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## PRIMARY PRODUCTS OF SYMBIOTIC NITROGEN FIXATION

I. SHORT-TERM EXPOSURES OF SERRADELLA NODULES TO  $^{15}\text{N}_2$ 

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## SUMMARY

1. The pattern of  $^{15}\text{N}_2$  incorporation by serradella nodules has been examined. After 45 sec exposure to  $^{15}\text{N}_2$ , several nitrogen compounds were enriched, including ammonia, glutamic acid and glutamine; aspartic acid, alanine and asparagine were less labelled.

2. Further enrichment of the uncombined ammonia of the nodules ceased after about 5 min fixation, apparently due to the saturation of an extremely small internal ammonia pool with excess isotope. The enrichment of amino acids and amides continued at a linear rate.

3. Though the data are qualitatively consistent with the formation of amino acids and amides from ammonia in a single metabolic pool, where  $\text{N}_2$  is fixed, a kinetic analysis indicates a more complicated situation than this. To preserve the position of ammonia as the key intermediate through which all newly-fixed  $\text{N}_2$  must flow, it is necessary to postulate the occurrence of two or more internal pools of ammonia active in early reactions of  $\text{N}_2$  fixation, in addition to the bulk of the nodule ammonia which remains unlabelled for more than 1 h. Other possible explanations are discussed.

## INTRODUCTION

Data from labelling of nitrogen compounds in nodules with  $^{15}\text{N}_2$  generally suggest the sequence:  $\text{N}_2 \rightarrow \text{NH}_3 \rightarrow$  amino acids<sup>1-3</sup>. Nevertheless, the identity of the primary nitrogen compounds and their quantitative importance in  $\text{N}_2$  assimilation remains uncertain. This doubt is reinforced by the lack of information of enzymes involved in  $\text{N}_2$  incorporation. Preliminary experiments in this laboratory consistently failed to reveal the presence of glutamate dehydrogenase in any of the centrifugal fractions from crushed lupin or serradella nodules. In addition, the relatively low labelling of ammonia by comparison with several other compounds<sup>2,3</sup> has been a problem—one not resolved by theories proposing dilution by a pool of unlabelled ammonia in nodules, since these other nitrogen compounds could also be present in more than one pool. Recently BERGERSEN<sup>4</sup> provided more convincing evidence that ammonia is the primary stable intermediate in  $\text{N}_2$  fixation by soybean nodules.

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In this paper a more extensive examination of  $^{15}\text{N}$ -labelling patterns during symbiotic  $\text{N}_2$  fixation is described, similar to the kinetic approach adopted by ALLISON AND BURRIS<sup>5</sup> for the study of  $^{15}\text{N}_2$  fixation by *Azotobacter*. In a model erected for quantitative treatment of the data, the flux of nitrogen through the ammonia pool of serradella nodules has been examined.

## METHODS

### *Nodule material*

Nodules for exposure to  $^{15}\text{N}_2$  were obtained from serradella (*Ornithopus sativus* Brot.) plants. *Rhizobium lupini* (D-25 strain, ref. 6) for inoculation of serradella seed was maintained on yeast-mannitol agar<sup>7</sup>. The inoculated seed was germinated in sterile sand and transferred to liquid culture when nodules were just visible in the upper roots. The seedlings were suspended on polythene slats in aerated solution held at 22° of the following composition ( $\mu\text{M}$  concentration):  $\text{KH}_2\text{PO}_4$ , 100;  $\text{K}_2\text{SO}_4$ , 200;  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ , 150;  $\text{CaCl}_2$ , 100;  $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$ , 10; sodium-ferric ethylenediamine di-(*o*-hydroxyphenyl-acetate), 100;  $\text{H}_3\text{BO}_3$ , 7.5;  $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$ , 0.15;  $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ , 0.6;  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ , 0.215;  $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$ , 0.075. The pH of the solution was maintained in the range 7.5–8.0 by an excess of solid  $\text{CaCO}_3$ . Ferric citrate at the same concentration can also serve as an iron source. The solution was changed at fortnightly intervals to maintain rapid growth of about 1000 plants in 400 l of solution. Water was deionised before preparation of the solution and no combined nitrogen sources were added.

