Purification and Some Properties of the Nitrogenase From Soybean (Glycine max Merr.) Nodules[1]

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Abstract. **The nitrogenase system in cell-free extracts of soybean nodule bacteroids was fractionated into 2 components by use of protamine sulfate or polypropylene glycol precipitation followed by chromatography on DEAE-cellulose. Iron and molybdenum were concentrated in 1 fraction and iron in the other. Combination of fractions resulted in a striking stimulation in activity relative to the activity of individual fractions. The effect of different proportions of the 2 fractions on specific activities was studied. The ratios of the rates of reduction of acetylene and N_2 by extracts or fractions of different purities were relatively constant. Extracts or fractions retained most of their nitrogenase activities when stored in liquid N_2.**

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The development of reproducible procedures for the preparation of cell-free extracts containing active nitrogenase has contributed greatly toward an understanding of the biochemistry of nitrogen fixation. As pointed out recently (11, 13) a major portion of the information from cell-free N_2 fixation experiments has been obtained with extracts of *Clostridium pasteurianum* and *Azotobacter vinelandii*. Cell-free extracts that are active in nitrogen fixation also have been prepared from several other organisms (11, 13). The nitrogenase systems from *C. pasteurianum* and *A. vinelandii* have been purified extensively by Mortenson *et al.* (20, 21) and by Bulen *et al.* (6) respectively. Nitrogenase from *C. pasteurianum* (21) has been resolved into 2 components, one having a molecular weight of approximately 100,000 and containing molybdenum, non-heme iron, and magnesium and a second containing non-heme iron and having a molecular weight of about 40,000. The molybdenum-iron component referred to by Mortenson *et al.* (21) as molybdoferredoxin was estimated to be 78% pure. Both fractions are required for ATP-dependent hydrogen evolution, electron-dependent ATP utilization, and nitrogen fixation.

Bulen *et al.* (5) have developed a simplified assay for measurement of nitrogenase in which sodium hydrosulfite functions as the electron donor. This assay has been used to monitor the progress in purifying the enzyme system from *A. vinelandii* (6). Nitrogenase from this source also was resolved into 2 components, 1 containing non-heme iron and molybdenum, and the other non-heme iron. Both of these were shown to be essential for N_2 reduction, ATP-dependent hydrogen evolution and the release of Pi associated with these reactions (6). An investigation (6) of the stoichiometry of the reaction revealed that 5 molecules of ATP were needed for each electron pair transferred.

Kelly, Klucas, and Burris (14) also have fractionated the nitrogen-fixing complex from *A. vinelandii* into 2 components and have described a method of storage whereby the activity of the purified fractions may be maintained for long periods of time. This method, which involves freezing and storing the preparations in liquid N_2, greatly facilitates further purification of the fractions.

The preparation of cell-free extracts containing active nitrogenase from nodules of legumes has been difficult because the enzyme from this source is unusually labile. By use of a press designed to operate under anaerobic conditions, Bergensen (3) demonstrated that a soybean nodule brei retained a low nitrogen-fixing capacity. The activity of this system was independent of exogenous reductant and an ATP-generating system. Further research (4, 17) established that the nitrogen-fixing activity in the brei was associated with the bacteroids. Active cell-free extracts of legume nodules were not prepared until Koch, Evans, and Russell (16, 17) developed a procedure utilizing a buffered ascorbate medium and insoluble polyvinylpolypyrrolidone to remove endogenous phenolics from macerated nodules under anaerobic conditions. Ammonia in crude extracts of bacteroids prepared by this method was removed by gel filtration and the essential components for the nitrogenase reaction were established. Activity of bacteroid nitrogenase required sodium hydrosulfite as the reductant and an ATP-generating system. More recently (15) an electron-donating

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system consisting of β-hydroxybutyrate, β-hydroxybutyrate dehydrogenase, NAD^+ and benzyl- or methylviologen has been shown to function in place of sodium hydrosulfite.

The purpose of this paper is to describe the partial purification and some properties of the nitrogenase from soybean nodule bacteroids.

