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THE NITROGEN FIXATION SYSTEM OF PHOTOSYNTHETIC BACTERIA

I. PREPARATION AND PROPERTIES OF A CELL-FREE EXTRACT FROM *CHROMATIUM*

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SUMMARY

The preparation and properties of cell-free extracts of the photosynthetic bacterium *Chromatium* which give consistent and appreciable nitrogen fixation are described. Nitrogen fixation required ATP and reducing power. Reducing power was supplied by reduced ferredoxin or, in its absence, by sodium dithionite or H_2, in the presence of catalytic amounts of a viologen dye. ATP was supplied by phosphocreatine and creatine phosphokinase, with a catalytic amount of free ATP.

The *Chromatium* nitrogenase complex was found to have the typical properties of other nitrogenases, *i.e.*, reduction of N_2 to ammonia, and of acetylene to ethylene, ATP-dependent H_2 evolution and reductant-dependent ATP hydrolysis.

INTRODUCTION

Nitrogen fixation by cell-free extracts of *Chromatium* was reported from this laboratory in 1961 (ref. 1). By showing that extracts of these obligate phototrophs can utilize H_2 to assimilate $^{15}N_2$ in the dark, these early experiments provided the first evidence that H_2 can serve as an electron donor for biological nitrogen fixation. (Fixation of $^{15}N_2$ in the presence of H_2 increased the atom % excess ^{15}N by 0.010–0.017, the detection limit[2] being 0.003.) By contrast, nitrogen fixation in whole *Chromatium* cells was strictly light dependent and its requirement for an electron donor was fulfilled by a relatively weak reductant such as thiosulfate[1]. These experiments suggested that the nitrogen-fixing enzyme complex (the nitrogenase system) of *Chromatium* requires a strong reductant and that illumination supplied energy needed to enhance the reducing power of electrons from thiosulfate to a level approaching that of H_2 (ref. 1).

Although $^{15}N_2$ fixation by *Chromatium* extracts was significant, its rate was very low[1], since it was measured before it was recognized that the reductant for nitrogen fixation must be supplemented with ATP[3–5]. Moreover, it was not known then that the "pyridine nucleotide reductase" discovered about the same time in *Chromatium*

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extracts[6] was a bacterial ferredoxin[7] similar to the ferredoxin of *Clostridium pasteurianum*[8], which is the electron donor for the nitrogenase system in that organism[9,10].

The purpose of this investigation was to study nitrogen fixation by extracts of photosynthetic bacteria in the light of present knowledge of the requirements for nitrogen fixation in cell-free systems. This communication reports the preparation and properties of *Chromatium* extracts with high nitrogen fixation activity.

METHODS

Culture of organisms

Chromatium Strain D was grown in the malate medium of ARNON *et al.*[11], modified by omission of NH_4Cl and sparged with 80% N_2–20% CO_2 gas mixture. Batch cultures of cells were grown for 4–5 days in 13-l carboys that were illuminated with a bank of incandescent lamps to give a light intensity of about 5000 lux. Initial growth was logarithmic with a generation time of about 30 h. The generation time could be reduced by supplemental illumination to about 16 h but this additional illumination was not usually provided since it had no effect on the final yield of cells or on their nitrogenase activity. Cells were harvested by a Sharples centrifuge. The cell paste was either used immediately or frozen at −20° and used within a few weeks.

C. pasteurianum cells were cultured from spore stock in the nitrogen-deficient medium of WESTLAKE AND WILSON[12]. Immediately after harvesting, the cell paste was dried in a Rinco rotary vacuum at 40–50° as described by CARNAHAN *et al.*[13].