### $^{15}\text{N}$ techniques

$^{15}\text{N}_2$  was produced from  $^{15}\text{NH}_4\text{Cl}$  (96%  $^{15}\text{N}$ , Isomet Co.) by oxidation over heated copper oxide<sup>8</sup>. The gas was stored over 20% (w/v)  $\text{Na}_2\text{SO}_4$  solution, containing 0.25 M  $\text{H}_2\text{SO}_4$  to remove impurities.

All nitrogen samples for  $^{15}\text{N}$  analysis were converted to ammonia for subsequent oxidation to  $\text{N}_2$  with hypobromite<sup>8</sup>. The hypobromite reagent was stabilised with KI to prevent  $\text{O}_2$  evolution<sup>9</sup>. The abundance of  $^{15}\text{N}$  in  $\text{N}_2$  samples was determined with an Associated Electrical Industries MS-3 mass spectrometer by measurement of the ratio of the ion currents at masses 28 and 29. Peaks at either mass 32 or mass 40 were used to correct for contaminating  $\text{N}_2$  from air. Tests showed that nearly all the air was probably derived from the solutions, since dry evacuated RITTENBERG tubes<sup>10</sup> did not contain enough gas to give measurable ion currents, though tubes containing aqueous solution did. The relative magnitude of these currents, after sensitivity corrections for each, did not agree with the known composition of the atmosphere, but agreed closely with the composition of dissolved air<sup>11</sup>. This was considered in derivation of correction factors for air contamination, which were employed in the analyses of  $^{15}\text{N}$  in separated amino acids and in uncombined ammonia. When more than 100  $\mu\text{g}$  of nitrogen was available for analysis, as after Kjeldahl digestion or reaction of nodule extracts with ninhydrin, air corrections were unnecessary. The atom per cent excess  $^{15}\text{N}$  was calculated from the atom per cent  $^{15}\text{N}$  data by subtraction of the abundance in the total-N of unlabelled nodule extracts. The natural abundance of  $^{15}\text{N}$  in such extracts was determined as 0.359–0.361 atom per cent  $^{15}\text{N}$ .

### *Classes of nitrogen*

The following methods were employed to recover different classes of nitrogen from nodule extracts.

(i) For determination of total enrichment in nodule extracts after exposure to  $^{15}\text{N}_2$ , organic nitrogen was converted to ammonia by Kjeldahl digestion with mercury catalyst<sup>8</sup>. Digested samples were then steam-distilled from 12 ml of 13 M NaOH containing 8% (w/v)  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 10 \text{H}_2\text{O}$  into 0.1 M  $\text{H}_2\text{SO}_4$ , and suitable aliquots taken for ammonia determination with Nessler's reagent<sup>12</sup>. Each  $\mu\text{g}$  of  $\text{NH}_3\text{-N}$  produced an absorbance in 1.0-cm cuvettes of 0.035–0.045 at 410  $m\mu$  in 6.0 ml final volume. Absorbance measurements were made 10 min after the addition of 1.0 ml of Nessler's reagent to a 5.0-ml aqueous ammonium sample. The nitrogen of ammonia recovered after Kjeldahl digestion is referred to as total-N.

(ii) A more selective method for release of organic nitrogen as ammonia is by reaction with ninhydrin<sup>13</sup>. Ninhydrin-N represents nitrogen from several sources, including uncombined ammonia, most of the amino-N of amino acids and a small proportion of the amide-N of glutamine<sup>13</sup>.

(iii) Uncombined nodule ammonia was recovered from nodule extracts by vacuum distillation<sup>14</sup> at pH 10 and 42°, trapped in 0.05 M  $\text{H}_2\text{SO}_4$  and determined by Nesslerisation.

(iv) Amino-N of amino acids was released as ammonia by the specific reaction with ninhydrin and recovered by distillation<sup>13</sup>.

