Short Communications

Reduction of Cyanide and Isocyanide by Nitrogenase of *Azotobacter chroococcum*

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Burns & Bulen (1965) showed that cell-free extracts of *Azotobacter vinelandii* contained an ATP-activated system capable of fixing nitrogen. When provided with sodium dithionite and an ATP-generating system, their preparations evolved less hydrogen under nitrogen than under argon, the difference being accountable as nitrogen reduced to ammonia. Hardy & Knight (1966b) showed that dithionite-dependent adenosine-triphosphatase activity was associated with nitrogen fixation. More recently preparations from *A. vinelandii* and *Clostridium pasteurianum* have been found to reduce a variety of additional substrate including acetylene (Dilworth, 1966), nitrous oxide, N_3^- and CN^- ions (Hardy & Knight, 1966a). We now report that nitrogen-fixing extracts of *Azotobacter chroococcum* reduce not only CN^- ions but also methyl isocyanide, and indicate the relevance of these reactions to the mechanism of nitrogen fixation.

Methods and Materials. Active cell-free extracts from *A. chroococcum* (N.C.I.B. 8003) grown on the nitrogen-free sucrose medium of Burk, Newton, Wilson & Harris (1953) were obtained by disruption at pH 7.4 with ultrasound by using a Dawe Soni-probe. Whole cells and debris were removed by centrifuging at 18000g for 30 min. and the supernatant was heated for 7 min. at 60° under an atmosphere of nitrogen. After cooling the extract was centrifuged at 18000g for 30 min. and the clear brown supernatant readjusted to pH 7.4 before use. The assay system contained 75 μ moles of tris buffer, pH 7.4, 5 μ moles of ATP, 5 μ moles of $MgCl_2$, 0.5 mg. of creatine kinase, approx. 3 mg. of $Na_2S_2O_4$, approx. 10 mg. of creatine phosphate, extract containing 5–10 mg. of protein and water to 2.4 ml. Reactions were carried out in Warburg flasks with double side arms modified for gas sampling by sealing one arm with a serum cap. After gassing, the reaction was started by tipping in solid dithionite and creatine phosphate from one side arm. Incubations were carried out in a water bath at 30° with shaking. Reactions were terminated by injecting 0.3 ml. of 30% (w/v) trichloroacetic acid into a side

arm through the serum cap and mixing this with the flask contents.

Gas samples were withdrawn from the flasks through the serum cap with a 5 ml. syringe; water was introduced into the flask as gas was withdrawn to maintain atmospheric pressure. Samples (2 ml.) of gas were analysed by vapour-phase chromatography in a Pye 104 instrument with flame ionization detector, on a column of Porapak R run at 73–74° and flow rate 75 ml./min.

Methyl isocyanide and ethyl isocyanide were prepared from the alkyl halides and AgCN (Shaw & Pritchard, 1966) and purified by preparative vapour-phase chromatography; methyl isocyanide was stored in liquid nitrogen and ethyl isocyanide was stored at –20°. Dilute aqueous solutions were prepared as required and were discarded after 4 hr.

Biochemicals were obtained from Sigma (London) Chemicals Ltd., London, S.W. 6. Other chemicals were the best obtainable commercial grades.

Results. With the complete reaction mixture under argon, hydrogen was evolved; under nitrogen, evolution of gas was partially inhibited. The differences were accountable as fixed nitrogen (Kelly, 1966). KCN or methyl isocyanide (5 mm) partially inhibited gas evolution under argon and the gas phase contained methane. The amount formed increased linearly with the time of incubation up to 30 min. No methane was formed if the ATP-generating system, the extract or the dithionite were omitted. Concentrations of methyl isocyanide greater than 5 mm caused inhibition of dithionite-dependent adenosine triphosphatase and there was a corresponding decrease in the amount of methane formed. Extracts from urea-grown *A. chroococcum* did not fix nitrogen, nor did they show ATP-dependent hydrogen evolution or reduction of methyl isocyanide.

