# AMMONIA—AN EARLY STABLE PRODUCT OF NITROGEN FIXATION BY SOYBEAN ROOT NODULES

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#### Summary

Experiments with detached soybean root nodules exposed to atmospheres containing very high enrichments of  ${}^{15}\mathrm{N}_2$  for short periods of time have shown that at about 1 min from the commencement of fixation more than 90% of the soluble fixed nitrogen could be accounted for as ammonia. The proportion of the newly fixed nitrogen in this form then rapidly diminished as the ammonia was rapidly converted to amino and other compounds. After fixation for 10 min, only about 20% of the newly fixed nitrogen was ammonia. Fixed nitrogen in amides rose steadily for 1 hr and after that time was equal in concentration to the fixed nitrogen in the form of ammonia.

### I. INTRODUCTION

Ammonia has been considered to be the most likely form in which fixed nitrogen enters assimilatory pathways in both free-living and symbiotic agents of nitrogen fixation (Burris 1956). However, hydroxylamine (Virtanen 1947), nitrate (or some analogue of it—Cheniae and Evans 1956), and other compounds have been suggested by various workers as being important early products of nitrogen fixation by legume root nodules.

More recently it has been shown conclusively with cell-free nitrogen-fixing preparations from free-living bacteria that ammonia was the main product (Mortenson, Mower, and Carnahan 1962). Failure to obtain active cell-free extracts from symbiotic systems and the relative insensitivity of the <sup>15</sup>N tracer methods have prevented the demonstration of a similar role for ammonia in the nodule system, although the distribution of <sup>15</sup>N among the components of the nitrogen pool of excised nodules exposed to labelled atmospheres for 1 hr was consistent with the <sup>15</sup>N entering assimilatory pathways by way of ammonia (Aprison, Magee, and Burris 1954).

In the experiments to be described, <sup>15</sup>N techniques and analytical methods were improved to enable short-term experiments to be carried out tracing incorporation of <sup>15</sup>N into ammonia in a similar way to the experiments of Allison and Burris (1957) with Azotobacter vinelandii.

### II. MATERIALS AND METHODS

### (a) Nodules

Nodules aged from 28–35 days were detached from glasshouse-grown soybeans cv. Shelby, which had been inoculated with strain CC711 of *Rhizobium japonicum* as previously described (Bergersen 1958). Incubations were done at  $23^{\circ}$ C in 100-ml

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conical flasks attached to a manifold with facilities for taking gas samples from the flasks.

### (b) Gas Mixtures

Gas mixtures in which the nodules were incubated contained 30% O<sub>2</sub>, 20% <sup>15</sup>N<sub>2</sub>, and 50% argon. They were prepared in reservoirs (2 l.) connected to a manifold equipped with a mercury manometer. The reservoirs were kept under pressure from a 30 cm head of water to prevent contamination from air and to facilitate rapid transfer of the gas to the evacuated incubation flasks. The <sup>15</sup>N<sub>2</sub> was 91–95 atoms % <sup>15</sup>N. Gas mixtures in the flasks were sampled 10 sec before the end of incubations which were timed from the point at which the gas mixtures reached 1 atm in the flasks. These samples were checked by mass spectrometry for composition and atoms % <sup>15</sup>N. All atoms % <sup>15</sup>N measured, in order to express the results in terms of 100 atoms % <sup>15</sup>N in the incubation gas mixture. In this way, corrections were made for small differences in the <sup>15</sup>N<sub>2</sub> concentrations occurring during the incubations due to differential exchange between residual dissolved nitrogen in the nodule tissues and the <sup>15</sup>N<sub>2</sub> of the gas mixtures.