### *Fractionation of amino compounds*

Amino acids were separated by a paper chromatographic technique. The supernatant from centrifugation of ethanol extracts of nodules was concentrated by vacuum evaporation at 30° and a sample applied as a band to the origin of 57 cm  $\times$  46 cm sheets of oxalic acid-washed Whatman No. 3 filter paper. Chromatograms were developed by descending solvent flow. A preliminary separation was achieved with 75 ml of phenol-water (80%, w/v). Papers were dried in an efficient fume-hood for 24 h and narrow strips were cut from the chromatogram and stained with 0.1% (w/v) ninhydrin in acetone. The strips were heated at 80° for 5 min for colour development, and unstained portions of the chromatogram were divided into five sections, each corresponding to a known group of the ninhydrin-positive compounds. Each group was eluted with 150 ml of 50% (w/v) aqueous ethanol at pH 3.5, except that the one containing glutamine was eluted with neutral ethanol solution. The eluates were concentrated by vacuum-evaporation to about 0.2 ml for a secondary separation in either butanol-acetic acid-water (60:15:25, v/v) or butanol-pyridine-water (1:1:1, v/v). 80 ml of solvent was employed per paper and amino compounds were located with ninhydrin solution. For release of the amino-N of ninhydrin-stained compounds as ammonia, the coloured bands were cut from the chromatograms, placed as rolls in 1-inch-diameter test tubes and reacted with ninhydrin<sup>13</sup>, after removal of contaminating ammonia by addition of 2 ml of 0.1 M  $\text{K}_2\text{HPO}_4\text{-KOH}$  buffer at pH 10.5 to each tube, with drying of the papers under vacuum over concentrated  $\text{H}_2\text{SO}_4$  for 48 h.

### *Exposure of nodules to $^{15}\text{N}_2$*

Serradella nodules were stripped from plants in the laboratory for exposure to  $^{15}\text{N}_2$ . To minimise variability, the nodules were thoroughly mixed, and samples of

7–9 g fresh-weight added to 50- or 100-ml exposure flasks. The nodules were twice flushed with argon by evacuation and then exposed to a gas mixture containing 0.10 atm enriched  $N_2$ , 0.25 atm  $O_2$  and 0.65 atm argon. When 1.0 atm pressure was reached each flask was isolated. Reaction was terminated by rapidly crushing the nodules in warm 80% (w/v) aqueous ethanol. The crushed nodule material was filtered and washed on polyvinylchloride filter paper by suction. The filtrate *plus* washings was centrifuged at 20000  $\times g$  for 15 min and the supernatant fluid concentrated by vacuum evaporation at 30°. The concentrate was stored at  $-20^\circ$  till required. Ammonia was not lost under partial vacuum, since extracts prepared in this way had a pH of 6 or less, declining to about pH 5 during concentration. Tests showed that the same quantity of ammonia was recovered per g of nodules whether extracts were concentrated previously or not, but it was desirable to remove ethanol before ammonia was distilled since ethanol in ammonium samples sometimes interfered with mass spectrometry<sup>13</sup>.

Ammonia was recovered by vacuum distillation<sup>14</sup> from duplicate samples of extract equivalent to about 1.5 g of fresh nodules. Amino acids were separated from aliquots equivalent to about 3 g of nodules. Amino-N was then converted to ammonia with ninhydrin, as described earlier. Total-N and ninhydrin-N were recovered from duplicate samples of extract equivalent to 0.5 g of fresh nodules.

## RESULTS

Serradella nodules proved reliable material for studies on  $^{15}N_2$  fixation. The rate of fixation remained linear for at least 90 min after excision (see Fig. 1), provided exposure flasks contained sufficient  $O_2$  to prevent significant decreases in  $O_2$  tension by respiratory consumption. Serradella nodules consumed 200–400  $\mu l$  of  $O_2$  per g fresh weight per h.

### *Enrichment of the ammonia pool of serradella nodules*

The pattern of enrichment in the total-N and the ammonia-N of serradella nodules is shown in Fig. 1. There was no detectable lag in enrichment of either.

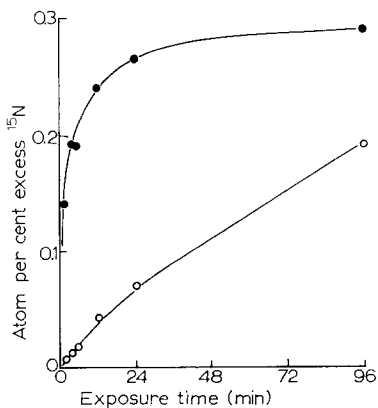


Fig. 1. Enrichment of ammonia and of total-N in serradella nodules exposed to  $^{15}N_2$ . Exposure 1: 10-g samples of nodules from plants cultured in solution for 8 weeks were exposed to a gas mixture containing 0.1 atm  $N_2$  (59.0 atom per cent  $^{15}N$ ), 0.25 atm  $O_2$  and 0.65 atm argon. Reaction was terminated as indicated by rapidly crushing nodules in 50 ml of warm 80% ethanol. Ammonia and total-N were recovered as described in METHODS. ●—●, ammonia; ○—○, total-N.