Materials and Methods

Source of Nodules. Soybean plants (*Glycine max* Merr. var. Merrit or Chippewa) inoculated with a commercial strain of *Rhizobium japonicum* (kindly supplied by Dr. Joe Burton of the Nitragen Co.) were cultured in a greenhouse or in growth chambers using a nitrogen-free nutrient solution (pH 6.7) in which phosphate was supplied as a mixture of KH_2PO_4 and K_2HPO_4 (1). Since large quantities of nodules were needed for purification of the nitrogenase at least 1000 cultures, each with 8 plants in an 8-inch pot containing perlite, were maintained continuously. Cultures were flushed twice daily with nutrient solution and every fourth day with water to remove any accumulated salts. When plants were 35-days old, an average of 800 soybean plants (100 cultures) were harvested and the root systems were washed with tap water. From such a harvest about 300 g of nodules were collected and washed with tap water. Within 1 hr after excision the nodules were used for the preparation of extracts or were frozen in liquid N_2 and stored at —70°.

Preparation of Crude Extract. All steps in the preparation of the extract were performed under anaerobic conditions. This was accomplished by sparging buffers or other reagents with purified N_2 or A (19), by transferring solutions with hypodermic syringes and by carrying out certain operations in a glove-box filled with N_2. In a typical experiment 150 g of either fresh or frozen nodules, 50 g of insoluble acid-washed polyvinylpolypyrrolidone (18) (Polyclar AT, General Aniline Corp.) and 400 ml of 0.02 M potassium phosphate buffer containing 0.2 M sodium ascorbate at pH 7.4 were placed in the vessel of an Omnimixer (Ivan Sorvall and Co.) fitted with gassing vents. The contents of the vessel were flushed with N_2 for 10 min at room temperature following which the nodules were macerated by blending for 5 min at a setting of 50 volts on a variable transformer. The macerated mixture was squeezed through 4 layers of cheesecloth and the filtrate centrifuged at 7000*g* for 10 min. The supernatant was discarded and the pellet containing the bacteroids was mixed with 80 ml of 0.025 M tris buffer at pH 7.4. After centrifuging at 7000*g* for 10 min, the pellet was suspended in 20 ml of the respective buffers specified in the legends of figures 1, 2, and 3. Some nodules contained an exceedingly active poly-β-hydroxybutyrate depolymerase which produced β-hydroxybutyric acid and thus a sharp decrease in pH of extracts. Solutions of 0.1 M tris or 0.05 M TES[N-tris(hydroxymethyl)methyl-2-amino-ethane sulfonic acid] buffer were used in some experiments (figs 2 and 3) to prevent this difficulty. Suspended cells were broken under anaerobic conditions with an Aminco French press at a pressure of 16,000 lb/sq inch. The macerated cells were collected under a stream of N_2 and centrifuged at 40,000*g* for 50 min. The supernatant was used immediately or was injected into a 50-ml polyethylene vial containing liquid N_2 and stored in a Dewar flask filled with liquid N_2. The frozen extract after storage was placed into a serum bottle which was subsequently capped and flushed with A, and the extract allowed to thaw.

Chromatography. After preliminary fractionation procedures (See figs 1, 2, and 3), the bacteroid nitrogenase was purified on columns of DEAE-cellulose (Whatman DE32) using anaerobic techniques essentially the same as those described by Munson *et al.* (22) and Kelly *et al.* (14). The DEAE-cellulose was precycled with 0.5 N HCl, 0.5 N KOH (15 volumes of each for each weight of dry DEAE-cellulose) and then with sufffficient distilled water until the pH was near neutrality. Columns were equilibrated with either 0.05 M TES at pH 8.0 or 0.025 M tris-Cl at pH 7.4 (See figs 1 and 2). Reservoirs of buffers for eluting the column were sparged with high-purity N_2 prior to use and a trace of sodium hydrosulfite was added to all buffered salt solutions just before the chromatographic procedure was initiated. The temperature of the columns was maintained at 9° by use of an external water jacket connected to a temperature-controlled water bath. The extract was layered on the surface of the anaerobic column with a hypodermic syringe, and 10 ml fractions were collected through a hypodermic needle injected into rubber-capped serum bottles maintained anaerobic by a stream of high-purity nitrogen flowing through the bottle. The flow rates of the columns were about 60 ml per hr.