## (c) Preparation of Nodule Extracts

It was previously shown (Bergersen 1960*a*) that in short experiments, the newly fixed nitrogen of soybean nodules was largely found in the soluble fraction, only small amounts being found (after incubation) in the membrane fraction and none in the bacteroids. In the present studies, nodules after incubation (20-g samples) were ground with glass pestles and mortars at  $0-4^{\circ}$ C and extracted twice with 50 ml of cold M/15 phosphate buffer, pH 7·0. The extract was filtered through organdie and centrifuged at 25,000 g in a refrigerated Servall centrifuge for 20 min. The clear supernatant was then quickly heated at 80°C for 5 min and quickly cooled to 10°C. This treatment inactivated enzymes and precipitated about 70% of the soluble protein. After centrifuging, the clear red-brown supernatant was made up to 100 ml with buffer. These extracts were uniform in all experiments, containing  $1 \cdot 8 - 2 \cdot 0$  mg nitrogen per gram (fresh wt.) nodules;  $40-50 \ \mu g$  of this nitrogen was recovered as NH<sub>3</sub>. It is considered that extracts prepared in this way contained all the nonbacterial, soluble, non-protein nitrogen of the nodules, together with some heat-stable soluble protein. Total nitrogen was determined by the Kjeldahl method.

### (d) Analysis of Ammonia Nitrogen

The soluble fraction prepared as above contained about 800  $\mu$ g NH<sub>3</sub> nitrogen in 100 ml phosphate buffer: 20 ml of this was used on the determination of total nitrogen and NH<sub>3</sub> nitrogen was assayed in two 40-ml aliquots as follows:

The samples were placed in 500-ml Quickfit distillation flasks fitted with capillary air inlets and connected to all-glass condensers and receivers. One drop of anti-foam (a 30% solution in liquid paraffin of Alkaterge C—Chemical Solvents Coorp., New York) was added followed by 40 ml of saturated borate buffer, pH 10.5 (final pH 10.0). The flask was held in a water-bath at 50°C and vacuum distillation

(50 mmHg) was continued for 1 hr, the NH<sub>3</sub> being trapped in 20 ml 2% boric acid in a 1-in. Quickfit tube to ensure complete collection of the NH<sub>3</sub>, which was measured by titration with 0.007 HCl. This method was adopted after trial of a number of methods had shown it to give the best recovery of NH<sub>3</sub> from the relatively large volumes of extract with the least hydrolysis of amides. Table 1 shows that with NH<sub>4</sub><sup>+</sup> concentrations of the order of those encountered in the extracts, recovery of NH<sub>3</sub> was better than 98% with less than 1% hydrolysis of amides.

The validity of the procedures used for extraction of nodules and analysis of free NH<sub>3</sub> depends on the absence of spontaneous or enzymatic breakdown to NH<sub>3</sub> of some precursor and on the absence of loss of NH<sub>3</sub> through conversion to amides or other compounds. The only precaution taken was the use of low temperatures of extraction and centrifugation followed by heat inactivation. That this was sufficient to prevent loss of NH<sub>3</sub> is shown by the following test: Two 21-g samples of nodules were ground as above and to one  $400 \ \mu g \ {}^{15}NH_4^4$  nitrogen (94 atoms %  ${}^{15}N$ ) was added

EVALUATION OF THE METHOD OF $\mathrm{NH}_3$ determination used
Samples were prepared in phosphate buffer ( $M/15$ , pH 7.0) and NH <sub>3</sub> was distilled into 20 ml 2% boric acid under vacuum (50 mmHg) at 50°C
for 1 hr after addition of an equal volume of saturated borate buffer
pH 10.5 (final pH 10.0)

TABLE 1

Sample	Amount of Nitrogen in Flask (mg)	Sample Volume (ml)	Amount of Nitrogen Distilled (mg)	Recovery (%)
NH <sup>+</sup>	$2 \cdot 0$	50	1.840	90.2
-	$1 \cdot 0$	50	0.984	$98 \cdot 4$
	$1 \cdot 0$	50	0.998	99.8
Asparagine	10	50	0	0
Glutamine	5	20	0.040	0.8

before the second 50 ml buffer extraction. Both extracts were then completed and free NH<sub>3</sub> determined as above. The control contained 815  $\mu$ g NH<sub>3</sub> nitrogen whose <sup>15</sup>N content was 0.365 atoms %. The other extract contained 1203  $\mu$ g NH<sub>3</sub> nitrogen with a <sup>15</sup>N content of 30.6 atoms %. These values theoretically should have been 1215  $\mu$ g NH<sub>3</sub> nitrogen; 31.2 atoms % <sup>15</sup>N. The agreement between these values is sufficient to justify the procedures used, although it cannot be demonstrated that the labelled NH<sub>3</sub> did not result from a very unstable precursor.