Examination of reduction of other cyanides and isocyanides by this system has produced the following information. When each substrate was present at 10 mm the relative rates of reduction were: methyl isocyanide, 100; KCN, 55; ethyl isocyanide, 9;

methyl cyanide, <0.2. Trifluoromethyl cyanide, ethyl cyanide, methyl iodide, methylamine and formaldehyde did not react to yield methane.

Besides methane, small amounts of ethylene and ethane were formed from cyanide and the isocyanides. These products were not observed if the ATP-generating system, dithionite or extract were omitted from the assay. Ethane and ethylene were true products of the reaction because they could not be detected as impurities in the substrates and the variations in their ratios according to the experimental conditions were inconsistent with their being formed from impurities. The relative amounts of methane, ethylene and ethane were different for the different substrates. At 5mm-methyl isocyanide gave the proportions 100:0.28:2.1, ethyl isocyanide gave 100:0.28:0.47 and cyanide ion gave the much different proportions 100:0.08:0.07. Temperature between 15° and 40° had no significant effect on these ratios, but the concentration of substrate did: with methyl isocyanide at 0.5mm the proportions were 100:0.05:0.21 but at 20mm they changed to 100:1.7:9.3.

Methylamine or ethylamine were also formed from methyl isocyanide or ethyl isocyanide respectively. These products were separated from the reaction mixture by Conway's (1962) micro-diffusion technique and identified by paper chromatography in butanol-acetic acid-water (40:10:50, by vol.).

At 5% carbon monoxide caused 100% inhibition of nitrogen fixation (Bulen, LeCompte, Burns & Hinkson, 1965). Methane formation was inhibited 90% by 10% carbon monoxide in argon, but the amount of ethylene formed slightly increased and ethane formation was only inhibited by 50%.

The reduction of these substrates in an environment of D₂O was examined by resuspending freeze-dried nitrogenase in 99.7% D₂O. The nitrogenase was sedimented by centrifuging for 4hr. at 200 000g and the pellet resuspended in more D₂O. The other reagents of the assay system were also prepared in D₂O; some of the freeze-dried material was put through a similar procedure with unlabelled water to produce control material.

With 5mm-methyl isocyanide the rate of methane formation observed in D₂O was about 60% of that observed in water but the rates of ethylene and ethane formation were about 300% and 150% respectively. The corresponding values for ethyl isocyanide were: methane, 75%; ethylene, 200%; ethane, 300%. For cyanide the values were: methane, 77%; ethylene, 200%; ethane, 100%. Examination of the methane formed in D₂O from methyl isocyanide with an MS9 mass spectrometer revealed only CD₄.

Comparison of the rates of methane formation from methyl isocyanide under argon or nitrogen

showed that 1 atm. of nitrogen caused 18% inhibition with 10mm-methyl isocyanide and 42% with 0.5mm-methyl isocyanide. Formation of ethylene and ethane was inhibited to similar extents.

Discussion. A variety of substrates, as mentioned in the introduction, interact with the ATP-activated nitrogenase from *Azotobacter*. This ability does not prove that a substrate such as methyl isocyanide is bound at the nitrogen-fixing site, but the inhibition of methyl isocyanide reduction by nitrogen makes this a reasonable presumption. It might be expected that binding of CN⁻ ions would occur through the nitrogen atom and therefore that methyl isocyanide would not be a substrate, but if substrate binding involved transition metals methyl isocyanide and cyanide would probably bind through the carbon atom. The reduction of methyl isocyanide but not methyl cyanide, the formation of CD₄ from isocyanide and the identification of methylamine as a reduction product make it clear that the substrate is bound by the isocyanide carbon atom.

