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SOME PROPERTIES OF PURIFIED NITROGENASE OF *AZOTOBACTER CHROOCOCCUM*

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SUMMARY

The nitrogenase of *Azotobacter chroococcum* was fractionated into two essential components by anaerobic chromatography and elution with $MgCl_2$. Fraction 1, which contained iron and molybdenum, was not damaged by exposure to air for 30 min at 20° but Fraction 2, which also contained iron but only traces of molybdenum, was completed inactivated by this treatment. The ratio of these fractions, giving maximum rate of nitrogen fixation, acetylene reduction or ATP-dependent hydrogen evolution was about 2:1 (mg protein of Fraction 1/mg protein Fraction 2) but for isocyanide or cyanide reduction the optimum ratio was about 6:1. $Na_2S_2O_4$-dependent ATPase activity catalysed by the fractions was not always proportional to the amount of acetylene or isocyanide reduced or hydrogen evolved and the amount of ATP hydrolysed per 2 electrons used for reduction varied. The relative amounts of methane, ethylene and ethane formed from methyl-, vinyl- or ethylisocyanide were different and were affected by isocyanide concentration and by the ratio of the two fractions. The amounts of products formed from methyl isocyanide were linear with time after a short lag and CO inhibited methane and ethane formation to about equal extents; ethylene production was only slightly inhibited. The observed difference in the optimum ratio for acetylene reduction compared with that for isocyanide is explained by assuming Fraction 2 contains the substrate complexing site, Fraction 1 provides electrons and isocyanide partially inhibits the reaction of Fraction 1 with 2.

INTRODUCTION

The nitrogen-fixing systems of *Clostridium pasteurianum*[1,2] and of *Azotobacter vinelandii*[3–5] have been partially purified into two components. One contained molybdenum and iron in a ratio of about 1:12 (ref. 3), the other contained iron. Nitrogen fixation or ATP-dependent hydrogen evolution[6] required both fractions as well as an ATP-generating system and source of reducing power. With nitrogenase of *C. pasteurianum*, pyruvate metabolised by the phosphoroclastic system provided both a source of ATP and reducing power[7,8]. ATP can conveniently be supplied to extracts

of *A. vinelandii*[6] or *A. chroococcum*[9] by low levels of ATP *plus* creatine phosphate and creatine phosphokinase; reducing power is provided by $Na_2S_2O_4$.

The two fractions of purified nitrogenase were unstable on storage overnight at 5° (ref. 3) but KELLY, KLUCAS AND BURRIS[5] reported that storage in liquid nitrogen allowed extracts to be kept indefinitely with full recovery of activity on thawing. Although the crude nitrogenase of *A. vinelandii* or *A. chroococcum* is not irreversibly inhibited by oxygen, BULEN *et al.*[3] reported that after purification and separation into two components the nitrogenase system was oxygen sensitive. The nitrogenase of *C. pasteurianum* is irreversibly damaged by oxygen, even in the crude state[10]. The role each fraction plays in nitrogen fixation is at present uncertain. BUI AND MORTENSON[11] have published data which indicate that Fraction 1 can react with ATP but it is not known if Fraction 1 or 2 contains the site for nitrogen binding and fixation nor why the nitrogenase of *A. vinelandii* becomes oxygen sensitive during purification.

The nitrogen-fixing systems of *C. pasteurianum*, *A. vinelandii* and *A. chroococcum* catalyse reduction of other compounds besides nitrogen, including N_2O to nitrogen and ammonia[12,13], acetylene to ethylene[14,15], cyanide to methane and ammonia[16], azide to ammonia and nitrogen[16,17], and isocyanide to methane, methylamine, ethylene and ethane[18,19]. Like nitrogen fixation, these reductions require an ATP supply, a source of reducing power and the nitrogenase system. CO, which inhibits nitrogen fixation[6] competitively inhibits isocyanide reduction[19]; nitrogen competitively inhibits isocyanide reduction[19]; acetylene competitively inhibits nitrogen fixation[14]; these observations, together with other data[20], including the requirements mentioned earlier, make it probable that other substrates are reduced at the same time as nitrogen itself. However, much of the published work with these substrates has used crude extracts, with which binding of a substrate at more than one site might occur but might be difficult to observe. Certain anomalies have been reported by KELLY, KLUCAS AND BURRIS[5] in the relative rates of reduction of cyanide, azide or acetylene with purified fractions of *A. vinelandii* nitrogenase, and JACKSON AND HARDY[21] have reported experiments on hydrogen/deuterium exchange reactions catalysed by nitrogenase in presence of nitrogen, but not other substrates, which they suggest indicate that the mechanism of nitrogen reduction is different from that of other substrates. The question whether these other substrates are reduced at the same site as nitrogen is of considerable importance since these alternative substrates, and acetylene in particular, are now being widely used as sensitive and convenient assays for nitrogenase[22–24]. Moreover, to understand the mechanism of biological nitrogen fixation, further data on the interaction of the two fractions with each other, with ATP, $Na_2S_2O_4$ and substrate are required. This paper presents a report of work carried out with purified fractions of nitrogenase from *A. chroococcum*, using a variety of substrates and assay conditions.

