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Incorporation of $^{15}N_2$ into Various Fractions of Soybean Root Nodules

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SUMMARY: Excised soybean root nodules exposed to an atmosphere containing an excess of $^{15}N_2$ incorporated the label first into the centrifugal fraction containing the intracellular membrane envelopes and small amounts of bacteroid cell walls. The ^{15}N concentration then increased in the soluble portion of the nodules. The bacteroid fraction was not labelled after 2 hr. exposure of the nodules to $^{15}N_2$. In ageing nodules incorporation of ^{15}N into the soluble fraction declined before incorporation into the membrane fraction. The inhibitory effects of CO, N_2O and H_2 on $^{15}N_2$ incorporation in the various fractions were studied; the results suggested differential inhibition. The membrane fraction contained 8·5 % (w/v) total N, 37 % (w/w) lipid, had a negligible O_2 uptake in the presence of substrates and had an absorption spectrum suggestive of the presence of porphyrin compounds. Further fractionation of the membrane fraction indicated that the ^{15}N was associated with the lighter particles and was only partially soluble in 3 N-HCl. The possibility that the membrane fraction contained the site of primary N_2 activation is discussed.

Aprison & Burris (1952) obtained consistent nitrogen fixation by exposing to $^{15}N_2$ soybean nodules which had been exised quickly from vigorous plants. Analysing only the 3 N-HCl-soluble portion for ^{15}N, they showed that fixation decreased with time, indicating depletion of an essential substrate, but could obtain no improvement with added substrates. Magee & Burris (1954) extended these findings to nodules of other legumes and showed the advantage of using large active nodules. Aprison, Magee & Burris (1954) used the same system to analyse the distribution of ^{15}N among the nitrogenous compounds of the nodules. Bergersen & Briggs (1958), studying the fine structure of soybean nodules, showed that the intracellular bacteroids were enclosed in a system of double-layered membrane envelopes in the host cytoplasm. A centrifugal preparation from crushed nodules was shown to contain these envelopes contaminated with a small amount of cell-wall material from the bacteroids.

The present paper reports the results of experiments following the course of incorporation of $^{15}N_2$ into the HCl-soluble portion of centrifugal fractions of crushed nodules which had been exposed for various times to an atmosphere containing a relatively large excess of $^{15}N_2$.

METHODS

Plant and bacterial material. Lincoln variety soybeans inoculated with *Rhizobium japonicum*, strain CC711, were grown in a glasshouse in a 1:1 (v:v) mixture of sand:vermiculite+McKnight (1949) nutrient solution. Nodule production and activity was essentially the same as described

previously (Bergersen, 1958). In order that the nodules should be as active as possible plants were given at least 6 hr. daylight before the nodules were quickly picked off into a beaker surrounded with ice (Virtanen, Moisio & Burris, 1955). Samples of nodules (5–7 g.) were exposed for various periods of time to an atmosphere containing $^{15}N_2$ (27 atoms % excess) 0·2 atm.; O_2, 0·2 atm.; He, 0·6 atm.; at 25°.

Fractionation of nodules. Immediately after exposure, the nodules were quickly frozen in stoppered test tubes immersed in solid CO_2: ethanol mixture. Each nodule sample was then fractionated at 1–4° as follows. After crushing in a mortar in M/15 phosphate buffer (pH 7·0) the nodule husks were removed by filtration through Whatman no. 1 filter-paper; preliminary tests showed that this material after washing contained no detectable ^{15}N. (In later experiments larger yields of membrane materials were obtained by replacing this step with filtration through four layers of cotton gauze.) The filtrate was then centrifuged to give the following fractions: (1) the bacteroids sedimented at 5000 *g* in 7–10 min.; (2) the pellet from 20 min. at 23,000 *g* containing membrane envelopes and small amounts of bacteroid cell walls (the membrane fraction); (3) the clear supernatant fluid from 20 min. at 23,000 *g* (the soluble fraction). Fractions (1) and (2) were washed twice in 25 ml. buffer and then suspended in 15 ml. buffer. All three fractions were then made to 3 N-HCl by addition of 6 N-HCl. The acidified suspensions were extracted overnight at 4° and then centrifuged; the acid-soluble nitrogenous material was converted to ammonia by Kjeldahl digestion and distilled into 10 ml. 0·1 N-H_2SO_4; N_2 was generated from the distillate by addition of alkaline hypobromite *in vacuo* and analysed for ^{15}N in a Consolidated-Nier mass spectrometer by determining the mass 29: mass 28 ratio.