Preparation of cell extracts

The preparation of *Chromatium* extracts was carried out anaerobically under argon or H_2. Cells were suspended in 0.005 M phosphate buffer (pH 6.8), chilled in an ice bath and sonicated with a Branson sonifier at full power for six successive 15-sec intervals, each followed by a 30-sec pause to prevent overheating. After sonication, the broken cells were centrifuged in capped tubes (previously gassed with argon) at 30000 × *g* in a Sorvall RC-2 centrifuge to remove any remaining unbroken cells and cellular debris. The dark red supernatant fluid (cell extract) contained about 40 mg protein per ml as determined by the biuret assay (with a bovine serum albumin standard). To prevent the pigments from interferring with the assay, the protein was precipitated by a solution of methanol–acetone (7:2, v/v).

In later experiments, *Chromatium* cells were broken in a Ribi cell fractionator (Ivan Sorvall, Inc.). The cells were suspended as before and passed through the valve at 25000–30000 lb/inch^2. The extract was kept cool and anaerobic by circulating refrigerated N_2 gas. The N_2-fixing activity and protein content of cell extracts prepared by Ribi fractionation was about 25% higher than in extracts prepared by sonication.

C. pasteurianum cells were extracted by the method of CARNAHAN *et al.*[13]. The hydrogen-donating system of these cells was prepared by the method of MORTENSON *et al.*[14]. The hydrogen-donating system rapidly evolved H_2 from dithionite, or H_2 and CO_2 from pyruvate, but had no N_2-fixing activity.

Assays

N_2 reduction was assayed by the formation of free ammonia[15], as described by BULEN *et al.*[16]. H_2 evolution was measured in conventional Warburg respirometers.

Reaction vessels were evacuated prior to the introduction of the appropriate gas. Creatine release from phosphocreatine hydrolysis was measured by the method of EGGLETON *et al.*[17]. The reduction of acetylene to ethylene[19,18] was measured by chromatography on an Aerograph 1200 gas chromatograph with a 2-m alumina column at 170° and a hydrogen flame ionization detector. Peak areas were established with an Infotronics CRS-11HSB digital integrator. Ethylene was quantitatively estimated from the ratio of the ethylene peak area (divided by the sensitivity factor 1.4) to the sum of the ethylene and acetylene peaks. The sensitivity factor is the ratio of the number of counts per μmole of ethylene to the number of counts per μmole of acetylene. The method requires adding a known amount of acetylene to the reaction vessel but is independent of the exact partial pressure of acetylene in the vessel, the amount injected into the chromatograph, or the reduction of a significant fraction of the added acetylene.

Ferredoxin preparation

Ferredoxins from acetone extracts of *C. pasteurianum*[20] and *Chromatium*[7] cells were purified by DEAE-cellulose chromatography essentially as previously described.

RESULTS

N_2 reduction

As with other nitrogenase systems, nitrogen fixation by *Chromatium* extracts in the dark required both ATP (in the presence of Mg^{2+}) and a strongly reducing electron donor which was supplied by the addition of sodium dithionite[16] (Table I). In experiments such as those in Table I, cell extracts prepared by sonication gave rates of N_2 fixation up to 2 μmoles NH_3 formed in 30 min.

TABLE I

REQUIREMENTS FOR N_2 REDUCTION BY CELL-FREE *Chromatium* EXTRACTS

Complete reaction mixture (final volume, 1.0 ml) contained: cell extract, 0.3 ml; creatine phosphokinase, 0.2 mg; and the following in μmoles: HEPES buffer (pH 6.8), 100; $MgCl_2$, 10; ATP, 5; creatine phosphate, 50; sodium dithionite, 20. Temp., 30°; time, 40 min. Gas phase, N_2 in all treatments except in a control under argon. Activity was measured as NH_3 present under N_2 less NH_3 present in the argon control.