METHODS AND MATERIAL

Growth

Batches of *Azotobacter chroococcum*, N.C.I.B. 8003, were grown on nitrogen-free medium[25] harvested and stored as described previously[19].

Assay of nitrogenase

The term nitrogenase is used in this paper for the nitrogen-fixing complex with

recognition that not all the components of this complex are necessarily active in binding or reducing nitrogen. Because of the oxygen sensitivity of purified nitrogenase, some modification of the procedures used with crude extracts[19] were necessary and techniques similar to those of KELLY, KLUCAS AND BURRIS[5] were used. The nitrogenase was assayed not only for nitrogen fixation but for reduction of cyanides, isocyanides or acetylene; for ATP-dependent hydrogen evolution and $Na_2S_2O_4$-dependent ATPase. For all substrates the final assay mixture contained 10 μmoles $MgCl_2$, 5 μmoles ATP, 40 μmoles creatine phosphate, 25 μmoles Tris–HCl buffer (pH 7.4), 0.2 mg creatine phosphokinase (EC 2.7.3.2), 20 μmoles of $Na_2S_2O_4$, nitrogenase, substrate and water to 1.5 ml. For convenience, ATP, $MgCl_2$, creatine phosphate and buffer were mixed in the appropriate concentrations; this reaction mixture (assay reaction mixture) was stored for up to 3 days at $-20°$ with no observed effect on assays.

Assays of nitrogen fixation were carried out in 20-ml serum bottles; 7-ml serum bottles were used for assays of cyanide, isocyanide and acetylene reduction. Assays of $Na_2S_2O_4$-dependent ATPase activity were carried out in 7.5-ml bottles or Warburg flasks. Bottles containing assay reaction mixture, creatine phosphokinase and water were thoroughly gassed with argon or nitrogen before being tightly stoppered with Suba-seals (W. Freeman and Co., Staincross, Barnsley, Yorkshire). Then, where appropriate, 0.2 ml of acetylene or required volume of isocyanide or cyanide solution were injected followed by Fraction 1 and freshly prepared $Na_2S_2O_4$ solution. Bottles were equilibrated at 30° for 5 min before adding Fraction 2 to initiate reduction. Reductions of acetylene, cyanide or isocyanide were stopped by injecting 0.1 ml of 30% (w/v) trichloroacetic acid unless ATPase determinations were also being made; in such cases 0.1 ml of 40% (w/v) KOH was used instead. Nitrogen fixation was stopped by addition of 3 ml of saturated K_2CO_3; microdiffusion and determination of the ammonia produced were done as previously described[27].

Assays of ATPase activity were made using a similar procedure to that of HARDY AND KNIGHT[26].

Hydrogen evolution assays were made by Warburg manometry at 30°; flasks containing assay reaction mixture, creatine phosphokinase and water in the main compartment were gassed with argon by means of 23-gauge hypodermic needles inserted through a serum cap sealing the side arm of each flask. After 5 min the needles were withdrawn and the gas exit taps immediately closed. $Na_2S_2O_4$, Fractions 1 and 2 were injected into each side arm and, after temperature equilibration, reactions were started by tipping the contents into the main compartment.

Gas chromatography

The products of reduction of cyanide, isocyanide or acetylene were determined by analysis of the gas phase by gas chromatography, using conditions similar to those described by KELLY[19].

Determination of protein

Protein concentrations were determined by the biuret method of GORNALL, BARDAWILL AND DAVID[28]. Fractions containing $Na_2S_2O_4$ were shaken in air for a few minutes before protein determinations to destroy the $Na_2S_2O_4$ which otherwise interfered with the assay.

Preparation and fractionation of nitrogenase

Crude extracts were prepared as described by KELLY[19] and partially purified

by selective precipitation with protamine sulphate under conditions very similar to those of BULEN, BURNS AND LECOMTE[6]. The partially purified material contained between 40 and 50 mg protein/ml and, when not used at once, was stored in pellet form in a cryostat of liquid nitrogen. Anaerobic chromatography at 5° on DEAE-cellulose 32 was carried out using slight modifications of the procedure of KELLY, KLUCAS AND BURRIS[5]. The iron/molybdenum protein (Fraction 1) was eluted by 40 mM $MgCl_2$ and had no nitrogen-fixing activity, acetylene reduction or ATP-dependent hydrogen evolution; the iron protein (Fraction 2) was eluted with 90 mM $MgCl_2$, it had slight activity alone and addition of Fraction 1 gave considerable enhancement. Fraction 1 contained iron and molybdenum: the ratio varied between 10–20 atoms iron per molybdenum atom; the total iron was approx. 0.44% of protein. Fraction 2 contained traces of molybdenum and about 0.46% iron.