The curve obtained for accumulation of excess  $^{15}\text{N}$  in nodule ammonia indicates the existence of an internal pool of ammonia in serradella nodules which is rapidly saturated with excess  $^{15}\text{N}$ , presumably to the extent of the enriched  $\text{N}_2$  gas. This result confirms the opinion of previous workers that the low labelling of the ammonia in soybean nodules<sup>2</sup> and non-legume nodules<sup>3</sup> was the result of dilution by ammonia not in equilibrium with that newly-fixed.

TABLE I

ENRICHMENT OF AMMONIA, NINHYDRIN-N AND TOTAL-N IN SERRADELLA NODULES EXPOSED TO  $^{15}\text{N}_2$

Serradella nodules from plants cultured in solution for 4.5 weeks were exposed at  $23^\circ$  to a gas mixture containing 0.1 atm  $\text{N}_2$  (69.8 atom per cent  $^{15}\text{N}$ ), 0.25 atm  $\text{O}_2$  and 0.65 atm argon. Reaction was terminated at times indicated by rapidly crushing nodules in 5 ml per g of warm 80% ethanol. Duplicate analyses, performed as described in METHODS, are shown. Total-N was recovered by Kjeldahl digestion of the ethanol-soluble material, ninhydrin-N by reaction with ninhydrin and ammonia by vacuum distillation.

Time of exposure (min)	Enrichment (atom per cent excess $^{15}\text{N}$ )		
	Ammonia	Ninhydrin-N	Total-N
3/4	0.042	—	0.006
	0.068		0.006
2 1/4	0.070	0.036	0.023
	0.089	0.052	0.023
4 1/4	0.132	0.096	0.041
	0.147	0.092	0.042
8 1/4	0.135	0.144	0.070
	0.169	0.130	0.072

A similar pattern of enrichment of ammonia was obtained in several other experiments, though the time taken for saturation of the internal ammonia pool was usually less than in Fig. 1. This may have been a function of nodule age, since those used in this experiment were at a more advanced stage than usual, containing a proportion with partly green interiors, instead of the normal pinkish-brown.

In a second experiment, nodules were sampled at an earliest time of 45 sec after exposure to  $^{15}\text{N}_2$ . The same pattern of enrichment of ammonia was obtained, though the rate of enrichment of both total-N and ninhydrin-N was linear (see Table I). The enrichment in ammonia was about the same at 4 1/4 min exposure as at 8 1/4 min. Assuming that the maximum enrichment in the internal ammonia pool which saturated approached that in the  $\text{N}_2$  gas supplied, this internal pool included only approx. 0.3% of the total nodule ammonia or about 10  $\mu\text{moles}$  ammonia per g. In the size of the internal pool, and the time taken for its saturation, these results are closely similar to those described recently<sup>4</sup> for fixation in soybean nodules.

#### *Enrichment in amino compounds*

The time course of enrichment in amino compounds is shown in Fig. 2. Glutamic acid was the most highly labelled compound, though at the earliest time of exposure, 45 sec, ammonia was slightly more enriched. The other important compound was glutamine. At 45 sec the enrichment in glutamine was greatest in the nitrogen released by ninhydrin, but since this degradation is not completely specific for the  $\alpha$ -amino-N

of this amide, some dilution from the amide group probably occurred. The residue is referred to as amide-N in Fig. 2, though the method is not specific. Since recoveries of nitrogen by Kjeldahl digestion of the residue were in the range, 90–130% of those obtained by reaction with ninhydrin, this should be approximately true.