#### (e) Analysis of Amide Nitrogen

After distillation of  $NH_3$  the water-bath was removed, 10 ml 40% NaOH was added, and the flasks were boiled for 15 min at 1 atm, the resulting  $NH_3$  being trapped in 2% boric acid as before. This procedure gave complete recovery of glutamine and asparagine amide nitrogen in preliminary tests.

	Tota	l Soluble Nitrogen			NH <sub>3</sub> Nitrogen		
Incubation Time (min)	Total Nitrogen (mg/g fresh wt.)	Corrected Atoms % <sup>15</sup> N Excess*	15N Excess (μg/g)	NH <sub>3</sub> Nitrogen (mg/g fresh wt.)	Corrected Atoms % <sup>15</sup> N Excess*	<sup>15</sup> N Excess $(\mu g/g)$	NH <sub>3</sub> <sup>15</sup> N Excess as Percentage of Total <sup>15</sup> N Excess
1.0	1.863	0.0025† 0.0030†	0 · 047 0 · 057	$\begin{array}{c} 0\cdot 0462\\ 0\cdot 0482\end{array}$	$\begin{array}{c} 0\cdot 106\\ 0\cdot 100\end{array}$	$\begin{array}{c} 0\cdot 049\\ 0\cdot 048\end{array}$	104 · 3 84 · 2
2.25	1.828 1.783	0.0126 0.0127	$\begin{array}{c} 0\cdot 230\\ 0\cdot 226\end{array}$	$0.0502 \\ 0.0497$	$\begin{array}{c} 0\cdot 213\\ 0\cdot 186\end{array}$	0.107 0.092	46 · 5 40 · 7
3.0	1 • 965 1 • 989	$0.0172^{+}_{-0.0189^{+}_{-0.0$	$\begin{array}{c} 0\cdot 331\\ 0\cdot 376\end{array}$	$\begin{array}{c} 0\cdot 0514\\ 0\cdot 0491 \end{array}$	$\begin{array}{c} 0\cdot 266\\ 0\cdot 264\end{array}$	0.137 0.130	41 · 1 34 · 6
<b>4 · 0</b>	$\frac{1\cdot873}{1\cdot952}$	0.02681 0.02681	0.502 0.523	$\begin{array}{c} 0\cdot 0469\\ 0\cdot 0457\end{array}$	$\begin{array}{c} 0\cdot 349\\ 0\cdot 378\end{array}$	$\begin{array}{c} 0\cdot 164 \\ 0\cdot 173 \end{array}$	32.7 33.1
* The nat	tural abundance of <sup>1</sup>	5N was taken from i	identical preparat	ions from nodules inc	ubated in air.		

† Mean of two determinations.

TABLE 2

RELATIONSHIP BETWEEN TOTAL SOLUBLE <sup>15</sup>N INCORPORATION AND <sup>15</sup>N INCORPORATION INTO NH3 BY NITROGEN-FIXING SOYBEAN ROOT NODULES Data for experiment 5 F. J. BERGERSEN

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### (f) Analysis of Amino Nitrogen

Aliphatic amino nitrogen was measured, after distillation of  $NH_3$ , by the method of Van Slyke (1929) in a magnetically stirred apparatus (Baird and Tatlock Ltd., England) which had been modified so that the Van Slyke nitrogen could be collected in sample tubes for <sup>15</sup>N determinations in the mass spectrometer. The Van Slyke gas was cooled in liquid air before admission to the mass spectrometer in order to condense traces of impurities remaining after alkaline permanganate absorption (Van Slyke 1929). These impurities interfere with mass 28 and 29 measurements. The method gave reproducible and correct values for amino nitrogen and for mass 29/28 ratios, when standard amino acids of known <sup>15</sup>N content were reacted in the buffer mixtures present in the nodule extracts. Full details of this method will be published elsewhere.