Inhibitors. Inhibition of nitrogen fixation by gaseous inhibitors was studied using 0·1 atm. $^{15}N_2$ (27 atoms % excess), and 0·2 atm. O_2, to which was added either CO (0·002 atm.), N_2O or H_2 (0·2–0·6 atm.); helium replaced the inhibitor in the controls and was used to bring the gas mixtures to one atmosphere. Freshly excised nodules were exposed to these mixtures and fractionated as described.

Analysis of membrane fraction. Total nitrogen and HCl-extractable nitrogen were estimated by nesslerization of Kjeldahl digests, and lipid by loss of weight of centrifuged pellets after acetone drying and ether extraction at 30°. Absorption spectra were determined with a Hilger Uvispek and respiratory activity with various substrates was measured manometrically. All measurements were made on the basis of dry weight/ml. of the membrane suspension used.

RESULTS

Time course of $^{15}N_2$ incorporation

Active nodules accumulated between 0·120 and 0·400 atoms % excess ^{15}N in the soluble fraction during 2 hr. of exposure to the isotope. A representative experiment is illustrated in Fig. 1. Significant concentrations of ^{15}N were found in the membrane fractions after 15 min. and these concentrations were

always higher than those in the soluble fractions of the 15 min. samples, except in one case in which nodule activity was so high (0·400 atoms % excess in the soluble fraction after 2 hr.) that the ^{15}N in the soluble fraction probably had already exceeded that of the membrane fraction at 15 min. The data for the 15 min. samples of six experiments are given in Table 1; in all these, fixation into the soluble fraction exceeded 0·150 atoms % excess ^{15}N after 2 hr. exposure. In no experiment in which active fixation occurred was any significant amount of label (i.e. less than 0·005 atoms % excess) found in the HCl-soluble portion of the bacteroids or in the unextracted bacteroids. The inclusion of the bacteroid fraction in assays for isotopic N in the nodule fractions provided a valuable internal negative control which increased the significance of the low concentrations of ^{15}N found in the membrane fraction.

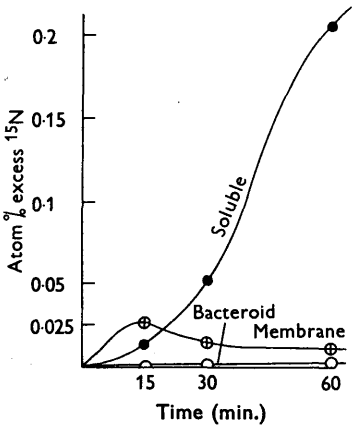

Fig. 1

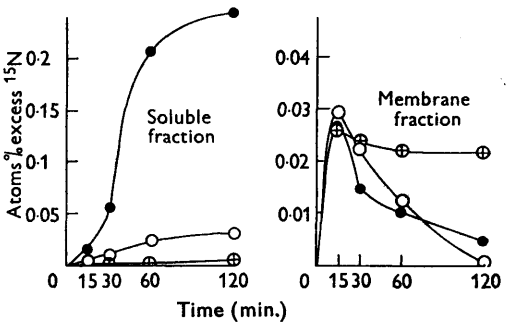

Fig. 2

Fig. 1. Time course of incorporation of $^{15}N_2$ into various centrifugal fractions of soybean nodules exposed to an atmosphere composed of $^{15}N_2$ (27 atom % excess) 0·2 atm.; O_2, 0·2 atm.; He, 0·6 atm. ●, Soluble fraction; ⊕, membrane fraction; ○, bacteroid fraction.

Fig. 2. Time course of incorporation of $^{15}N_2$ into fractions of soybean nodules of various ages. ●, 4 weeks; ○, 5½ weeks; ⊕, 6 weeks.