Omission	*Activity (% of complete system)*
None	100
$MgCl_2$	75
Initial ATP	<5
ATP-generating system (creatine phosphate–creatine phosphokinase)	<5
Sodium dithionite	<5

In the early experiments, many preparations showed a low apparent nitrogenase activity. The low activity was found to be the result of assimilation (up to 1 μmole) of newly formed ammonia. This assimilation of newly formed ammonia was dependent on the presence of ATP but not of dithionite. Fig. 1 shows that unwashed cells assimilated added ammonia (under argon) and, in a parallel treatment, gave a

low apparent rate of N_2 fixation. Washing the cells eliminated the assimilation of free ammonia (regardless whether ammonia was added or formed from N_2), presumably by removing compounds that served as ammonia acceptors. To increase the measurable rate of cell-free N_2 fixation in subsequent experiments, cells were routinely washed before being disrupted.

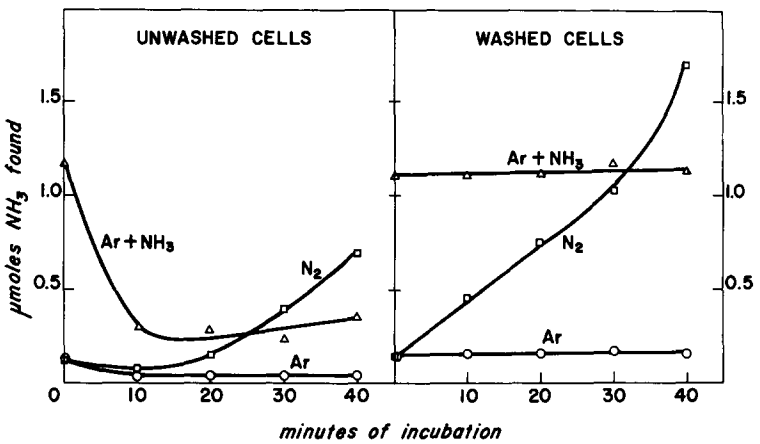

Fig. 1. Effect of washing cells on time course of formation and assimilation of ammonia. The basic reaction mixture was the same as in the complete treatment of Table I. 1.0 μmole NH_4Cl added where indicated.

After sonication, centrifugation of the crude cell extract for 90 min at 144000 × *g* yielded a sediment and a light reddish-brown supernatant containing about 25 mg protein per ml. The N_2-fixing activity remained in the 144000 × *g* supernatant extract and was not found in the 144000 × *g* precipitate particles, which contained bacteriochlorophyll and the photochemical activity associated with chromatophores (Table II).

Table II also shows that the nitrogenase activity of the cell extract preparation had the same sensitivity to heat as the nitrogenase of *C. pasteurianum*[14]. The *Chromatium* nitrogenase enzyme system was almost completely inactivated by heating for 10 min at 60°.

TABLE II

EFFECT OF CENTRIFUGATION AND HEAT TREATMENT ON N_2 FIXATION BY *Chromatium* CELL EXTRACTS

Assay conditions were the same as the complete treatment in Table I.

Cell fraction	% *of* N_2 *reduction activity*
Cell extract	100
144000 × *g* supernatant	99
144000 × *g* precipitate*	1
Cell extract, heated 10 min, 40°	104
Cell extract, heated 10 min, 50°	59
Cell extract, heated 10 min, 60°	2

* These particles suspended in 0.3 ml 0.005 M phosphate buffer (pH 6.8) were 2.4 times as concentrated as in the starting cell extract preparation.

Acetylene reduction

The most sensitive assay of the nitrogenase activity of *Chromatium* extracts proved to be the reduction of acetylene to ethylene, a nitrogenase activity first observed by DILWORTH[18] and SCHÖLLHORN AND BURRIS[19] in *C. pasteurianum* and *Azotobacter vinelandii*. Table III gives a comparison of ammonia formation and acetylene reduction in *Chromatium* extracts. In these experiments, the ratio of ethylene to ammonia formed was slightly higher than the theoretical value of 1.5. However, considering the limitations of the methods, these ratios are reasonably close.