Assays. Nitrogen fixation was assayed by the method of Dilworth *et al.* (9). The standard assay mixture in a final volume of 1.5 ml contained: 100 μmoles of TES buffer at pH 7.4, 10 μmoles of $MgCl_2$, 50 μmoles of creatine phosphate, 5 μmoles of ATP, and 0.5 mg of creatine phosphokinase. All reagents were adjusted to pH 7.4 with KOH. Each bottle (21 ml volume) was flushed 5 times with high-purity N_2 or A. Subsequently, 40 μmoles of $Na_2S_2O_4$ from an 80 mM solution in 0.05 M TES buffer at pH 7.4 were injected into each bottle. The reaction was initiated by injecting an appropriate amount of extract into the bottle through the rubber serum cap. After incubation with shaking for 20 min in a water bath at 30°, the reaction was terminated by injection of 2 ml of saturated K_2CO_3 solution. Ammonia was distilled into 1 N sulfuric acid by the procedure described by Dilworth *et al.* (9), and after 2 hr the amount was estimated colorimetrically by the use of Nessler's reagent (24).

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Acetylene reduction was measured in reactions by the procedure reported by Kelly *et al.* (14) and HCN and azide reduction by the method of Hardy and Knight (12). After 1-ml aliquots of the extract were digested with concentrated H_2SO_4 and 30 % H_2O_2 solution as described by Bulen and LeComte (6), iron content was determined colorimetrically by the use of *o*-phenanthroline (2), and molybdenum by the dithiol procedure (7).

After solubilization of particulate material by the addition of 0.1 ml of a 10 % sodium deoxycholate solution to each 0.1 ml of extract, the protein content of the extracts was estimated by use of the biuret reagent of Gornall *et al.* (10).

Results and Discussion

Purification. For the experiment illustrated in figure 1 the crude extract in 0.05 M TES buffer, pH 7.4, was treated with protamine sulfate, heated and centrifuged before application to a column of DEAE-cellulose equilibrated with 0.05 M TES buffer at pH 7.4. Some proteins failed to adhere to the DEAE-cellulose column and were eluted rapidly. The addition of 0.025 M $MgCl_2$ to the buffer eluted a fraction (between 100 and 110 ml) that contained a relatively high iron content and some hemoprotein. A major peak of protein, greenish-brown in appearance, was removed from the column between 170 and 190 ml of eluate. This fraction contained 95 % of the nitrogenase in the eluate and a relatively high concentration of iron and molybdenum. The application of 0.2 M $MgCl_2$ solution to the column resulted in the elution of additional proteins that also were relatively high in iron content. This fraction (210–230 ml of eluate) contained about 5 % of the nitrogenase in the total volume of eluate. Assays of the various fractions for nitrogenase provided no evidence of separation of the enzyme system into more than 1 component.

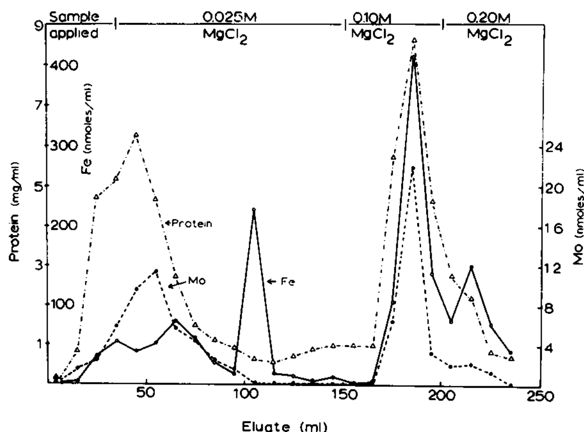

FIG. 1. Chromatography of soybean bacteroid nitrogenase in TES buffer. In preparation for the chromatography, 40 ml of crude bacteriod extract (680 mg of protein) in 0.05 M TES buffer at pH 8 was treated with 50 mg of protamine sulfate per g of protein. The tube was sealed under A, heated for 5 min at 55°, cooled to 5°, and then centrifuged at 35,000*g* for 30 min. The supernatant was transferred to an anaerobic DEAE column (2.5 × 7 cm) that previously had been equilibrated with 0.05 M TES buffer at pH 7.4. The column was eluted with 0.025 M $MgCl_2$, 0.1 M $MgCl_2$ and 0.2 M $MgCl_2$, each in 0.05 M TES at pH 7.4, as indicated. Fractions of 10 ml were collected and assayed for protein, nitrogenase activity, iron, and molybdenum.

The major colored fraction from the experiment (170–190 ml of eluate, fig 1) was centrifuged for 16 min at 52,000 rpm in a Spinco Model E centrifuge. From the Schlieren patterns it was indicated that approximately 88 % of the protein in the extract could be accounted for by the major colored fraction. After centrifugation for 12 min the major peak in the Schlieren pattern separated into 2 components with estimated apparent s values of 8.0 and 4.5.