Table 1. *Atom % excess ^{15}N in fractions of soybean nodules exposed to $^{15}N_2$ for 15 min.*

In all six experiments the soluble fractions contained more than 0·150 atom % excess after the nodules had been exposed to the isotope-containing atmosphere for 2 hr. Gas-phase: 0·2 atm. $^{15}N_2$ (27 atom % excess); 0·2 atm. O_2; 0·6 atm. He.

Experiment	Soluble fraction	Membrane fraction	Bacteroid fraction
	atom % excess ^{15}N		
1	0·014	0·026	0·000
2	0·004	0·029	0·000
3	0·002	0·026	0·000
4	0·011	0·031	0·000
5	0·000	0·019	−0·002
6	0·022	0·030	0·000

In experiments in which the atom % excess ^{15}N of the soluble fraction rose steadily with time for more than 2 hr. the ^{15}N content of the membrane fraction remained almost constant at between 0·020 and 0·030 atoms % excess. In some experiments incorporation of label into the soluble fraction declined after 1 hr., the ^{15}N content of the membrane fraction decreasing from its maximum at 15 min. to nearly zero at 2 hr.

Effects of nodule age

It has been shown previously (Bergersen, 1958), that nodules of the type used in this work cease to function when they are 6 weeks old. Fractionation of $^{15}N_2$-exposed nodules aged 4, 5½, and 6 weeks, gave the data illustrated in Fig. 2. The 4-week-old nodules gave the typical patterns of fixation, 5½-week-old nodules fixed much less ^{15}N into the soluble fraction and this fraction of 6-week-old nodules contained no detectable ^{15}N. The isotopic content of the membrane fractions of the 15 min. sample of nodules of all ages, however, was the same, and that of the 6-week-old nodules remained at this concentration, indicating that in these nodules, nitrogen fixed in the membrane fraction was not released into the soluble fraction.

Effect of CO, N_2O, *and* H_2

Typical results of these tests are given in Table 2. Incorporation of ^{15}N into the soluble fraction was completely inhibited by 0·002 atm. CO and the concentration of isotope in the membrane fraction was decreased to about one-third that of the control, a concentration which is just significant and which was obtained on several occasions. The ^{15}N in the membrane fractions did not decrease with time, an effect similar to, but not as marked, as that in old nodules when nitrogen from the membrane fraction was not transferred to the soluble fraction. Both N_2O and H_2 at a partial pressure of 0·6 atm. inhibited fixation, N_2O completely in all fractions; H_2 decreased the ^{15}N

Table 2. *Effect of gaseous inhibitors of nitrogen fixation on the incorporation of* ^{15}N *into soybean nodule fractions*

For gas phase composition see text.

Treatment	Fraction	Atom % excess ^{15}N after exposure of nodules for 15 min.	Atom % excess ^{15}N after exposure of nodules for 2 hr.
Control	Soluble	0·000	0·229
	Membrane	0·019	0·018
0·002 atm. CO	Soluble	0·005	−0·004
	Membrane	0·007	0·007
Control	Soluble	0·022	0·195
	Membrane	0·030	0·014
0·6 atm. N_2O	Soluble	0·000	0·005
	Membrane	0·003	0·004
0·6 atm. H_2	Soluble	−0·002	0·023
	Membrane	0·005	0·003

of the membrane fraction below the limits of significance, and after 2 hr. the soluble fraction contained only about one-eighth the isotope of the same fraction of the control.

Analysis of the membrane fraction

The most notable feature shown by analysis of the membrane fraction was the high value for lipid of 37% (w/v). Total N was 8·5% (w/v); respiration was negligible at 3 μl./hr./mg. dry weight, and was not increased by added succinate, malate, citrate or glucose; there were no detectable cytochrome pigments. These findings make it unlikely that plant mitochondrial material is included in the membrane fraction. The absorption spectrum showed peaks at 420, 440, 503, 535, 600, 630 and 680 mμ and is suggestive of a mixture of porphyrins and/or porphyrin complexes. Soybean nodules are known to contain relatively large quantities of free porphyrins (Klüver, 1948; Dr J. E. Falk, private communication). Most of the peaks in this absorption spectrum can be attributed to porphyrins (Lemberg & Legge, 1949), perhaps strongly absorbed on the membrane material. In preliminary attempts to obtain particles from the membrane fraction which contained a higher ^{15}N content, it was found that material which sedimented at 23,000 *g* in 20 min. consistently had more ^{15}N than that of particles sedimenting at 12,000 *g* in 10 min. Further work on this has not as yet been possible because of the limited amount of membrane fraction available for analysis; 15 g. nodule yields only *c.* 20 mg. of washed fraction. Digestion of the membrane fraction residue after 3N-HCl extraction showed that only a portion of the ^{15}N was extracted: e.g. a sample whose acid extract contained 0·026 atoms % excess still had 0·016 atom % excess in the residue nitrogen. In contrast, the precipitated material from the acid-treated soluble fraction contained no detectable ^{15}N.