Re-chromatography of Fractions 1 and 2

Some further purification of Fraction 2 was obtained (as determined by assays of Fraction 2 alone, compared with Fraction 1 *plus* 2) by re-chromatography at pH 7.4 on DEAE-cellulose 32 using conditions of elution similar to those of the first separation. The activity of this rechromatographed Fraction 2 was enhanced about 24 fold by addition of Fraction 1; it had a specific nitrogen-fixing activity of approx. 180 nmoles nitrogen fixed per mg protein (of Fraction 2) per min or of 85 based on total protein. Fraction 1 was also re-chromatographed on DEAE-cellulose 32 at pH 7.4 and some inactive material was eluted by 32 mM $MgCl_2$. This material was pink in colour and had a spectrum with maxima at 558, 529 and 428 mμ; on exposure to air a precipitate formed and the absorption peaks disappeared. The peaks did not reappear on addition of $Na_2S_2O_4$, and from these observations the material was probably an unstable *b*-type cytochrome[30]. Fraction 1 was eluted as one sharp peak with 60 mM $MgCl_2$; it had about 12 atoms of iron per molybdenum atom.

Storage and handling of Fractions 1 and 2

Fractions 1 and 2 were handled anaerobically at all times unless otherwise stated. They were stored as frozen pellets in liquid nitrogen. For assays, the required amount was transferred under a stream of argon to a bottle kept well flushed with argon. A Suba-seal was then tightly inserted, the material allowed to thaw out and, in an ice bath, it maintained full activity for at least 8 h. Fractions were transferred to assay bottles using syringes well flushed with argon, and fitted with 21- or 23-gauge needles.

For other details of chemicals, gases, *etc.*, reference should be made to KELLY[19].

RESULTS

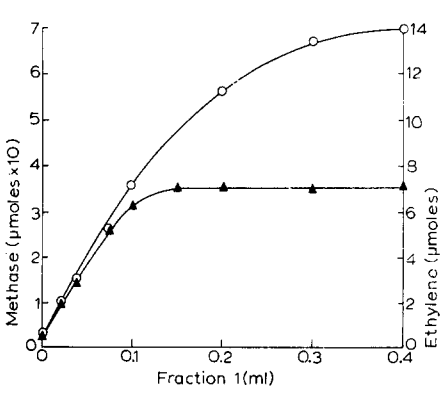
Sensitivity of Fractions 1 and 2 to oxygen

During assays of ATPase activity of Fraction 1 alone, it was briefly exposed to air to destroy $Na_2S_2O_4$, so that non-$Na_2S_2O_4$-dependent ATPase activity could be measured. No activity was observed but the material showed no loss of activity in reduction assays with Fraction 2 despite the fact that it became cloudy. To determine the degree of sensitivity of the fractions to oxygen, each was exposed to air for 30 min at 20°. Fraction 2 remained clear, Fraction 1 became turbid. A sample was centrifuged to remove precipitate, which was resuspended in an equivalent volume of 25 mM Tris–HCl buffer (pH 7.4). Uncentrifuged Fraction 1, supernatant or re-

suspended pellet were assayed separately with untreated Fraction 2 for acetylene reduction and ATPase. The air-treated Fraction 2 was assayed in a similar way with untreated Fraction 1. Controls of untreated Fraction 1 *plus* untreated Fraction 2, and untreated Fraction 2 alone were included. Fraction 1 was completely active after exposure to air and the activity was entirely in the supernatant. The nature of the pellet was not determined but preliminary tests indicated it contained sulphur but not protein or lipid, nor did it contain a significant amount of iron, molybdenum or phosphate. By contrast, Fraction 2 had lost all its reduction activity on exposure to air, and did not show any ATPase activity. In similar experiments, air-treated Fraction 2 was inactive in ATP-dependent hydrogen evolution assays or reduction of isocyanide, whereas Fraction 1 was unaffected. Exposure of Fraction 2 to 6% oxygen for 30 min caused about 50% reduction in all measured activities. MOUSTAFA AND MORTENSON[30] reported that Fraction 2 from *C. pasteurianum* is cold labile and they suggested that such treatment was a convenient way of producing material free of active Fraction 2, but since the oxygen treatment is quicker and equally selective, this might be a preferable procedure.

Titration of Fraction 2 with Fraction 1

Assays of the nitrogenase were carried out with a fixed amount of Fraction 2 and varied amounts of Fraction 1 and *vice-versa*, to determine the ratio of the two which gave maximum amount of product in unit time. The results obtained with cyanide or acetylene as substrate are given in Fig. 1. They show that, for acetylene, the maximum amount of reduction was obtained with 1.1 mg protein of Fraction 1: 0.53 mg Fraction 2, a ratio of about 2:1. However, with cyanide as substrate, the observed optimum ratio was about 3.2 mg of Fraction 1: 0.53 mg of Fraction 2, a ratio of about 6:1. Nitrogen fixation or ATP-dependent hydrogen evolution had the same optimum ratio as that observed for acetylene, whereas methyl isocyanide