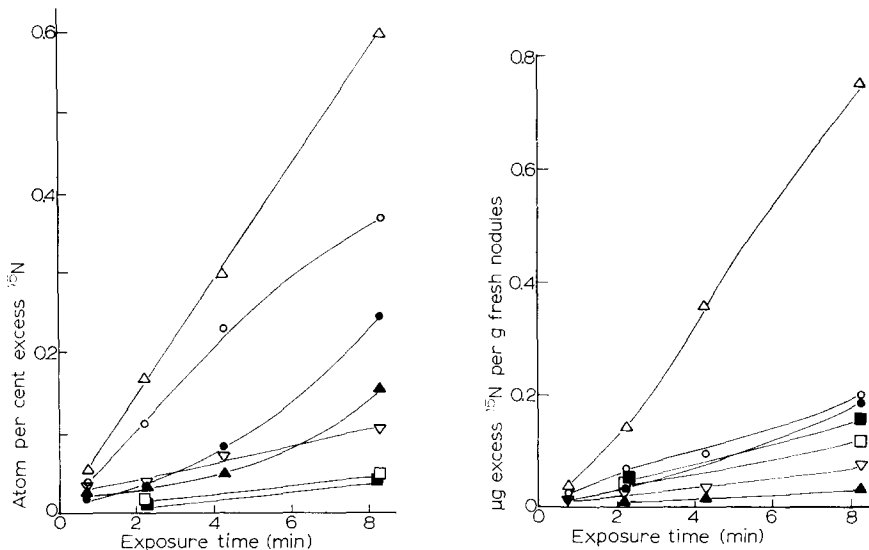


Fig. 2. Time course of atom per cent excess  $^{15}\text{N}$  in amino compounds. Exposure 2: Conditions for exposure of nodules were as described in Table I. Amino acids and amides were separated by paper chromatography and amino-N released with ninhydrin. The enrichment in amide-N is that in the nitrogen remaining after reaction of separated amides with ninhydrin.  $\triangle$ — $\triangle$ , glutamic acid;  $\circ$ — $\circ$ , glutamine amino-N;  $\bullet$ — $\bullet$ , glutamine amide-N;  $\blacktriangle$ — $\blacktriangle$ , alanine;  $\nabla$ — $\nabla$ , aspartic acid;  $\square$ — $\square$ , asparagine amino-N;  $\blacksquare$ — $\blacksquare$ , asparagine amide-N.

Fig. 3. Time course of  $\mu\text{g}$  of excess  $^{15}\text{N}$  in amino compounds. Exposure 2: Conditions for exposure were as in Table I. The data are derived from those shown in Fig. 2, taking into account the total nitrogen concentration of each compound in nodules.  $\triangle$ — $\triangle$ , glutamic acid;  $\circ$ — $\circ$ , glutamine amino-N;  $\bullet$ — $\bullet$ , glutamine amide-N;  $\blacktriangle$ — $\blacktriangle$ , alanine;  $\nabla$ — $\nabla$ , aspartic acid;  $\square$ — $\square$ , asparagine amino-N;  $\blacksquare$ — $\blacksquare$ , asparagine amide-N.

The enrichment of the compounds is plotted as  $\mu\text{g}$  of excess  $^{15}\text{N}$  per g of fresh nodules in Fig. 3. The pre-eminent position of glutamic acid is accentuated in this plot. Of the other compounds, asparagine is increased in stature in spite of the relatively low enrichment in atom per cent excess of its nitrogen atoms, since it was quantitatively the major compound in the extracts. Apart from glutamic acid, glutamine and asparagine, only alanine and aspartic acid contained significant excess  $^{15}\text{N}$  after  $8\frac{1}{4}$  min reaction. In arriving at this conclusion all ninhydrin-positive compounds were examined for excess  $^{15}\text{N}$  by reaction with ninhydrin, followed by Kjeldahl digestion of the residue.

The enrichment in the significant compounds as a percentage of the total  $\mu\text{g}$  of excess  $^{15}\text{N}$  in the extracts is shown in Fig. 4. This figure demonstrates that ammonia, glutamine and glutamic acid were the most important vehicles of excess isotope after 45 sec. The excess  $^{15}\text{N}$  in ammonia as a percentage of the total fell rapidly as the ammonia pool saturated. The percentage in glutamic acid increased slightly up to  $8\frac{1}{4}$  min at the conclusion of the exposure. The share of other compounds in the total

excess  $^{15}\text{N}$  remained approximately constant, indicating that their rate of enrichment was linear.

In Fig. 5 is shown the  $\mu\text{g}$  of excess  $^{15}\text{N}$  in various classes of nitrogen bondings as a percentage of the excess  $^{15}\text{N}$  in total-N. Also plotted is the deficit of excess tracer,

