NITROGEN FIXATION: CELL-FREE SYSTEM WITH EXTRACTS OF AZOTOBACTER*

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A reproducible cell-free nitrogen fixation system associated with pyruvate oxidation was first described by Carnahan et al, (1960) using extracts of the anaerobe, Clostridium pasteurianum. Recent experiments (Mortenson, 1964; Hardy and D'Eustachio, 1964; D'Eustachio and Hardy, 1964) have shown that an ATP-generating system is required and that hydrogen gas can serve as the electron donor.

Attempts to obtain a cell-free fixation system from aerobic bacteria have met with less success. Low levels of N^{15}-incorporation were previously observed in this laboratory (unpublished) using the molybdenum-rich particulate fraction from Azotobacter described by Keeler, Bulen, and Varner (1956). Nitrogen fixation has been reported for a system using extracts of Azotobacter cells prepared in the medium in which they grew (Nicholas and Fisher, 1960; Nicholas, Silvester, and Fowler, 1961; Nimeck, Wilson, and Nicholas, 1963). Cell-free fixation experiments conducted in a similar manner in our laboratory following cell rupture with a sonic oscillator or French

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press have failed to show any significant N^{15} enrichment. In this report we describe a reproducible, cell-free system in which the nitrogenase activity of Azotobacter extracts is supported by a crude hydrogenase preparation from C. pasteurianum and an ATP-generating system.

METHODS

Azotobacter agilis (A. vinelandii O) was maintained in liquid culture as previously described (Bulen, 1961). For enzyme isolation, three or six liter cultures were incubated for 16 hr under high aeration. The cells were separated by centrifugation, and suspended in 0.025 M potassium phosphate pH 7 at a ratio of 22 g cell paste to 60 ml buffer. Crude extracts were prepared by disrupting the cells in a precooled French pressure cell and removing unbroken cells by centrifugation at 10,000 g. Sedimentable fractions obtained by successive centrifugations at 35,000 g for 1/2 hr, 144,000 g for 1 hr and 144,000 g for 6 hr are designated as P_{35}, P_{144-1}, and P_{144-6}, respectively. The corresponding supernatant fractions are designated S_{35}, S_{144-1}, and S_{144-6}. C. pasteurianum was cultured on N_2 in a nitrogen-free medium or on ammonia in a medium containing 800 mg $(NH_4)_2SO_4$ per l. Extracts were prepared in 0.02 M potassium phosphate pH 7 by autolysis of dried cells (Carnahan et al, 1960), heated under H_2 in a 70° bath for 15 min and centrifuged. The supernatant fraction, designated 70° HECP, serves as a crude hydrogenase and ferredoxin preparation.

Fixation reactions were conducted anaerobically in Warburg flasks for 1 hr at 30° under a gas mixture composed of 0.2 atm N_2

containing 95% N^{15}, 0.3 atm H_2 and 0.5 atm helium. The entire contents of the reaction mixtures were analyzed for N^{15}.

RESULTS

Cell-free fixation was observed with systems composed of Azotobacter extracts, 70° HECP, and an ATP-generating system (Table I). Initial experiments indicated that a large portion of the

Table I: Nitrogen Fixation with Azotobacter Extracts

Experiment	Fraction	µg N Fixed	Specific Activity*
1	Crude Extract †	4.22	.36
2	"	5.14	.37
	"	5.13	.37
3	S_{144-1} †	5.18	.46
4	" ††	6.60	.68

Reaction mixtures contained in 2 ml: 80 µmoles KH_2PO_4, pH 7; 10 µmoles ATP; 60 µmoles creatine phosphate; 0.5 mg creatine kinase; 5 µmoles $MgCl_2$; 1 ml HECP (ca. 3 mg protein); and 0.5 ml of crude extract (ca. 14 mg protein) or 0.5 ml of S_{144-1} (ca. 10 mg protein) as indicated. *µg N fixed per mg protein in Azotobacter extract. †Prepared under H_2. ††Prepared in air.

activity of the crude Azotobacter extract remained in the supernatant fraction obtained after a 1 hr centrifugation at 144,000 g (S_{144-1}) and that preparation in air did not measurably alter the activity.

Requirements for the complete system are shown in Table II. Fixation observed in the absence of added ATP is attributed to the presence of endogenous ADP. No fixation occurs in the absence of other components of the ATP-generating system, S_{144-1}, or 70° HECP. With the crude extract, fixation is not linear with extract concentration (Figure 1). The extent of the reaction may be limited by the amounts of other components used. The inhibition observed at higher extract concentrations is tentatively ascribed to the ATPase activity

Table II: Requirements for Cell-free Fixation

System	μg N Fixed
Complete	4.69
Minus ATP	3.40
" CP and CK	0
" S_{144-1}	0
" 70°-HECP	0
" ATP and CK	0

Reaction mixture as given in Table I except 0.3 ml of S_{144-1} and 0.6 ml HECP were used. CP, creatine phosphate; CK, creatine kinase.