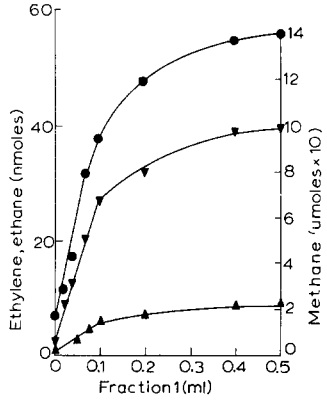


Fig. 1. Titration of Fraction 2 with Fraction 1. Activity regained for reduction of acetylene to ethylene (▲—▲) or cyanide (2 mM) to methane (○—○) upon recombination of Fractions 1 and 2. All assays contained 0.53 mg protein Fraction 2 and varied amounts of Fraction 1 (7.3 mg protein/ml).

Fig. 2. Titration of Fraction 2 with Fraction 1. Activity regained for reduction of 5 mM methylisocyanide to methane (●—●), ethylene, (▲—▲) and ethane (▼—▼). Other conditions as for Fig. 1.

(Fig. 2) resembled cyanide. The amounts of methane and ethane formed from methyl isocyanide increased with increasing amounts of Fraction 1 at approximately corresponding rates, whereas ethylene showed a slower rate of increase: the ratio of ethane to ethylene with Fraction 1:2 at 2:1 was about 4, but at 6:1 (Fraction 1:2) the ratio was 5. Reverse titrations with a fixed level of Fraction 1 and varied Fraction 2 gave the same optimum ratios and confirmed the difference between isocyanide or cyanide and nitrogen, acetylene or hydrogen evolution.

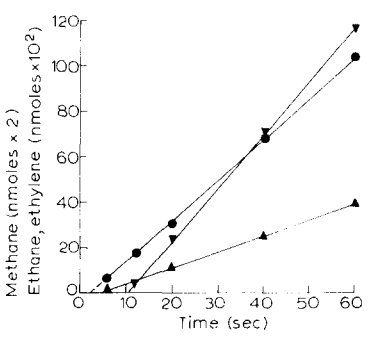
Time course of reaction

A time-course experiment was set up with two levels of Fraction 1 against one of Fraction 2. Methane, ethylene and ethane were produced at linear rates at both levels of Fraction 1; the rate of production of the three products was again faster with the higher level of Fraction 1. Corresponding experiments with acetylene as substrate showed that reduction was also linear over 10 min, but the rates with the two levels were approximately the same.

In a further time-course experiment, reactions were stopped after very short periods of time (Fig. 3). The slight lag before methane production became linear was probably insignificant, but the lag before ethylene and ethane production became linear was considered significant since it was observed in repeat experiments.

Effect of isocyanide concentration on the rate of reduction

One possible explanation of the above mentioned phenomenon is that isocyanide or cyanide caused slow irreversible inactivation of the purified nitrogenase. Isocyanide at high concentrations does inhibit crude nitrogenase[19]. The effect of isocyanide concentration was determined by setting up assays with various levels of isocyanide, a fixed level of Fraction 2 and two levels of Fraction 1. Fig. 4 shows that inhibition of methane formation did not occur until the isocyanide concentration was greater than 5 mM and that a higher level of Fraction 1 did not affect this inhibition though



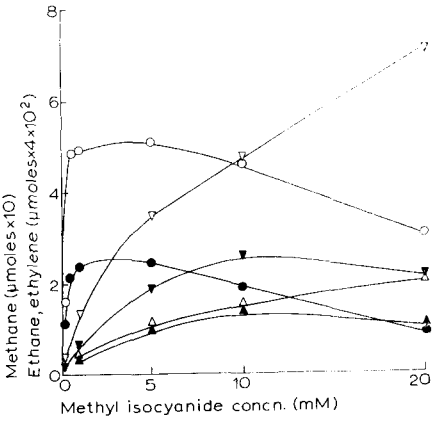


Fig. 3. Time course of reduction of 10 mM methyl isocyanide. Fraction 1, 1.46 mg; Fraction 2, 1.06 mg. ●—●, methane; ▲—▲, ethylene, ▼—▼, ethane.

Fig. 4. Effect of methyl isocyanide concentration on the amount of products. ●—●, methane; ▲—▲, ethylene; ▼—▼, ethane, with Fraction 1, 0.73 mg; ○—○, methane; △—△, ethylene; ▽—▽, ethane, with Fraction 1, 2.92 mg; Fraction 2, 0.53 mg per assay in all cases.

isocyanide reduction was faster at all concentrations with the higher level of Fraction 1. For methane production, a plot of $1/v$ against $1/S$ (ref. 32) gave a K_m for methyl isocyanide of about 0.1 mM with low level of Fraction 1, about 0.2 mM with the higher level compared with a figure of 0.18 mM for crude extracts of nitrogenase[19]. The amounts of ethylene and ethane were also higher at all isocyanide concentrations with the higher level of Fraction 1 and no inhibition of ethylene or ethane formation was observed. Other experiments have shown that inhibition of by-product formation does occure above 30 mM isocyanide with high level of Fraction 1. Differences in the calculated K_m values of isocyanide with respect to these products were obtained: K_m for ethylene was 16 mM with low level of Fraction 1, but 33 mM with the higher level. The corresponding figures for ethane were 50 mM and 11 mM.