Re-examination of the bacteroid fraction showed that about 30% of the cells still had intact cell walls, and it was found that a cell-wall preparation which sedimented at 5000 *g* in 10 min. could be obtained from the bacteroids after 20 min. sonic treatment in a 10 k.c. Raytheon magnetostrictive oscillator. When such a preparation was made from bacteroids from nodules exposed to $^{15}N_2$ no excess of isotope was found in the HCl-soluble extract, although that of the membrane fraction of the same nodules contained 0·030 atom % excess ^{15}N.

DISCUSSION

The data presented in this paper strongly suggest that the site of the primary reactions of N_2 fixation in soybean root nodules is located in some component of the membrane fraction, since this has been shown to attain its maximum atom % ^{15}N excess before the soluble fraction, while the bacteroid fraction was unlabelled after exposure for 2 hr. to an isotope-enriched atmosphere. Additional evidence is provided by the ageing and inhibition data which show that, under certain circumstances, the membrane material may take up N_2 without the formation of any detectable fixation products, as shown by the absence of ^{15}N from the soluble fraction. It is recognized that there is a possibility that these results may be misleading in that the early labelling of the

membrane fraction may be due to the accumulation of ^{15}N compounds formed in low concentrations elsewhere, but undetected because of the large amounts of unlabelled HCl-extractable N compounds in the other fractions. Further development of this work will not be easy because of the small amounts of material involved and the low sensitivity of the ^{15}N techniques, as compared with radioactive isotopic methods. However, the identification of the labelled N-compounds of the membrane fraction and a study of the properties of the active portion of this material may provide a starting-point for further study of the chemistry of the first stages of the N_2 fixation phenomenon.

The membrane fraction, as seen in electron micrographs of sections of embedded centrifuge pellets, consists largely of fragments of the intracellular membrane envelopes, which in the intact nodule contain the bacteroids (Bergersen & Briggs, 1958). The high lipid content of the fraction is consistent with this, and the fact that the ^{15}N was associated with the lighter particles suggests that the newly fixed N is found in the membrane fragments rather than in the small amounts of bacteroid cell walls in the fraction. This is confirmed by the absence of ^{15}N excess in the cell walls of bacteroids isolated from nodules exposed to an isotopically enriched atmosphere. The finding that the bacteroids contained no label in these experiments is in agreement with Turchin (1957) who found that the fixed N was associated with the plant material rather than with the bacteroids.

In nodules ceasing to function because of age the data indicate that the removal of fixed N from the membrane fraction is the point at which breakdown first occurs, since the membrane fraction initially contained the same amount of ^{15}N, irrespective of the amount of isotope which finally appeared in the soluble fraction. When no ^{15}N was transferred to the soluble fraction the ^{15}N of the membrane fraction remained constant.

The gaseous inhibitors, at the concentrations used, gave results which suggest that they may provide a means of selectively blocking various reactions. Thus the results with CO suggest that the primary steps in the fixation process were not affected as much as the subsequent transfer of fixed products to the soluble fraction. In contrast, N_2O and H_2 affected both membrane and soluble fractions to the same extent at all concentrations used, H_2 being less inhibitory at 0·6 atm. than N_2O.

If it be conceded that the site of primary fixation is in the membrane envelopes the nodule bacteriac an no longer be regarded as being nitrogen-fixing agents; they play an essential part in the development of the structures of the fixing organs, but their metabolic role must be somewhat removed from the activation and transformation of molecular nitrogen. However, because of the bacteroid cell-wall component of the membrane fraction the bacteroid surface cannot be definitely excluded as the site of these reactions, although the evidence so far obtained favours the membrane envelope.

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