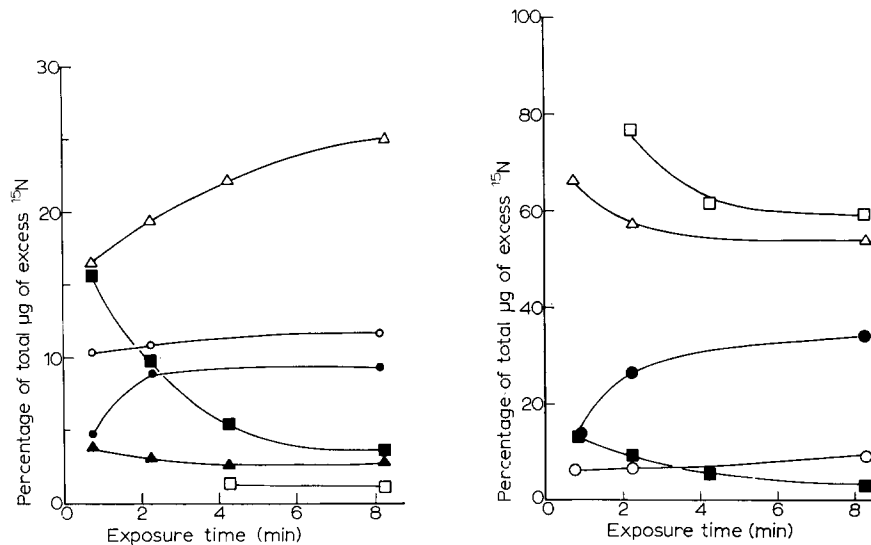


Fig. 4. Percentage of  $\mu\text{g}$  of excess  $^{15}\text{N}$  in total-N contained in ammonia, and in amino compounds. Exposure 2: Conditions for exposure of nodules were as in Table I.  $\Delta$ - $\Delta$ , glutamic acid;  $\circ$ - $\circ$ , glutamine;  $\bullet$ - $\bullet$ , asparagine;  $\blacktriangle$ - $\blacktriangle$ , aspartic acid;  $\square$ - $\square$ , alanine;  $\blacksquare$ - $\blacksquare$ , ammonia.

Fig. 5. Percentage of  $\mu\text{g}$  of excess  $^{15}\text{N}$  in total-N recovered in ammonia, amino-N and amide-N. Exposure 2: Reaction conditions were as in Table I. Amino-N was obtained from the sum of the excess  $^{15}\text{N}$  recovered in individual amino acids separated by paper chromatography. Ninhydrin-N (see METHODS) is also shown, but this was not used in estimation of the deficit, obtained by subtraction of the  $\mu\text{g}$  of excess  $^{15}\text{N}$  in ammonia, amino-N and amide-N from that in total-N.  $\blacksquare$ - $\blacksquare$ , ammonia;  $\bullet$ - $\bullet$ , amino-N;  $\circ$ - $\circ$ , amide-N;  $\Delta$ - $\Delta$ , deficit;  $\square$ - $\square$ , ninhydrin-N.

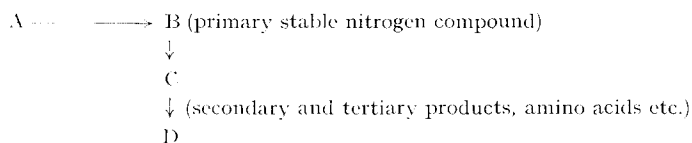
obtained by subtraction of excess  $^{15}\text{N}$  in ammonia, amino-N and amide-N from that in total-N. This deficit was 13% of the excess  $^{15}\text{N}$  in total-N after 15 sec, increasing to about 34% after 8¼ min. Part of this can be ascribed to inevitable losses of amino acids during paper chromatography. The loss with authentic samples of glutamic acid, alanine,  $\gamma$ -aminobutyric acid and isoleucine was 12–25% of each amino acid taken through all the phases of the chromatographic procedure. This does not account for more than part of the deficit, however, and excess  $^{15}\text{N}$  must have been present in other ethanol-soluble compounds of unknown identity.

#### DISCUSSION

A feature of the enrichment of nitrogen compounds in fixation by serradella nodules was the rapidity with which several compounds obtained significant excess  $^{15}\text{N}$ . Thus at 45 sec exposure there was little difference between ammonia, glutamic acid and glutamine in enrichment. It was unlikely that more definite information could be gained of the precursor-product relationship from briefer exposures of

serradella nodules to  $^{15}\text{N}_2$ . A further problem in interpreting labelling data from legume nodules is their complexity of structure. This makes possible the occurrence of many sources of molecules of each compound obtained by extraction<sup>15, 16</sup>. Consequently, direct comparisons of enrichment data may be misleading for deciding which compounds are precursors and which their products.