ATPase activity

The amounts of ATP hydrolysed by nitrogenase during reduction has been determined by a number of workers[4,33,34] and used to calculate the μmoles of ATP hydrolysed per 2 electrons used for reduction. Most of these calculations produced figures of about 5 μmoles ATP hydrolysed per 2 electrons whatever the substrate, though R. H. BURRIS and his colleagues (personal communication) have calculated a figure of about 3 μmoles ATP per 2 electrons for *A. vinelandii* preparations fixing nitrogen and a low figure has been observed in our laboratory with acetylene as substrate (M. G. Yates, personal communication).

With the increased level of Fraction 1, a greater amount of methane was produced from methyl isocyanide (Figs. 2 and 4). This observation required either that more ATP was hydrolysed with the higher level of Fraction 1, or that fewer μmoles ATP were used per μmole of methane formed. ATPase activity was therefore measured in Warburg manometers with (a) methyl isocyanide, (b) acetylene and (c) no added substrate and compared with substrate reduction at two ratios of the active fractions. Because CO is an inhibitor of substrate reduction but not of H_2 evolution, flasks containing 100% CO were also included. ATPase of Fraction 1 alone was also determined. Amounts of hydrogen evolved over 10 min were determined before stopping the reaction and analysing for P_i, methane or ethylene. The results of the experiments and calculations of ATP used per μmole of product are given in Table I. The calculations do not include a correction for a slight ATPase activity of Fraction 1 alone, which was apparently $Na_2S_2O_4$ dependent. The amount of ATP hydrolysed in the absence of added substrate was not affected by the amount of Fraction 1 or by CO; 3.5–3.7 μmoles ATP were hydrolysed per μmole hydrogen produced (requiring 2 electrons per molecule). The amount of ATP used in presence of acetylene (also requiring 2 electrons per molecule) was about the same, but if allowance for the hydrogen evolved was made, *i.e.*, 3.7 μmoles ATP per 1 μmole evolved, then, with the lower level of Fraction 1, 2.9 μmoles ATP were required per 2 electrons. With the higher level of Fraction 1, this figure dropped to about 0.7. For hydrogen and acetylene reduction together, the mean amount of ATP required per electron pair was 3.2 or 2.1. With isocyanide as substrate, an increase in ATP hydrolysis was observed on increasing the level of Fraction 1. The corresponding figures for ATP requirements, assuming isocyanide needs 6 electrons for reduction, were 18 and 10. Again, if reduction of hydrogen and isocyanide were considered together, mean figures of 13.2 and 6.7 μmoles ATP per electron pair were obtained. In the presence of 100% CO,

TABLE I

DETERMINATION OF HYDROGEN EVOLUTION, ATP HYDROLYSIS AND SUBSTRATE REDUCTION WITH ACETYLENE OR METHYL ISOCYANIDE AND CALCULATIONS ON THE AMOUNT OF ATP HYDROLYSED PER 2 ELECTRONS

A, B and C measured; D, E, F and G calculated. (a) 0.73 mg Fraction 1, 0.53 mg Fraction 2; (b) 2.9 mg Fraction 1, 0.53 mg Fraction 2. For other conditions assay see text.

	Substrate added									
	None		*None*		*Acetylene*		*Methyl-isocyanide, 1 mM*		*Methyl isocyanide, 1 mM*	
Gas phase	*Argon*		*CO*		*Argon*		*Argon*		*CO*	
	(a)	*(b)*	*(a)*	*(b)*	*(a)*	*(b)*	*(a)*	*(b)*	*(a)*	*(b)*
(A) μmoles H_2 evolved	4.7	4.6	4.8	4.8	1.9	3.7	0.42	1.84	3.9	4.8
(B) μmoles methane formed							0.31	0.57	<0.02	<0.02
μmoles ethylene formed					3.7	3.8				
(C) μmoles P_i formed	16.8	16.0	17.9	17.9	17.6	16.0	18.2	24.0	20.2	23.0
(D)* Calculated μmoles ATP hydrolysed/μmole hydrogen: C/A in absence of substrate	3.6	3.5	3.7						5.2	4.8
μmoles ATP used for H_2 evolution in presence of substrate. (A × 3.6)					6.8	13.3	1.5	6.6	7.0	4.8
(E) μmoles ATP hydrolysed for reduction of other substrates (C − D)					10.8	2.7	16.7	17.4		
(F) μmoles ATP used/2 electrons (E/B*)	3.6	3.5	3.7	3.7	2.9	0.7	18.0	10.0	5.2	4.8
(G) μmoles ATP used/2 electrons (C/[A + B])					3.2	2.1	13.2	6.7		

