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Short Communication

Reduction of Acetylene and Nitrogen Gas by Breis and Cell-free Extracts of Soybean Root Nodules

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Cell-free extracts of *Clostridium pasteurianum* (8) and of *Azotobacter vinelandii* (2) in the presence of an ATP generating system and an appropriate electron donor, catalyze the reduction of N_2 gas to ammonia. In recent investigations (3, 4, 9) of the properties of nitrogenase from these organisms, it has been established that the enzyme system is non-specific for electron acceptor and will catalyze the reduction of nitrous oxide, cyanide, azide and acetylene in addition to N_2. No reproducible procedures have been reported for the preparation from legume nodules of cell-free extracts capable of catalyzing the reduction of N_2 gas. Bergerson (1) has described a method for the demonstration of nitrogen-fixation with a brei from soybean root nodules. In his procedure, strictly anaerobic conditions were necessary for the preparation of the brei and the sensitive ^{15}N technique was used for the detection of N_2 fixation. In most of the experiments no more than 6 mμ atoms of N were fixed per mg of brei protein.

We have approached the problem of nitrogen fixation by nodules of symbionts by measuring the rate of reduction of acetylene to ethylene. Intact soybean nodules were shown to catalyze the reduction of acetylene to ethylene at a rapid rate and the conditions optimum for the reduction determined (5). The purpose of this communication is to report conditions necessary for catalysis of acetylene reduction by breis and cell-free extracts of nodules and the catalysis of N_2 reduction to ammonia by a cell-free extract of a nodule bacteroid preparation.

Soybean plants were cultured in the greenhouse or in growth chambers and supplied with a nitrogen-free nutrient solution as described previously (5). When plants were 36 to 40 days old, nodules were harvested, washed with tap and distilled water and utilized for the various experiments. The production of ethylene from acetylene was assayed by a gas chromatographic procedure (5) and the reduction of N_2 gas to ammonia was determined by the method of Mortenson (7).

Whole soybean root nodules consistently catalyze the reduction of acetylene to ethylene (5) but nodules macerated under aerobic or anaerobic conditions in buffers of various types catalyze little or no acetylene reduction. The possibility that phenolic compounds might inhibit enzymes in nodule extracts was suggested by the observation that a DEAE column removed large quantities of green to brown pigments from nodule extracts. These observations led to an investigation of procedures for the removal of phenolics or related compounds from nodule extracts. Loomis and Battaile (6) have shown that a solid preparation of polyvinylpyrrolidone (Polyclar AT from the General Aniline Corp.), (PVP) effectively removed phenolic compounds from plant extracts prepared in a buffered ascorbate solution and thus prevented phenolic oxidation products from inactivating certain plant enzymes. These procedures (6) were incorporated into a method for the preparation of nodule extracts for use in acetylene and N_2 reduction experiments.

In a typical experiment 100 g of soybean nodules were collected, washed, and placed in a glove bag which was evacuated and flushed with argon 3 times. The nodules were macerated in 150 ml of 20 mM potassium phosphate buffer (pH 7.2) containing 1 mM $MgCl_2$ and 200 mM ascorbate adjusted to pH 7.0 with KOH. The macerate was squeezed through cheese cloth and mixed with 50 g of acid-washed PVP. After 10 minutes the PVP and macerate was squeezed through 100-mesh bolting cloth and the resulting brei utilized for the experiment described in table I. For the preparation of cell-free extracts the brei, maintained under anaerobic conditions, was centrifuged at 34,000 $\times$ *g* for 15 minutes, the supernatant discarded and the bacteroid fraction suspended in a volume of either 20 mM potassium phosphate or 50 mM potassium cacodylate buffer at pH 7.2, (tables II and III) equal to the volume of bacteroid pellet. The suspension was placed in a French press under an atmosphere of argon, cells were forced through

Table I. *Acetylene Reduction by Soybean Nodule Brei as Influenced by Polyvinylpyrrolidone and Ascorbate in the Brei Preparation*

Each reaction tube (32 ml) contained 10 ml of nodule brei (25 mg protein per ml by microkjeldahl) representing 2.5 gms of 36 day-old nodules. The brei with or without ascorbate and PVP as indicated was prepared in a solution of 20 mM potassium phosphate buffer at pH 7.2 and 1 mM $MgCl_2$. The gas volume (22 ml) in each reaction tube was composed of 0.25 atm of oxygen. 0.1 atm of acetylene and 0.65 atm of argon. Reaction mixtures were incubated at 28° on a reciprocating shaker. At the times indicated, 1 ml samples of gas were removed through the serum cap of each tube and analyzed for ethylene.

Preparation of brei	Ethylene produced Incubation time (min) 30	45	60
	μmoles	μmoles	μmoles
With PVP	0.97	5.93	7.52
Without PVP	0.04	0.06	0.08
With PVP and 0.3 M ascorbate	5.34	13.92	14.11
Without PVP, with 0.3 M ascorbate	0.21	0.46	0.48

the orifice at a pressure of 24 tons per square inch and broken cells were collected under argon. The suspension of broken cells was centrifuged at 2° for 20 minutes at 34,000 × *g* and the supernatant fraction kept under argon for use in experiments described in tables II and III.

The effect of PVP and ascorbate treatments on the capacity of the brei to catalyze the reduction of acetylene is presented in table I. Brei prepared with PVP but without ascorbate catalyzed the reduction of acetylene to ethylene at a rate of 7.5 μmoles per hour. When PVP was omitted in the procedure the resulting brei and reactants produced only 0.08 μmoles of ethylene per hour. In contrast nodule brei prepared with PVP and 0.3 M ascorbate in buffer catalyzed the reduction of acetylene at a rate of over 14 μmoles per hour. The brei prepared in a medium with ascorbate but without PVP showed weak activity. Although all breis were prepared under anaerobic conditions, oxygen was essential in reaction mixtures for acetylene reduction. In other experiments the optimum concentration of ascorbate in the brei preparation medium proved to be 0.2 M.