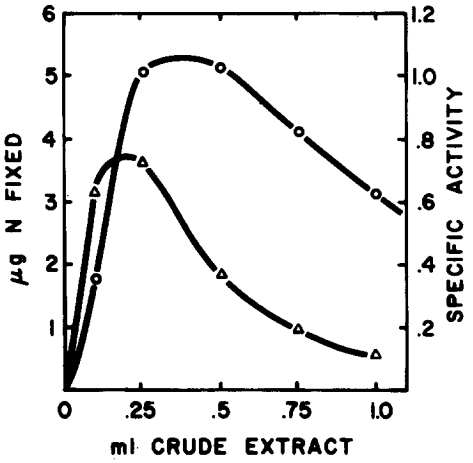

Figure 1. Variation of nitrogen fixation with crude extract concentration.
Reaction mixture as given in Table I. ▲—▲, μg N Fixed; O—O, Specific Activity.

present in these extracts. A non-linear response was also observed with the S_{144-1} fraction.

The 70° HECP provides a crude preparation of hydrogenase and ferredoxin. The data in Figure 2 show optimal amounts for the system described and the lack of inhibition by an excess. In a hydrogen atmosphere, 1 ml of a typical preparation catalyzed the reduction

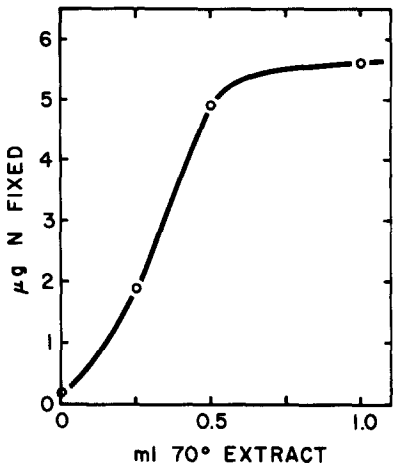

Figure 2. Variation of nitrogen fixation with 70° HECP concentration.
Reaction mixture as given in Table I but using 0.25 ml of crude extract.

of 2.8 μmoles of methylene blue and 2.6 μmoles of ferricyanide per minute. This extract is inactive in the fixation reaction if the hydrogenase is destroyed by exposure to air or by heating at 80°. Available evidence indicates that the 70° HECP does not contribute to the nitrogenase activity. Repeated control experiments in which S_{144-1} was omitted failed to show any N^{15} enrichment. Seventy degree heated extracts from ammonia grown cells, which contain hydrogenase but no nitrogenase, effectively support fixation by the Azotobacter extracts.

In the absence of creatine phosphate and creatine kinase, ATP at levels up to 25 mM gave no significant N^{15} enrichment. Mortenson (1964) has shown that ATP levels above 5 mM inhibit cell-free fixation with extracts of C. pasteurianum. A similar inhibition could account for our observations. With the generating system, 5 mM ADP was as effective as 5 mM ATP but at 12.5 mM ADP was inhibitory.

The distribution of nitrogenase activity in fractions obtained by differential centrifugation is shown in Table III. Localization of the activity in the P_{144-6} fraction is indicated by the activity in this fraction and its near absence from S_{144-6}. The P_{144-6} fraction is the molybdenum-rich fraction previously shown to contain a large portion of Mo^{99} taken up by the cells (Keeler, Bulen, and Varner, 1958).

Table III: Nitrogenase Activity in Fractions Obtained by Differential Centrifugation

Fraction	Mg Protein/flask	Specific Activity
Crude Extract	13.9	.368
P_{35}	11.8	.184
P_{144-1}	12.2	.205
S_{144-1}	11.2	.460
P_{144-6}	9.6	.717
S_{144-6}	7.7	.072

Reaction mixtures as given in Table I.

SUMMARY AND DISCUSSION

Cell-free nitrogen fixation with extracts of Azotobacter has been observed. Hydrogen gas serves as the electron donor in the presence of a hydrogenase and ferredoxin preparation obtained from C. pasteurianum. Low levels of ATP are supplied with an ATP-generating system.

The fact that O_2 is not required for fixation by the nitrogenase of this aerobe rules out the frequently hypothesized formation of an oxidized intermediate by reaction with molecular oxygen. The occurrence of the reaction under strongly reducing conditions makes it unlikely that any oxidation reactions are involved and emphasizes the similarity of this system to those obtained from anaerobes.

This fixation system has two potential advantages for future study: (1) the electron donor system and the ATP-generating system can be added exogenously to purified fractions and (2) the nitrogenase containing fractions can be handled in air. Enzyme purification is in progress.

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