* Making the assumption that reduction of methyl isocyanide requires 6 electrons, that of acetylene or hydrogen 2.

isocyanide reduction was inhibited almost completely, but more ATP was hydrolysed than in the control without isocyanide. Thus the ATP consumption was not affected by CO but was increased by methyl isocyanide without a corresponding increase in reduction or hydrogen evolution. In experiments to determine acetylene reduction, hydrogen evolution and ATPase activity with Fraction 2 alone (which has slight activity), 1.34 μmoles acetylene were reduced, 0.1 μmole hydrogen evolved and 6.4 μmoles ATP hydrolysed. Considering hydrogen evolution and acetylene reduction together, this gives a mean figure of 4.5 μmoles ATP per electron pair. Though the accuracy of the phosphate determination in the presence of somewhat labile creatine phosphate limits the precision of this type of experiment, the data in Table I clearly

indicate an increased efficiency in use of ATP with increased amounts of Fraction 1. The significance of these data is considered in the discussion.

ADP inhibition

Fraction 1 alone had slight $Na_2S_2O_4$-dependent ATPase activity. ATP at high levels inhibits nitrogen fixation, and it is for this reason that a low level of ATP *plus* a generating system is generally used. However, MOUSTAFA AND MORTENSON[31] have recently shown, by using the sensitive test of acetylene reduction, that the inhibitory species is ADP. It is possible that the higher level of Fraction 1 might produce inhibitory steady-state concentrations of ADP despite the presence of creatine kinase and creatine phosphate and, since there are no published data on the subject, comparative degrees of inhibition by ADP of reduction of various substrates were determined. For such assays, no creatine kinase or creatine phosphate was added; ATP (5 μmoles) alone, or with various levels of ADP, was added, and the reactions were stopped after 1 min to minimise ADP formation. Acetylene reduction was inhibited to the same extent as cyanide or isocyanide reduction by ADP: 2.5 μmoles ADP caused 45% inhibition, 5 μmoles 78%. The ATPase activity of Fraction 1 alone was confirmed by preincubating it with ATP. After 30 min preincubation, 30% inhibition of acetylene reduction occurred, corresponding to that observed with about 1.3 μmoles ADP. Thus, 0.1 ml Fraction 1 alone hydrolysed 0.018 μmole ATP/min.

Effect of CO or NO on isocyanide reduction

$N^{12}O$ or CO inhibit nitrogen fixation but do not inhibit ATP-dependent hydrogen evolution. If the purified nitrogenase has more than one site which can bind and reduce cyanide or isocyanides, then inhibition studies with CO or NO might distinguish between these sites. Assays were set up with and without CO or NO at various partial pressures and the percent inhibition of isocyanide reduction determined. Correction for a small amount of ethylene present in commercial CO was made. The formation of methane or ethane was inhibited to about the same extent by either CO or NO, but ethylene was inhibited to a smaller extent: 40% CO inhibited methane formation by 48%, ethane formation by 47%, but did not affect ethylene production. Correspondingly, 15% NO inhibited methane production by 45%, ethane production by 50% but ethylene production by only 8%. CO caused the same percent inhibition with Fraction 1 at a higher level (ratio 6:1) with various concentrations of isocyanide. Very similar results were obtained with vinylisocyanide as substrate with the low level of Fraction 1.

HARDY AND JACKSON[35] suggested that ethylene could be formed by insertion of CO into a methyl–enzyme complex formed from isocyanide and nitrogenase, but this would not happen with NO.

Reduction of nitriles

HARDY AND JACKSON[35] tested the reduction of a variety of higher nitriles by nitrogenase and used their data to speculate on the amount of space around the active site. It seemed desirable to check some of the data with the purified fractions from *A. chroococcum*. Acetonitrile, ethylnitrile or acrylonitrile 1 mM were incubated for 30 min and assayed for methane production. Ethylnitrile was not reduced at a detectable rate; acetonitrile was reduced at only 0.0075% of the rate of methyl

isocyanide and acrylonitrile at 0.008%. Since HARDY AND JACKSON tested some of these nitriles at 500 mM, small amounts of impurities or slow rates of spontaneous decomposition might be significant; the interpretation of data with such poor substrates is difficult and consequently speculations could be misleading.

Origin of by-products from isocyanide

KELLY, POSTGATE AND RICHARDS[18] suggested that the ethylene and ethane formed as by-products from methyl isocyanide reduction arise as a result of C_1 radical interaction, but HARDY AND JACKSON[35] pointed out that kinetic data indicated that free isocyanide played a role in the formation of such by-products. They suggested that isocyanide reacts with nitrogenase to form a methyl–enzyme intermediate into which free isocyanide may then insert to produce the observed ethylene and ethane, and they also detected propylene, propane and possibly C_4 hydrocarbons as additional by-products.