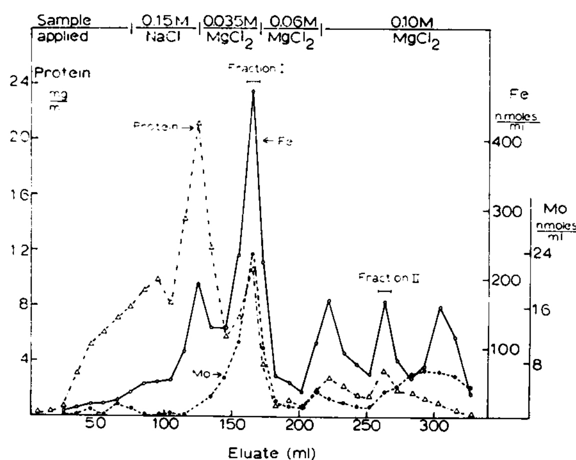

FIG. 2. Fractionation of bacteroid nitrogenase into 2 components. In the preparation for chromatography, 74 ml of crude extract (950 mg protein in 0.025 M tris-Cl pH 7.4) was treated with protamine sulfate (50 mg/g of protein) and centrifuged at 35,000*g* for 10 min. The supernatant liquid was placed on an anaerobic DEAE column (2.5 × 13 cm) that previously was equilibrated with 0.025 M tris-Cl at pH 7.4. The column was eluted with salt solutions, all prepared in 0.025 M tris-Cl at pH 7.4, as shown in the diagram. Fractions of 10 ml were collected and assayed for nitrogenase activity, protein, iron and molybdenum as indicated in the Materials and Methods.

Although further purification is essential, these results provide a tentative suggestion that the 2 major components observed in Schlieren patterns may represent 2 components of the bacteroid nitrogenase.

Another fractionation procedure was utilized that was essentially that employed by Kelly *et al.* (14) for the resolution of *A. vinelandii* nitrogenase into 2 fractions. The major differences between this procedure and that described in figure 1 are as follows: the extract was prepared in 0.025 M tris-Cl. pH 7.4, the column equilibrated with tris rather than

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Table I. *A Summary of the Fractionation of Bacteroid Nitrogenase Into Two Components*
Details of the purification procedure are presented in figure 2.

	Total protein	Total activity	Specific activity	Activity recovery	Fe	Mo
	mg	*units*[1]	*units/ mg protein*	*%*	*nmoles/ mg protein*	*nmoles/ mg protein*
Crude extract	950	8700	9.2	100	8	1.1
Fraction 1	106	0	0	0	44	2.2
Fraction 2	34	190	5.6	2	50	1.0
Fractions 1 and 2	27	2500	72.5[2]	29	...	...

[1] One unit is equivalent to 1 nmole of N_2 reduced per min.
[2] This value is based upon the protein content of fraction 2 which was limiting. In this reaction 0.2 ml of each fraction (2.1 mg of protein from fraction 1 and 0.68 mg from fraction 2) was utilized in the assay.

TES buffer, and NaCl solution was used for elution prior to the addition of solutions of $MgCl_2$. The addition of 0.15 M NaCl to the column resulted in the elution of considerable protein including some hemoproteins (fig 2). A major greenish-brown component was removed between 160 and 170 ml of eluate. This fraction contained considerable Fe and Mo and is referred to in figure 2 as fraction 1. The addition of 0.06 M $MgCl_2$ followed by 0.1 M $MgCl_2$ resulted in the elution of other colored fractions containing appreciable Fe, but only the 255 to 265 ml eluate (fraction 2, fig 2) proved to show activity when combined with fraction 1. The final protein collected from the column between 280 and 330 ml of eluate was blue-gray in appearance and did not possess any nitrogenase activity. The results summarized in table I show that fraction 1 exhibited no nitrogenase activity when assayed alone. Weak activity was shown by fraction 2 alone, but a combination of equal volumes of fractions 1 and 2 resulted in a striking increase in specific activity (table I). The only other fraction with activity was the eluate between 205 to 240 ml (fig 2) but this activity was dependent upon the addition of fraction 2 to the assay. From these results it appears that the nodule bacteroid nitrogenase consists of at least 2 protein components. Investigation of the homogeneity of the 2 fractions in the Spinco Model E centrifuge has been complicated by precipitation of the fractions during the operation. Gel electrophoresis revealed a major component and several minor components in each of the 2 fractions but air was not excluded during the procedure and thus the extent of denaturation during electrophoresis is not known. From analyses and from spectra showing fairly broad absorption bands in the region near 415 mμ, these fractions are similar to the iron-molybdenum protein and the iron protein identified as components of the nitrogenase from *A. vinelandii* (6) and *C. pasteurianum* (21).