TABLE III

AMMONIA FORMATION AND ACETYLENE REDUCTION BY *Chromatium* FRACTIONS

Reactions were run in 15-ml serum bottles containing in 1.0 ml total volume: cell fraction indicated, 0.3 ml; creatine phosphokinase, 0.2 mg; and the following in μmoles: HEPES buffer (pH 6.8), 100; $MgCl_2$, 10; ATP, 5; creatine phosphate, 50; sodium dithionite, 20. Gas phase: N_2 or acetylene, 100 mm Hg, argon to 1 atm. Reactions were run for 40 min at 30°.

Cell fraction	NH_3 *formed* (*μmoles*)	*Acetylene reduced* (*μmoles*)	*Acetylene/*NH_3 *ratio*
Cell extract	1.95	3.37	1.71
144000 × *g* supernatant	1.66	2.90	1.74
144000 × *g* precipitate*	0	0	—

* These particles suspended in 0.3 ml 0.005 M phosphate buffer (pH 6.8) were 2.4 times as concentrated as in the starting cell extract preparation.

Stability of nitrogenase activity

Nitrogenase activity of the cell extracts or of the 144000 × *g* supernatant fluid free of chromatophores was not markedly decreased by storage at 3° for at least 3 days (Table IV). Thus, *Chromatium* nitrogenase (at least the unpurified enzyme) does not exhibit the cold lability of the nitrogenase of *C. pasteurianum*[21] and *Rhodospirillum rubrum*[22]. Despite some variation, *Chromatium* extracts with high initial activity showed generally greater stability. A slight increase in activity was frequently seen after 24 h storage in the cold. Extracts prepared in a Tris, phosphate,

TABLE IV

STABILITY OF NITROGENASE ACTIVITY IN *Chromatium* EXTRACTS

Assays for N_2 reduction were as in the complete system in Table I. The same reaction conditions were used for acetylene reduction except that the gas phase was 10% acetylene–90% argon. Values indicate the range observed in different experiments.

Time of storage at 3° (days)	*% of activity remaining*	
	N_2 *reduction*	*Acetylene reduction*
0	100	100
1	77–114	98
2		66–100
3	63–96	74
4		59

or HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 6.8–7.8) had similar activity and storage properties.

Effect of the electron donor system

Several electron donor systems were tested for their ability to couple to the nitrogenase system of *Chromatium*. Since *Chromatium* extracts contained hydrogenase[1,23] (hydrogen acceptor oxidoreductase, EC 1.12.1.1), they were able to use hydrogen gas (in the presence of catalytic amounts of benzyl viologen) as a source of electrons for acetylene reduction (Table V). The catalytic role of benzyl viologen could also be performed by methyl viologen or the hydrogen-donating system from *C. pasteurianum* extracts[14]. Without catalytic amounts of either a viologen dye or hydrogen-donating system, H_2 was ineffective as an electron donor, even in the presence of clostridial or *Chromatium* ferredoxin. The effectiveness of H_2 (in the presence of hydrogen-donating system) was usually increased by the addition of *Chromatium* or *C. pasteurianum* ferredoxin. With hydrogen-donating system and clostridial ferredoxin, H_2 was about as effective an electron donor as dithionite (Table V).

Attempts to demonstrate N_2 fixation with NADH as the electron donor were unsuccessful. NADH by itself, or together with benzyl viologen, methyl viologen or ferredoxin, was ineffective.

TABLE V

EFFECT OF ELECTRON DONOR SYSTEM ON ACETYLENE REDUCTION BY *Chromatium* EXTRACTS

Assays were run in 5-ml micro-Fernbach flasks, containing in 1.1 ml total volume: *Chromatium* cell extract, 0.3 ml; creatine phosphokinase, 0.2 mg; and the following in μmoles: HEPES buffer (pH 7.2), 100; $MgCl_2$, 10; ATP, 5; creatine phosphate, 50; and, where indicated, sodium dithionite, 20; methyl viologen or benzyl viologen, 0.1; *C. pasteurianum* ferredoxin, 196 μg*; *C. pasteurianum* hydrogen-donor system[15], 0.2 ml. Gas phase, argon or H_2, 1 atm with 0.5 ml acetylene injected.