The variation in the ethylene: ethane ratio (Fig. 2), differential inhibition by CO and the time course of reduction (Fig. 3) suggest that ethylene may be produced by a mechanism which differs from that responsible for ethane production. Isocyanides can react not only by insertion-type mechanisms[36] involving two isocyanide carbons, but by end-on attack, the isocyanide of a free molecule reacting with the hydrocarbon end of another molecule[37]. Experiments in ^2H_2O (refs. 18, 19) gave completely deuterated methane, indicating that this product arises entirely from the isocyanide carbon, but complete analysis of the deuterated ethylene and ethane has not been made. In this work, acetylene was detected as a product of spontaneous decomposition of methyl, ethyl or vinyl isocyanide (10 mM in buffer) in absence of reducing agent. No other short-chain hydrocarbon gas was observed, even with added $Na_2S_2O_4$. Small amounts of acetylene were observed during reaction of methyl isocyanide by nitrogenase, but never more than appeared in the blank vessel without enzyme. Even if

TABLE II

EFFECT OF SUBSTRATE CONCENTRATION AND LEVEL OF FRACTION 1 ON PRODUCTS FORMED FROM METHYL, ETHYL OR VINYL ISOCYANIDE

(a) 0.73 mg Fraction 1, 0.53 mg Fraction 2; (b) 2.9 mg Fraction 1, 0.53 mg Fraction 2. Figures represent nmoles × 10.

Substrate	*Product*	*Concentration*							
		20 mM		*10 mM*		*5 mM*		*1 mM*	
		(a)	*(b)*	*(a)*	*(b)*	*(a)*	*(b)*	*(a)*	*(b)*
Methyl isocyanide (CH_3NC)	Methane	800	1890	1790	4500	2400	4840	2400	4840
	Ethylene	20.6	42.5	28.5	31.0	18.8	18.8	5.6	4.7
	Ethane	44.6	143.0	54.2	97.5	37.5	70.5	11.7	21.7
Ethyl isocyanide (C_2H_5NC)	Methane	945	182	1040	2240	755	1460	177	—
	Ethylene	3.4	6.4	2.8	4.7	11.7	1.65	0.47	—
	Ethane	2.2	5.5	1.3	3.2	6.7	1.3	0.32	—
Vinyl isocyanide (C_2H_3NC)	Methane	300	410	610	1040	1300	2560	770	1440
	Ethylene	6.3	17.6	6.1	20.6	6.8	17.9	2.3	4.0
	Ethane	13.0	26.5	21.4	52.0	35.0	89.0	22.1	52.5

nitrogenase catalysed acetylene production from isocyanide and subsequently reduced this to ethylene, then CO or NO would be expected to inhibit ethylene production to a similar extent as they inhibit formation of the other products. This was not observed, nor did acetylene accumulate in the presence of CO. To obtain further data which might indicate which mechanisms predominate in nitrogenase action, vinyl or ethyl isocyanide at several concentrations were tested as substrate with two ratios of Fraction 1:2. The results in Table II include figures for methyl isocyanide for comparison. With all the isocyanides more products were formed with higher ratio of Fraction 1, but no C_3 or C_4 products were detected. The concentration of isocyanide had an effect on the amounts and ratio of ethylene and ethane; this was particularly noticeable for vinyl isocyanide: at about 20 mM about four times as much ethylene was formed and about half as much ethane as appeared at 1 mM. The maximum rates of reduction of vinyl and ethyl isocyanide were about 52 and 46% of methyl isocyanide, respectively. The K_m for vinyl isocyanide was about 0.8 mM compared to 0.2 mM for methyl isocyanide.

DISCUSSION

The difference in the optimum ratio of Fractions 1 and 2 for maximum rate of acetylene reduction compared with that for isocyanide reduction does not appear to be due to binding and reduction of the isocyanide at more than one site on the nitrogenase, unless these sites have very similar properties with respect to isocyanide binding and inhibition by CO or NO. Isocyanide can bind to a variety of transition elements and complexes containing one or more methyl isocyanide molecule per metal atom have been prepared by Dr. R. L. RICHARDS of this Unit. These complexes are reduced by borohydride to give methane and C_2 by-products no matter whether the complexes contain 1 or 2 molecules per metal. Phenyl isocyanide complexed to metals can give methane and ethane on reduction with borohydride, indicating that a free alkyl group is not necessary for by-product formation.

The observation that methane and ethane formation are more sensitive to CO or NO inhibition than ethylene production suggests that the site of formation of this compound may be distinct but, since there is no clear correlation between product formation and chemical structure of the substrate, *e.g.* vinyl isocyanide (H_2C–CHNC) gave considerably more ethylene and ethane than ethyl isocyanide (C_2H_5NC), it is likely that during isocyanide reduction by nitrogenase several mechanisms are operating simultaneously to produce ethylene and ethane. Experiments with specifically labelled [^{14}C]isocyanides might elucidate this question.