Recombination of Fractions. A third fractionation was employed that was similar to the one described in figure 2, with the exception that the protamine sulfate precipitation step was omitted and a preliminary polypropylene glycol (PPG) precipitation was carried out (fig 3). Chromatography of the PPG fraction on DEAE-cellulose separated the nitrogenase system into 2 components, neither of which exhibited activity alone. An experiment was performed (fig 3 and Expt A table II) where the concentration of fraction 1 was held constant at 1.5 mg of protein per reaction and fraction 2 was added to a series of reactions in increments to obtain a maximum of 2.7 mg of protein per reaction. In

Table II. *Effect of Different Combinations of Fractions 1 and 2 on Specific Activities*
Data were calculated from results presented in figure 3. Assays were conducted by the standard procedure in Materials and Methods.

Expt	Nitrogenase components added: Fraction 1	Fraction 2	Specific activities: Based on protein in varied fraction	Based on total protein
	mg protein		*nmoles N_2 fixed per mg protein per min*	
A	1.5	0.2	104	13
	1.5	0.4	115	26
	1.5	0.9	86	32
	1.5	1.8	54	29
	1.5	2.7	36	23
B	0.4	0.9	140	42
	0.8	0.9	84	39
	1.5	0.9	51	32
	3.0	0.9	26	21
	4.6	0.9	15	13

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another series (fig 3 and Expt B table II) fraction 2 was held constant at 0.9 mg of protein per reaction, and increasing increments of fraction 1 were added. Increasing the concentration of the varied fractions yielded typical enzyme saturation curves. From the results presented in figure 3 and table II, it is essential, in the determination of the specific activity of a fraction, to provide an excess of one fraction and estimate the specific activity of the other fraction by varying this fraction in the range where the response approaches linearity. Optimum combinations of the 2 fractions can be obtained by observing maximum specific activities from combinations based on total protein (table II). Such data however are of little value for the assessment of purification of individual fractions.

Relation Between Acetylene and N_2 Reduction. The capacity of soybean nodules and nodule extracts to carry out nitrogenase-dependent acetylene reduction was used as a sensitive assay leading to the initial demonstration of cell-free nitrogen fixation

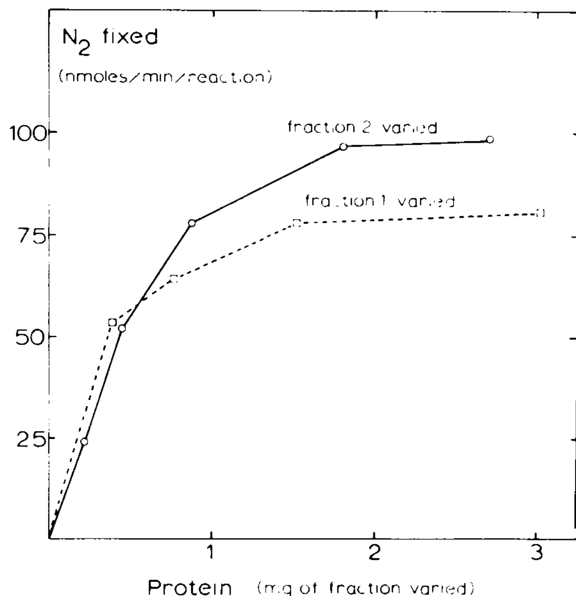

FIG. 3. Nitrogenase activity as influenced by different combinations of fractions. The fractionation procedure was described in figure 2 with the exception that protamine sulfate precipitation was omitted and instead the extract was fractionated with polypropylene glycol, (PPG) (P400 Matheson Coleman and Bell Corp.). Sufficient PPG was added to obtain a concentration of 6.25 ml per g of protein. The precipitated protein was dissolved in 0.05 M tris-Cl buffer at pH 8.0 and the chromatography performed as described in figure 2. Each reaction in the experiment where fraction 2 was varied contained a constant amount of fraction 1 (1.5 mg protein) and the concentrations of fraction 2 indicated. In the experiment where fraction 1 was varied, a constant amount of fraction 2 (0.9 mg protein) was added and fraction 1 was varied as indicated. Nitrogenase activity was assayed by the standard assay procedure (Materials and Methods).