Ethylene and ethane were consistently produced in small amounts, suggesting that C₁ radicals were formed during the reduction that interacted to a limited extent to give C₂ by-products. In principle this process could occur by two mechanisms: either a C₁ radical could react with a free molecule of isocyanide or, if, as Bulen *et al.* (1965) have suggested, the nitrogenase has two adjacent binding sites normally capable of fixing one nitrogen molecule, then two isocyanide molecules might interact at these sites. The C₂ by-products would then be formed by interaction of adjacent C₁ radicals. This second mechanism is supported by two lines of evidence. (1) The CN⁻ ion, which could bind across two sites, gave only traces of C₂ products and its rate of reaction was just over half that of methyl isocyanide. (2) D₂O decreased the rate of methane formation but did not influence the formation of ethylene and ethane proportionally. If reduction of methyl isocyanide involved successive attacks on the bound radical by hydrogen, the rate of methane formation could be considerably slower in D₂O (Falk & Halpern, 1965), but the interaction of two C₁ radicals would be unaffected and therefore ethylene and ethane could be produced at a relatively faster rate in D₂O than in H₂O.

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Menachromanol from a Photosynthetic Bacterium

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Chlorobium thiosulphatophilum has been shown to contain two naphthaquinones, menaquinone-7 and 'chlorobiumquinone' (Frydman & Rapoport, 1963). The possibility that other related compounds are also present has been investigated.

C. thiosulphatophilum (N.C.I.B. 8346) was grown anaerobically as described by Larsen (1953). The harvested organism was extracted with acetone, the acetone extract diluted with water and the lipid extracted into ether. After the ethereal extract had been evaporated to dryness *in vacuo* the lipid was adsorbed on a silicic acid-Celite (2:1, w/w) column. The less-polar lipid constituents were eluted with 25% (v/v) diethyl ether in light petroleum (b.p. 40-60°), leaving the chlorophyll and other polar lipids adsorbed on the column.

A small sample of the eluted lipid was chromatographed on thin layers of silica gel G with 10% (v/v) di-isopropyl ether in light petroleum. The developed chromatoplates were sprayed with 0.002% (w/v) fluorescein in ethanol and examined under ultraviolet light. Strongly ultraviolet-absorbing compounds detected were 'chlorobiumquinone' (R_f 0.30) and menaquinone-7 (R_f 0.74). However, a compound that showed a strong blue fluorescence in ultraviolet light (R_f 0.56) was also observed. This compound gave an immediate Emmerie-Engel positive reaction, suggesting that it may be phenolic.

To obtain more of this compound the lipid eluted from the first column was rechromatographed on a second silicic acid column. The fluorescent compound was eluted along with the menaquinone by 2.5% (v/v) diethyl ether in light petroleum, the γ -carotene having been previously eluted with 0.5% (v/v) diethyl ether in light petroleum. The

fluorescent compound was separated from the menaquinone by thin-layer chromatography as before. The compound in cyclohexane showed λ_{max} at 245 (intense) 320 and 338 m μ (weak), which are very similar to those shown by naphthachromanol (Mamont, Cohen, Azerad & Vilkas, 1965).

To test the possibility that this compound was menachromanol, phylochromanol was prepared from phyloquinone (Chen & Dallam, 1963). On comparison, the two compounds were extremely similar, showing identical spectra and fluorescence in ultraviolet light. However, they differed in their polarity; the *Chlorobium* compound had R_f 0.44 on thin-layer chromatography with 7% (v/v) di-isopropyl ether in light petroleum, whereas that of phylochromanol was 0.30.

Comparison of the infrared spectra of the two compounds showed many points of similarity, the differences being accounted for by the presence of an unsaturated side chain in the compound isolated from *Chlorobium* (Table 1).

From this evidence the compound appears to be a menachromanol, and, as we have confirmed that the menaquinone from *Chlorobium* is menaquinone-7 by nuclear-magnetic-resonance spectrometry, it is likely to be menachromanol-6. To prove this completely it would be necessary to synthesize the compound; unfortunately, published methods for cyclizing the corresponding quinone were found to lead to further cyclization in the side chain.

Russell & Brodie (1961) have shown exogenous phyloquinone to be cyclized to phylochromanol by extracts of *Mycobacterium phlei*, and, moreover, have demonstrated chromanol formation from the endogenous menaquinone-9(2H) in phosphorylating preparations. There have been many