#### (g) Mass Spectrometer Measurements

Isotope ratio measurements on samples of  $NH_3$ , Kjeldahl  $NH_3$ , and amide  $NH_3$  in boric acid after distillation and titration were made as described previously (Bergersen 1962) with an M86 (Atlas-Werke, Bremen) mass spectrometer. In all experiments, instead of using air  $N_2$  the natural abundance of <sup>15</sup>N was determined from the isotopic ratios of nitrogen prepared from equivalent fractions of nodules incubated in air. This measure compensated for small differential isotopic fractionations which occurred during analyses and was a factor in enabling reliable measurement of very small enrichments in short experiments. It also compensated for the abundance of <sup>15</sup>N derived from the HNO<sub>2</sub> in the Van Slyke nitrogen. All <sup>15</sup>N excess values were calculated as micrograms in order to express the quantitative roles of the various labelled components in the total incorporation of <sup>15</sup>N.

Partial pressures of components of gas mixtures were measured from the magnitudes of the peaks of the masses present corrected by the source calibration for each component gas.

In the short experiments, the major limitation was the difficulty in analysing low enrichments of <sup>15</sup>N in the total soluble fraction. At 1 min these enrichments were of the order of 0.003 atoms % excess. This is only one-fifth the minimum level considered to be reliable evidence of  $^{15}N$  incorporation (Burris 1956). In this laboratory 0.005 atoms % excess has been considered until now to be the limit of reliable In the experiments described above, however, by using standard measurement. nitrogen carefully prepared from equivalent unlabelled nodule fractions, adequate replication of measurements on each sample and by reading the mass 29/28 ratios at equal pressures and at equal intervals after admission of samples to the analyser, greater precision was possible. For example, five ratio determinations on a single sample of one soluble fraction gave a mean of 0.0018 atoms % excess before applying any corrections. This enrichment was statistically significant at the 1% level when five similar ratio determinations, performed on a similar sample of nitrogen from untreated nodules, were used to determine the standard natural abundance of <sup>15</sup>N. The variations between duplicate nodule samples were, however, much larger. In the 1-min incubations of experiment 5 this variation was 0.0005 atoms %, which, because of the low enrichment in the nitrogen of the total soluble material, amounted to about 20% of the measured excess (Table 2). The variation between the values

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for the NH<sub>3</sub> was, in contrast, only about 6% and this was reduced further when atoms % were converted to  $\mu g$  <sup>15</sup>N. These considerations illustrate the difficulties of precise determinations of <sup>15</sup>N in samples containing large amounts of extraneous nitrogen and explain the scatter between duplicates encountered in samples with low enrichment. The use of increased replication in short experiments is thus obviously desirable, but difficulties in quickly obtaining large enough batches of detached nodules prevented this in the present work. An alternative might be the removal of more extraneous nitrogen than was accomplished in the relatively mild treatments used in the preparation of the soluble fraction.



Fig. 1.—Relationship between total soluble <sup>15</sup>N incorporation and incorporation into NH<sub>3</sub> and amides as a function of time. Data for experiments  $1 (\bigcirc)$  and  $3 (\bullet)$ .

#### III. RESULTS

Six experiments were carried out. In experiment 1,  $^{15}$ N excess in the various components of the soluble fraction of the nodules was measured between 10 min and 1 hr from commencement. All other experiments were of shorter duration, exposure to the labelled gas mixture being from 1 to 6 min for experiments 2, 4, and 5, from 2 to 10 min for experiment 3, and from 1 to 10 min for experiment 6.

Fixation of  ${}^{15}N_2$  by detached nodules proceeded at a constant rate for at least an hour from about 1 min after commencement of each experiment. Figure 1 shows data from two comparable experiments (1 and 3). The maximum amount of fixed  ${}^{15}N$  in the form of NH<sub>3</sub> was reached in 10 min and after a slight drop, remained constant until 1 hour. Labelled amide nitrogen continued to rise until at 1 hr it was approximately equal to the NH<sub>3</sub> nitrogen. In these and subsequent experiments, fixation into NH<sub>3</sub> was linear with time from 0 to 6 min; i.e. there was no discernible lag. When both total <sup>15</sup>N incorporation and <sup>15</sup>N incorporation into NH<sub>3</sub> were plotted as a function of time, as in Figure 2, the extension of the total line intersected the NH<sub>3</sub> line at 0.9, 1.4, 0.7, and 1.0 min respectively in experiments 2–5. Figure 2 shows this type of plot for experiment 3. This suggested that at about 1 min all the fixed nitrogen in the soluble fraction was in the form of NH<sub>3</sub>. This was demonstrated in experiment 5 (Table 2), in which an average of 94% of the newly fixed nitrogen