Table III. *Relationship Between the Reduction of Acetylene and Nitrogen by Extracts and Fractions of Nodule Bacteroids*

The extracts of bacteriods were prepared by the method described in figure 3. Assays for acetylene reduction and for nitrogenase activity are described under Materials and Methods. When fractions 1 and 2 were assayed together, 0.05 ml of each fraction (0.29 mg protein from fraction 1 and 0.20 mg from fraction 2) was utilized and the results expressed on the basis of the protein in fraction 2.

Type of extract	Rate of reduction		Ratio of reduction C_2H_2 : N_2
	C_2H_2	N_2	
	nmoles per mg protein per min		
Crude	35.2	11.8	2.98 :1
25–55 % PPG ppt	76.1	27.4	2.78 :1
Fraction 1	0.0	0.0	...
Fraction 2	74.4	19.3	3.85 :1
Fractions 1 and 2	607.4	192.6	3.15 :1

extracts of nodule bacteroids (16, 17). Since the reduction of acetylene to ethylene requires 2 electrons per molecule whereas 6 electrons are required for the reduction of N_2 to 2 molecules of NH_3, it is of considerable interest to investigate the relationship between acetylene reduction and N_2 reduction by crude extracts and by partially purified fractions. As indicated in table III, crude extract, a polypropylene glycol precipitate and a combination of fractions 1 and 2 all rapidly catalyzed the reduction of both acetylene and N_2. Fraction 1 alone exhibited no activity in either assay, but fraction 2 alone exhibited considerable activity in both assays and therefore must have been contaminated with fraction 1. An average of ratios of rates of acetylene reduction to rates of N_2 reduction gives a value of 3.19, which is close to the theoretical value of 3.0. With the exception of the ratio obtained with fraction 2, ratios of rates of acetylene reduction to N_2 reduction were very close to 3.0. The absolute amount of N_2 reduced was very low in the test where fraction 2 was assayed alone and thus considerable error might have been associated with this measurement. These data support the conclusion that acetylene reduction and N_2 reduction are catalyzed by the same enzyme. Apparently the flow of electrons to the substrate is limiting the rate of the reaction rather than the interaction of the substrate with the active site (11).

The nitrogenase system from nodule bacteroids not only reduces acetylene and N_2 but also catalyzes an ATP-dependent reduction of KCN and NaN_3 and in this respect behaves like the nitrogenase from *C. pasteurianum* and *A. vinelandii* (12, 23).

Enzyme Stability. Experimentation with nitrogenase from legumes requires an adequate source of nodules and also methods for maintaining activities of partially purified extracts. From table IV it is clear that soybean nodules that were frozen in liquid

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Table IV. *The Stability of Nitrogenase From Soybean Nodules*

The methods for the preparation of nodules and crude extracts are described under Materials and Methods. Nodules were frozen, where indicated, by immersing in liquid N_2, then removed and stored at —70° for 30 days. Extracts were frozen and kept in liquid N_2 as described in Materials and Methods. Fractions of the bacteroid nitrogenase were prepared by the procedure outlined in figure 2.

Materials	Conditions	Specific activity Before storage	After storage	Recovery
		nmoles N_2 fixed per mg protein per min		%
Crude extract	From fresh nodules	7.6	...	...
Crude extract	From frozen nodules	7.2	...	...
Crude extract	Stored under A at 0° for 24 hr	7.6	6.4	84
Crude extract	Stored under A at 25° 24 hr	9.8	6.6	67
Crude extract	Stored in liquid N_2 for 7 days	7.2	7.6	105
Fractions 1 and 2	Stored in liquid N_2 for 7 days	72.0[1]	50.0[1]	69

[1] Specific activity is based on the protein content of fraction 2 since fraction 1 was added in excess.

N_2 and then stored at −70° for 30 days retained most of their activity. Crude extracts that were kept anaerobic and stored at 0° for 24 hr maintained 84 % of the original activity whereas comparable extracts stored in liquid N_2 for 7 days lost no activity. Storage of crude extracts under A at 25° for 24 hr resulted in a loss of 33 % of the activity. Fractions of the enzyme also were fairly stable when stored in liquid N_2. Other experiments have shown conclusively that activities of fractions or crude extracts are lost rapidly when exposed to air.

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