Table II. *Requirements for Catalysis of Acetylene Reduction by a Cell-free Extract of a Nodule Bacteroid Preparation*

The complete reaction mixture in a final volume of 6 ml contained: 100 μmoles of potassium phosphate buffer, pH 7.2; 15 μmoles of $MgCl_2$; 6 μmoles of potassium ATP; 360 μmoles of creatine phosphate; 2.4 mg creatine kinase; 120 μmoles of $Na_2S_2O_4$ and 1.5 ml of cell-free extract (see text) containing 61 mg protein per ml. The procedures for gassing, incubation and sampling were those described in table I with the exception that oxygen was omitted and replaced by argon.

Reaction conditions	Ethylene produced (μmoles/hr)
Complete	9.63
Without ATP	<0.01
Without creatine phosphate	<0.01
Without creatine kinase	7.27
Without $Na_2S_2O_4$	<0.01
Complete, but extract boiled	<0.01

Nodule brei was separated by centrifugation into a bacteroid fraction and a soluble protein supernatant containing leghemoglobin. The supernatant solution alone exhibited no capacity to catalyze the reduction of acetylene but the suspended pellet containing bacteroids and other particulates, rapidly catalyzed the reduction of acetylene. Combination of the supernatant fraction with the bacteroid preparation resulted in an increase in the rate of acetylene reduction but the evidence indicated that the stimulation was due to oxidizable substrates in the supernatant. Since the capacity to catalyze acetylene reduction remained in the bacteroid fraction after washing in buffer to remove leghemoglobin and other components it was concluded that the enzymes necessary for acetylene reduction were located in the bacteroid preparation and not in the supernatant.

A series of experiments were conducted to determine whether cell-free extracts of the bacteroid fraction would catalyze the reduction of acetylene and N_2 gas. A complete reaction mixture containing cell-free extract and lacking O_2 (table II) produced about 9.6 μmoles of ethylene per hour. Reactions lacking ATP or creatine phosphate or $Na_2S_2O_4$ or active extract produced less than 0.01 μmole of ethylene per hour. The omission of creatine kinase reduced the rate of the reaction only slightly and in some experiments the reaction was not completely dependent upon ATP. In all experiments, however, the reaction has been dependent upon creatine phosphate, $Na_2S_2O_4$ and extract. Similar results have been obtained in 4 successive experiments.

Since all cell-free extracts of the bacteroid fraction catalyzed an ATP and $Na_2S_2O_4$ dependent reduction of acetylene the capacity of the extract to catalyzed N_2 gas reduction was expected. Experiments were conducted, therefore, that were similar to the one described in table II with the exception that acetylene was omitted in the reaction and the gas volume in each tube was made to 1 atm with N_2 or argon (table III). From the data in table III it is apparent that the complete reaction mixtures with an ATP generating system, $Na_2S_2O_4$ and N_2 gas produced ammonia at a fairly rapid rate. The omission of the ATP generating system, $Na_2S_2O_4$, or N_2 gas resulted in little ammonia production. In these and other experiments crude extracts alone contained about 200 mμmoles of ammonia per mg of protein. To date all attempts to remove the endogenous ammonia has resulted in

Table III. *Reduction of Nitrogen Gas by Cell-free Extracts of Soybean Nodule Bacteroid Preparations*

The complete reaction mixture in a final volume of 6 ml (26 ml tube) contained the quantities of reaction components listed in table II with the exception that 150 μmoles of potassium cacodylate buffer at pH 7.2 was used instead of potassium phosphate buffer. A volume of 1.5 ml of bacteroid extract (61 mg/ml, Exp I and II and 41 mg/ml, Exp III) was added to each reaction. Prior to incubation each reaction mixture was evacuated and flushed 3 times with N_2 or Ar as indicated. Samples were assayed for NH_3 by the method of Mortenson (7).

		Ammonia in reaction					
		Exp I		Exp II		Exp III	
Reaction system	Gas phase	Total assayed	Total less NH_3 in Ext*	Total assayed	Total less NH_3 in Ext	Total assayed	Total less NH_3 in Ext
		mμmoles/1½ hr/mg protein					
Complete	N_2	328	120	332	124	402	202
Without ATP system**	N_2	227	19	235	27	...	...
Without ATP	N_2	...	...	...	...	406	206
Without $Na_2S_2O_4$	N_2	212	4	220	12	223	23
Without N_2 gas	Ar	...	...	220	12	...	...

* The crude cell-free extract contained a relatively high content of endogenous NH_3; therefore, NH_3 in extracts, after incubation at 28° during the experiment was determined and subtracted from the total NH_3 assayed. In most extracts about 200 mμmoles per mg protein were found. A complete reaction mixture with extract boiled prior to incubation contained 165 mμ moles of NH_3 per mg of protein (Exp III).

** Creatine phosphate, creatine kinase and ATP.

inactivation of the nitrogenase system. Despite the high concentration of endogenous ammonia in extracts, complete reaction mixtures produced at least 7 μmoles of ammonia during the incubation period. This quantity could be accurately measured in presence of the endogenous ammonia in the extract. The evidence that cell-free extracts of a bacteroid fraction (bacteroids and other particulates) from soybean nodules catalyze the reduction of both acetylene and N_2 gas and that the reduction of both of these gases is dependent upon $Na_2S_2O_4$ and an ATP generating system is considered conclusive. The properties of the enzyme system are being investigated.

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