Fig. 2.—Data for experiment 3. ○ Total soluble <sup>15</sup>N incorporation. ● NH<sub>3</sub>
<sup>15</sup>N excess. The vertical lines indicate the range of four replicates for the total and two replicates for the NH<sub>3</sub>.

was in the form of NH<sub>3</sub>. The data from four experiments are summarized in Figure 3(a) in which NH<sub>3</sub> <sup>15</sup>N excess is plotted as a percentage of the total soluble <sup>15</sup>N excess. The dotted lines show the extrapolations to the intercept points of data plotted as in Figure 2. Figure 3(b) gives similar data for experiment 6 in which <sup>15</sup>N excess in amino nitrogen, amide nitrogen, and NH<sub>3</sub> nitrogen was measured as a percentage of the total <sup>15</sup>N excess.

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These results show that  $NH_3$  is the primary stable product of fixation appearing in the soluble fraction of these nodules and that after a very brief lag this product is very rapidly incorporated into amino and other compounds with slower incorporation into amides. Thus, even while the absolute amount of  $NH_3$  <sup>15</sup>N was still increasing, the percentage of total fixed <sup>15</sup>N in this form was very rapidly diminishing.



Fig. 3.—(a) NH<sub>3</sub> <sup>15</sup>N excess plotted as a percentage of the total soluble <sup>15</sup>N excess: • experiment 2;  $\bigcirc$  experiment 3;  $\odot$  experiment 4;  $\blacksquare$  experiment 5. (b) Data for experiment 6:  $\bigcirc$  NH<sub>3</sub>; • aliphatic amino;  $\bigcirc$  amide;  $\blacksquare$  residue.  $\mu g$  <sup>15</sup>N excess in each plotted as a percentage of total soluble  $\mu g$  <sup>15</sup>N excess.

### IV. DISCUSSION

The speed with which  $NH_3$  is incorporated into amino compounds (probably amino acids—Aprison, Magee, and Burris 1954) and other soluble compounds has made it difficult to demonstrate that  $NH_3$  is a major early product of nitrogen fixation in detached soybean nodules. However, the data presented above constitute the first strong evidence that  $NH_3$  is the first major stable soluble product of fixation as has been shown with other nitrogen-fixing agents. Any earlier soluble product must, in fact, be present in amounts undetectable by the methods used, or be very rapidly transformed to  $NH_3$ , during the 20–30-min manipulation period at  $0-4^{\circ}C$ .

Extracts of nodules used in these experiments contained about 50  $\mu$ g NH<sub>3</sub> nitrogen per gram fresh weight but the newly fixed NH<sub>3</sub> reached a maximum concentration of only  $0.1-0.3 \mu$ g NH<sub>3</sub> nitrogen per gram in about 6 min, while total incorporation continued in a linear manner for at least 1 hr. In other words, the bulk of the nodule NH<sub>3</sub> was not in equilibrium with the newly fixed NH<sub>3</sub>, at least in experiments of up to 1 hr duration. This was suggested by Aprison, Magee, and Burris (1954) as the probable reason for the relatively low <sup>15</sup>N excess of NH<sub>3</sub> from nodules exposed to labelled gas mixtures for 1 hr in their experiments.

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The site at which nitrogen is bound and transformed by way of bound intermediates to  $NH_3$  remains in doubt. It was proposed previously on the basis of slightly longer experiments (Bergersen 1960*a*, 1960*b*) that this may take place within the membrane envelope which encloses bacteroids and leghaemoglobin in the nodule tissue. The incorporation of  ${}^{15}N_2$  into  $NH_3$  proceeded at a constant rate from the beginning of each of these experiments. If the membrane was the source of this  $NH_3$  one would expect that it would contain a constant amount of  ${}^{15}N$ . Studies of the relationship between  ${}^{15}N$  of the membrane fraction and  $NH_3$   ${}^{15}N$  are now necessary.

### V. Acknowledgments

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