Electron donor system	*Acetylene reduced (μmoles)*	
	Expt. A	*Expt. B*
1. H_2 + methyl viologen	1.06	0.422
2. H_2 + benzyl viologen	—	0.319
3. H_2 + hydrogen-donating system	0.99	—
4. H_2 + hydrogen-donating system + *Chromatium* ferredoxin	1.82	—
5. H_2 + hydrogen-donating system + clostridial ferredoxin	3.78	1.97
6. Sodium dithionite	3.91	—

* Determined by Lowry–Folin assay with spinach ferredoxin as standard.

ATP requirement of the Chromatium nitrogenase complex

The nitrogenase reactions are noted for their requirement of appreciable amounts of ATP and, at the same time, for being inhibited by high concentrations of either ATP or ADP in the reaction mixture[24]. To provide the needed combination of a large reservoir and a low concentration of ATP, a low initial amount of ATP *plus* an ATP-generating system comprising creatine phosphate and creatine phosphokinase[25] was used in our experiments. When larger amounts of ATP were supplied initially without an ATP-generating system, only a small amount of acetylene was reduced and the activity of the system became dependent on the Mg/ATP ratio

(Fig. 2). The optimum Mg/ATP ratio was 1:2, in agreement with that reported by KENNEDY *et al.*[24]. Even at the optimum ratio, however, the amount of acetylene reduced was very small in relation to the amount of ATP present, assuming a requirement of 4–5 ATP/$2e^-$, similar to that found with other nitrogenase systems (refs. 21, 24, 26, *cf.* also ref. 27). The low level of acetylene reduction was not due to rapid utilization and depletion of ATP by other reactions since the rate of acetylene reduction was linear for at least 10 min.

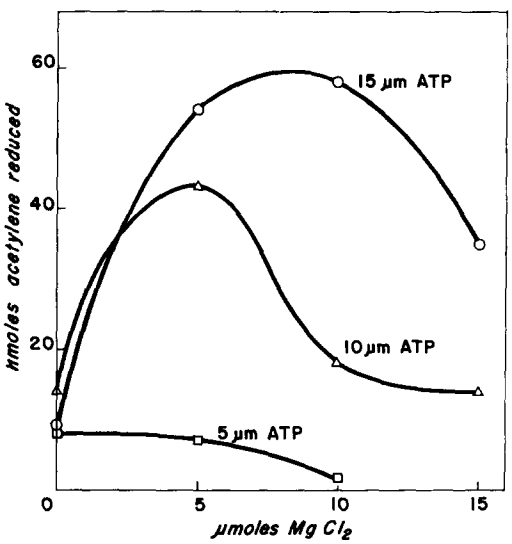

Fig. 2. Influence of Mg^{2+} concentration on acetylene reduction by *Chromatium* cell extracts in the presence of substrate levels of ATP. Reaction mixtures (in 5-ml micro-Fernbach flasks) contained, in a final volume of 1.1 ml: cell extract, 0.3 ml; HEPES buffer (pH 7.2), 100 μmoles; *C. pasteurianum* hydrogen-donating system[15], 0.2 ml; *C. pasteurianum* ferredoxin, 196 μg; and $MgCl_2$ and ATP (μmoles) as indicated. Gas phase, H_2 + 0.5 ml acetylene. Time, 30 min; temp., 30°.