The well-defined pattern of ammonia enrichment in serradella nodules encouraged a kinetic analysis of the data in an attempt to determine the flux of nitrogen through the ammonia pool (along lines which have been employed for study of intermediates by  $^{14}\text{C}$ -labelling). The intermediate stages of  $\text{N}_2$  assimilation can be depicted:



In this scheme, the concentration of free intermediates at the site of fixation is represented by A, B, C, D. The labelling pattern of ammonia indicates the existence of a discrete compartment in the nodules in which the primary products are formed. For a model system in which Compound B acts as an intermediary compound for all fixed  $\text{N}_2$ , the estimated turnover of nitrogen in B should equal the rate of fixation.

ZILVERSMIT<sup>17</sup> discusses a model for labelling of a metabolite from a labelled precursor of constant specific activity. The relationship

$$S_B = S_A (1 - e^{-kt}) \quad (1)$$

is derived, in which the specific activity of Compound B at any time  $t$  is represented by  $S_B$ , the specific activity of precursor A by  $S_A$ , and  $k$  denotes the fractional turnover rate (the proportion of the total of B renewed per unit time). From (1)

$$\ln \frac{S_A}{S_B - S_A} = kt \quad (2)$$

A reasonable interpretation of the results for labelling of the ammonia pool would be that ammonia is the primary stable intermediate in symbiotic fixation, and that other nitrogen compounds such as glutamic acid and glutamine are formed directly from it. When the data for early enrichment of ammonia were applied in Eqn. 2 and turnover constants calculated for each time, however, a marked discrepancy approaching one order of magnitude between calculated rates of turnover and the observed fixation rate was found (Table II). Even errors of up to 25 % in  $^{15}\text{N}$  analysis make little difference to the order of the discrepancy. Since the possible error was considerably less, the data are inconsistent with the model. Other explanations need to be advanced to explain the labelling pattern, though these must take note of the clear qualitative evidence for the existence of an internal ammonia pool active in early reactions of fixation.

An explanation for the failure of the model could be that ammonia is not the sole vehicle of nitrogen flow to amino acids and other organic compounds. For instance, intermediates between  $\text{N}_2$  and ammonia could be directly involved in amino acid formation as BACH<sup>19</sup> suggested in a scheme for  $\text{N}_2$  fixation by *Azotobacter*, in which hydrazine combined directly with  $\alpha$ -ketoglutarate, producing either glutamine or



TABLE II

TURNOVER CONSTANTS FOR THE AMMONIA POOL OF SERRADELLA NODULES

$S_B = S_A \times \frac{S_{\text{Ammonia at } t}}{S_{\text{Ammonia at saturation}}}$ . The turnover time ( $t_t$ ), the inverse of  $k$ , is the period required for the flux of nitrogen through the hypothetical ammonia pool to equal its magnitude. The size of the active ammonia pool was derived by proportion from the final enrichment of total nodule ammonia, the enrichment of the  $N_2$  gas supplied ( $S_A$ ), and the ammonia concentration; this is equivalent to the turnover per  $t_t$ .

	Exposure time $t$ (sec)	$S_A$	$S_{\text{Total nodule ammonia at time } t}$ (atom per cent excess $^{15}N$ )	$S_{\text{Total nodule ammonia when saturated}}$	Ammonia-N concentration ( $\mu g/g$ fresh weight)
Exposure 1	120	58.6	0.142	0.275	31.7
Exposure 2	45	69.4	0.055	0.152	55.9
	135	69.4	0.080	0.152	55.9

	Calculated $S_B$ (atom per cent excess $^{15}N$ )	$k_{(\text{sec}^{-1})}$	$t_t$ (sec)	Calculated turnover per $t_t$ ( $\mu g N/g$ fresh weight)	Actual total fixation ( $\mu g N/g$ fresh weight)
Exposure 1	30.3	0.00606	165	0.149	0.888
Exposure 2	25.1	0.00998	100	0.123	0.552
	36.5	0.00554	181	0.123	0.998

glutamic acid plus ammonia. While the present results do not eliminate this possibility for symbiotic  $N_2$  fixation, the lack of correspondence between the pattern of labelling of glutamic acid and ammonia is at variance with it. Another possibility not completely excluded by these results is one involving hydroxylamine in amino acid formation, *via* oximino acids. However, no specific evidence for this has been presented, and the route from  $N_2$  to hydroxylamine is thermodynamically unfavourable<sup>20</sup>. Also, BERGERSEN<sup>4</sup> has shown that almost 100% of the excess  $^{15}N$  can be recovered in ammonia alone after 1 min exposure of soybean nodules to  $^{15}N_2$ —unlike serradella nodules, soybean nodules apparently show a lag before excess  $^{15}N$  is incorporated in amino acids. Moreover, KIDBY AND DILWORTH<sup>21</sup> have recently shown glutamate dehydrogenase in bacteroids of *R. lupini* isolated from both lupin and serradella nodules. The activity recorded was adequate to agree with the hypothesis that synthesis from ammonia is the unique route of amino acid formation, judged by the rates of  $^{15}N_2$  fixation by detached nodules.