Although the rate of acetylene reduction and ATPase activity was not affected by the higher level of Fraction 1 (Table I and Fig. 1), calculations of the amount of ATP used for acetylene reduction under the two sets of conditions indicated that less ATP was required per μmole acetylene reduced with the higher level of Fraction 1. ATPase activity was affected by the level of Fraction 1 in the presence of methyl isocyanide but again calculations indicated that less ATP was required per μmole of methane formed at the higher level of Fraction 1. However, in all these calculations a correction was made for the amount of hydrogen evolved, which increased with increased amount of Fraction 1, whether acetylene or methyl isocyanide was substrate, but not in the absence of added substrate. This correction was based on the

assumption that μmoles of ATP used per μmole of hydrogen evolved was always the same and figures calculated in the absence of substrate could therefore be subtracted from the total to obtain the amount of ATP used for reduction of substrate. However, the data of Table I suggest that this assumption, which has been widely made, is invalid. The figure of 5 μmoles ATP per 2 electrons quoted is high but if this figure is comprised of (a) ATP carefully used, *i.e.*, in producing reducing power by interaction with nitrogenase and electrons from $Na_2S_2O_4$, and (b) ATP hydrolysed without producing reducing power, then the various figures can be reconciled. The idea that ATP may be hydrolysed without interaction with electrons to give reducing power could be considered as the reverse of uncoupling of electron transfer and oxidative phosphorylation by NAD^+ in mitochrondia (*cf.* LEHNINGER[38]).

Various suggestions have been advanced for the role of ATP in nitrogen fixation. One is that it is involved in structural changes of the nitrogenase[6]. Another is that it is used to develop special reducing power required for fixation, possibly involving hydride formation[39,40]. Most hypotheses suggest that the site of substrate reduction and ATP utilization are separate; the observation that CO inhibits nitrogen fixation but not hydrogen evolution has been used as evidence that the site of fixation and evolution are separate. Two distinct sites with transfer of reducing power from the hydrogen evolving site to that of nitrogen fixation offers a reasonable working hypothesis, which has been favoured by HARDY AND BURNS[20].

To explain the data presented in this paper, it is postulated that Fraction 2 has both the site of fixation and hydrogen evolution, that ATP *plus* electrons provided by Fraction 1 from $Na_2S_2O_4$ react with Fraction 2 to produce a reduced site (site A). In the absence of added substrate, each site A is reduced, proton attack then produces hydrogen. In the presence of substrate most of the reducing power is transferred to the fixation site (site B) and is used for reduction of bound substrate.

At a ratio of Fraction 1 to 2 of 2:1, the supply of electrons from Fraction 1 determines the maximum amount of reducing power obtained. Addition of more Fraction 1 did not catalyse more hydrogen evolution in the absence of substrate (Table I) because this was already at the maximum, determined by concentration of reduced site A. However, in the presence of acetylene, some of the A sites were not reduced, consequently addition of more Fraction 1 caused increased reduction of A and more hydrogen was evolved. No more acetylene was reduced because the availability of site B was rate limiting. To explain why the amount of ATP used in the presence of acetylene was the same at the two levels of Fraction 1 (Table I) but more products were formed, it is assumed that when the supply of electrons to Fraction 2 was limited, some ATP was hydrolysed without reducing power being developed. It was this 'non-useful' ATP hydrolysis that methyl isocyanide affected, so that most of the ATP hydrolysed in the presence of isocyanide did not cause reduction of site A or B. The efficiency of reduction was therefore very low and neither site A nor B limited hydrogen evolution or isocyanide reduction. Addition of more Fraction 1 provided more electrons and though methyl isocyanide partially inhibited the formation of reduced nitrogenase, more A sites were reduced therefore, more hydrogen was evolved, also more reducing power was transferred to site B and more methyl isocyanide was reduced. This hypothesis is supported by the observation (Table I) that isocyanide, in the presence of CO (which prevents reduction by competitively blocking binding of substrate) showed higher ATPase than the controls. Thus the

postulated inhibition by isocyanide did not depend on its being bound at the site of reduction. The view that Fraction 1 is concerned with the provision of electrons is supported by the fact that, with Fraction 1 to Fraction 2 in the ratio 6:1, relatively more ethane than ethylene was formed from all isocyanides than at ratio 2:1 (Table II).

All the determinations in this paper have been carried out using excess $Na_2S_2O_4$; under conditions where the electron balance could be determined accurately, it should be possible to show differences in the electrons utilised per μmole ATP hydrolysed in the presence or absence of methyl isocyanide. The data in Table I suggest that, at best, 2 ATP per electron pair are required.

The sensitivity of *A. chroococcum* nitrogenase to oxygen was due to Fraction 2; Fraction 1 was stable in air. The observation that particulate nitrogenase of Azotobacter was relatively insensitive to oxygen implies that the oxygen-sensitive sites are protected by material which is removed during fractionation. Cytochromes of the *b* or *c* group may possibly be the protective agents. Slow loss of activity does occur with particulate nitrogenase of *A. chroococcum*, which could be due to oxygen damage. Preparations damaged in this way would have, in effect, a different ratio of Fraction 1:2 compared with undamaged nitrogenase, and this might explain some of the reported variations of ATP used per electron pair.

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