adequate supply of carbon skeletons. The occurrence of isocitrate dehydrogenase in bacteroids and in the soluble fraction of serradella nodules[15], producing α-ketoglutarate, whilst also providing reducing power, may be significant. The subsequent transfer of amino-N from glutamic acid and glutamine could then be mediated by transaminases, which are present at high activity in *R. lupini* bacteroids[15].

ACKNOWLEDGEMENTS

I am indebted to Dr. C. A. Parker for advice and encouragement, to C.S.I.R.O. for a Studentship and to the University of Western Australia for financial assistance.

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