TABLE VI

REDUCTANT-DEPENDENT HYDROLYSIS OF ATP BY CELL EXTRACTS OF *Chromatium* CELLS

Reaction mixtures contained in 1.2 ml: cell extract, 0.5 ml; creatine phosphokinase, 0.2 mg; and the following in μmoles: HEPES buffer (pH 6.8), 100; $MgCl_2$, 10; ATP, 5; creatine phosphate, 50. Other additions where indicated: sodium dithionite, 20; methyl viologen, 0.2; benzyl viologen, 0.2; *C. pasteurianum* ferredoxin, 200 μg. Gas phase, argon in the dithionite treatment and H_2 elsewhere. Temp., 32°. Time, 45 min. Reaction stopped with 1.0 ml satd. K_2CO_3 solution for assay of creatine.

Reductant	*Reductant-dependent creatine released**
H_2	0.5
H_2 + clostridial ferredoxin	1.7
H_2 + benzyl viologen	3.1
H_2 + methyl viologen	5.8
Dithionite	13.6

* Values have been corrected for 8.7 μmoles of creatine released in the absence of reductant.

Another feature of the nitrogenase complex of *Chromatium* observed earlier in nitrogenase of *C. pasteurianum*[4,28,29] and *A. vinelandii*[30] is a hydrolysis of ATP in the presence of reductants of nitrogen fixation—a phenomenon called a reductant-dependent ATPase by HARDY *et al.*[28]. Table VI shows that the extent of ATP hydrolysis (determined by assaying the creatine released from phosphocreatine) corresponded to the effectiveness of the reductants in N_2 or acetylene reduction: ATP hydrolysis with dithionite was much greater than with H_2 (in the presence of viologen).

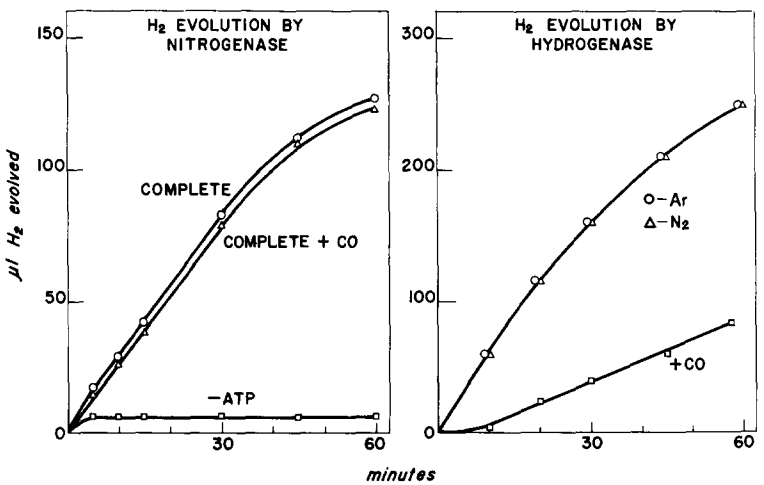

Fig. 3. A (left). ATP-dependent H_2 evolution by extracts of *Chromatium* cells. The reaction mixture in Warburg vessels contained (a final volume of 2.0 ml): cell extract, 1.0 ml; creatine phosphokinase, 0.2 mg; and the following in μmoles: HEPES buffer (pH 6.8), 200; $MgCl_2$, 10; ATP, 5; creatine phosphate, 50; sodium dithionite, 20 (in side arm). Center well, 0.2 ml 4 M NaOH and a strip of filter paper. Gas phase, argon and, where indicated, 10% CO. Temp., 30°. B (right). ATP-independent H_2 evolution by extracts of *Chromatium* cells. The reaction mixture in Warburg vessels contained (in 2.0 ml final volume): cell extract, 1.0 ml; and the following in μmoles: HEPES buffer (pH 6.8), 200; methyl viologen, 0.2; sodium dithionite, 20 (side arm). Center well, 0.2 ml 4 M NaOH and a strip of filter paper. Gas phase: upper curve, argon or nitrogen; lower curve, argon + 10% CO.