The discrepancy between this view and the calculated flux of nitrogen through the ammonia pool of serradella nodules can be resolved by postulating the existence of more than one internal pool of ammonia saturating with excess  $^{15}N$ , with only one directly concerned in amino acid formation. This explanation is supported by the results of pulse-labelling experiments with serradella nodules<sup>18</sup>, in which there was a rapid displacement of about half the excess  $^{15}N$  in ammonia within 15 sec of removal of  $^{15}N_2$  and substitution with  $^{14}N_2$ ; the remainder of the excess  $^{15}N$  in ammonia was displaced at a greatly reduced rate. Thus, though the kinetic analysis fails to provide rigorous proof of the ammonia hypothesis of  $N_2$  assimilation, the results, in general,

are consistent with it. No attempt was made to calculate the turnover of nitrogen in glutamic acid or glutamine, since the size of the active pools could not be estimated. Unlike the ammonia pool, these were probably unbounded, due to translocation from the region of fixation. It is unnecessary that other major "ports of entry" for nitrogen into organic combination occur: between them, glutamic acid and glutamine contained more than half the excess  $^{15}\text{N}$  in amino compounds.

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#### REFERENCES

- 1 I. ZELITCH, P. W. WILSON AND R. H. BURRIS, *Plant Physiol.*, 27 (1952) 1.
- 2 M. H. APRISON, W. E. MAGEE AND R. H. BURRIS, *J. Biol. Chem.*, 208 (1954) 29.
- 3 G. LEAF, I. S. GARDNER AND G. BOND, *J. Exptl. Bot.*, (1958) 320.
- 4 F. J. BERGERSEN, *Australian J. Biol. Sci.*, 18 (1965) 1.
- 5 R. M. ALLISON AND R. H. BURRIS, *J. Biol. Chem.*, 224 (1957) 351.
- 6 C. A. PARKER AND A. E. OAKLEY, *Australian J. Exptl. Agr. Animal Husbandry*, 3 (1963) 9.
- 7 P. H. GRAHAM, *Antonie van Leeuwenhoek, J. Microbiol. Serol.*, 30 (1964) 68.
- 8 R. H. BURRIS AND P. W. WILSON, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 4, Academic Press, New York, 1957, p. 355.
- 9 A. P. SIMS AND E. C. COCKING, *Nature*, 181 (1958) 474.
- 10 D. B. SPRINSON AND D. RITTENBERG, *J. Biol. Chem.*, 180 (1949) 707.
- 11 C. D. HODGMAN, *Handbook of Chemistry and Physics*, 38th ed., Chemical Rubber, Cleveland 1957, p. 1606.
- 12 A. I. VOGEL, *Quantitative Inorganic Analysis*, 3rd ed., Longmans, London, 1961, p. 783.
- 13 I. R. KENNEDY, *Anal. Biochem.*, 11 (1965) 105.
- 14 G. W. PUCHER, H. B. VICKERY AND C. S. LEAVENWORTH, *Ind. Eng. Chem. Anal. Edition*, 7 (1935) 152.
- 15 D. B. COWIE AND F. T. MCCLURE, *Biochim. Biophys. Acta*, 31 (1959) 236.
- 16 D. H. MACLENNAN, H. BEEVERS AND J. L. HARLEY, *Biochem. J.*, 89 (1963) 316.
- 17 D. B. ZILVERSMIT, *Am. J. Med.*, 29 (1960) 832.
- 18 I. R. KENNEDY, *Biochim. Biophys. Acta*, 130 (1966) 295.
- 19 M. K. BACH, *Biochim. Biophys. Acta*, 26 (1957) 104.
- 20 N. S. BAYLISS, *Australian J. Biol. Sci.*, 9 (1956) 364.
- 21 D. K. KIDBY AND M. J. DILWORTH, to be published.