ATP-dependent H_2 evolution

An ATP-dependent hydrogen evolution by the nitrogenase enzyme complex, first observed by BULEN *et al.*[16,31] in extracts of *A. vinelandii*, is now known to be a property of the nitrogenase systems of *C. pasteurianum*[28,32], *R. rubrum*[22], and of legume nodule extracts[33]. As shown in Fig. 3A, *Chromatium* extracts, supplied with dithionite as a substrate, also exhibited an ATP-dependent H_2 evolution. Unlike the ATP-independent H_2 evolution catalyzed by the conventional *Chromatium* hydrogenase[1,23] (Fig. 3B) which was unaffected by N_2 (not shown), required methyl viologen and was inhibited by CO, the ATP-dependent H_2 evolution due to nitrogenase did not require the presence of methyl viologen and was insensitive to CO (*cf.* ref. 32).

The ATP-dependent H_2 evolution was substantially lowered by the presence of N_2 (Table VII). This decrease appears to result from a utilization of a portion of the total ATP-dependent electron flow in N_2 reduction. Total electron transfer from dithionite could be followed directly by the evolution of H_2 in an argon atmosphere

TABLE VII

COMPARISON OF TOTAL ELECTRON FLOW DURING N_2 FIXATION AND DURING ATP-DEPENDENT H_2 EVOLUTION UNDER ARGON

The reactions were carried out in 15-ml Warburg vessels, which contained a 2.0-ml final volume: cell preparation, 1.0 ml; creatine phosphokinase, 0.2 mg; and the following in μmoles: HEPES buffer (pH 6.8), 200; $MgCl_2$, 10; ATP, 5; creatine phosphate, 50; sodium dithionite (side arm), 20. Center well contained 0.2 ml 4 M NaOH and a strip of filter paper. Gas phase, N_2 or (in the last column) argon. Aliquots of the reaction mixture were withdrawn for NH_3 determination immediately after terminating the reaction.

Preparation	*N_2 atmosphere*				*Argon atmosphere*
	N_2 fixed (*A*) (μl)	*H_2 required to fix N_2* (*B*) (μl)	*H_2 evolved* (*C*) (μl)	*A + B + C* (μl)	*H_2 evolved* (μl)
A. Cell extract prepared by sonication	33.5	101.5	46.8	182	165
B. Cell extract prepared by Ribi fractionation	15.9	47.6	57.3	121	118
C. 144000 × *g* supernatant fluid from B	18.6	55.7	48.0	122	109

or, in an N_2 atmosphere, by the combined fixation of N_2 and evolution of H_2. Note in Table VII that the total H_2 evolved in the N_2 atmosphere is accounted for by the sum of Columns C and A. This sum *plus* the computed hydrogen requirement for N_2 reduction (Column B) give the total electron flow during N_2 fixation. Table VII shows that, in general, the total electron flow during N_2 fixation corresponded to the total ATP-dependent H_2 evolution under argon (*cf.* ref. 21).

CONCLUDING REMARKS

The *Chromatium* sp. used here is the first of the purple sulfur bacteria to yield a cell-free nitrogen-fixing preparation that was consistently active, without exhibiting the rapid pH drop or sensitivity to the presence of chromatophores that inhibit nitrogen fixation by extracts of *R. rubrum*[22].

The activity of the *Chromatium* nitrogenase complex depended on the availability of reducing power and ATP. As in Clostridium species, reduced ferredoxin donates electrons to *Chromatium* nitrogenase and is probably the immediate natural reductant. In the absence of ferredoxin, reducing power for nitrogenase can be supplied by dithionite or by H_2 in the presence of catalytic amounts of methyl or benzyl viologen. Like other nitrogenases, *Chromatium* nitrogenase also shows a large requirement for ATP, best supplied by phosphocreatine and creatine phosphokinase, with a catalytic amount of ATP.

The *Chromatium* nitrogenase complex was found to have the typical properties of other nitrogenases, *i.e.*, reduction of N_2 to ammonia and of acetylene to ethylene, ATP-dependent H_2 evolution and reductant-dependent ATP hydrolysis.

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