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The Kinetics of the Reduction of Isocyanides, Acetylenes and the Cyanide Ion by Nitrogenase Preparation from *Azotobacter chroococcum* and the Effects of Inhibitors

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1. Nitrogen-fixing preparations from *Azotobacter chroococcum* reduced substrates with the following K_m values: methyl isocyanide, $1{\cdot}8 \times 10^{-4}$M; ethyl isocyanide, $2{\cdot}5 \times 10^{-2}$M; cyanide ion, $1{\cdot}4 \times 10^{-3}$M; acetylene, $1{\cdot}2 \times 10^{-4}$M. 2. Nitrogen, carbon monoxide or hydrogen competitively inhibited isocyanide reduction with the following K_i values: hydrogen, $1{\cdot}3 \times 10^{-3}$M; carbon monoxide, $6{\cdot}8 \times 10^{-6}$M; nitrogen, $4{\cdot}3 \times 10^{-4}$M. 3. Living nitrogen-fixing bacteria, and isolated clover nodules, formed methane from methyl isocyanide. 4. These results are discussed in relation to other work and possible mechanisms of nitrogen fixation.

Cell-free fixation of nitrogen was first reported by Carnahan, Mortenson, Mower & Castle (1960), with extracts of *Clostridium pasteurianum*. Bulen, Burns & LeComte (1965*a*) showed that particulate preparations from *Azotobacter vinelandii* not only fixed nitrogen but catalysed hydrogen evolution anaerobically; both nitrogen fixation and hydrogen evolution required ATP and sodium dithionite. The amount of hydrogen evolved in unit time under argon was greater than that evolved under nitrogen and the difference was related to the amount of nitrogen fixed. Hydrogen evolution and nitrogen fixation were accompanied by a specific adenosine triphosphatase, which was distinguished from non-specific adenosine triphosphatase by its heat stability, insensitivity to fluoride inhibition and requirement for dithionite (Hardy & Knight, 1966*a*). Carbon monoxide at low partial pressures inhibited nitrogen fixation but not hydrogen evolution (Bulen, LeComte, Burns & Hinkson, 1965*b*), and consequently hydrogen evolution under nitrogen plus carbon monoxide was the same as that under argon with or without carbon monoxide. Nitrogen fixation during growth of *Azotobacter* spp. was known to be inhibited by the azide ion (Wilson & Roberts, 1954) or nitrous oxide (Rakestraw & Roberts, 1957), and inhibition of cell-free nitrogen fixation by the azide or cyanide ion, or by nitrous oxide, was demonstrated by Lockshin & Burris (1965). Schölhorn & Burris (1966, 1967*a*) reported that azide was reduced by nitrogenase, and Hardy & Knight (1966*b*, 1967) reported that cyanide or nitrous oxide was also reduced by nitrogenase preparations. Nitrogenase preparations were shown to reduce acetylene, though only as far as ethylene (Schölhorn & Burris, 1966, 1967*b*; Dilworth, 1966). Although the cyanide or azide ion, or nitrous oxide, interact with the relevant site of nitrogenase through a nitrogen atom, binding by a nitrogen atom is clearly not essential, since acetylene could interact only through a carbon atom. Isocyanides ($R{-}N{\geq}C$) were therefore considered as possible substrates for, or inhibitors of, nitrogenase activity. A short communication on the research has already been published (Kelly, Postgate & Richards, 1967), and further details of the work and some kinetic data are presented here.

METHODS AND MATERIALS

Growth of Azotobacter chroococcum *N.C.I.B.* 8003. Most of the material used in this work was provided by the Department of Biochemistry, Imperial College of Science and Technology, London, from a 3000l. batch grown in nitrogen-free medium (Newton, Wilson & Burris, 1953). Small quantities were grown in 20l. fermenters containing about 16l. of the same medium, which was sterilized by autoclaving for 30min. at 15lb./in.^{2}, inoculated with 100ml. of 2-day culture (0·8–1mg. dry wt./ml.) and flushed with air at approx. 5l./min. for 48hr. at 30°. Cells were harvested by centrifuging for 20min. at 3000*g* in an MSE 6l. centrifuge. The cell paste was washed once with water and then stored at −20°. No significant differences in the activities of nitrogenase preparations from such material were observed during 9 months. Bacteria grown on a fixed-nitrogen source were obtained by adding sterile urea (final concn. 50mM) to a nitrogen-free medium; otherwise conditions of growth, harvesting and storage were the same as for nitrogen-fixing bacteria.

Preparation of nitrogen-fixing extracts. Frozen cells were thawed, an equal volume of water was added and 2N-KOH was added in drops with careful stirring until the pH,

initially between 5·5 and 6·0, was about 7·0. Batches (30ml.) of cell suspension, cooled in ice, were subjected to ultrasound from a Dawe stainless-steel Soniprobe at 8A for 2min. The probe was cooled in ice for 2min. and the batch then subjected to a further 2min. treatment. Batches were combined and centrifuged at 36000*g* for 30min. and the turbid supernatant, containing 30–40mg. of protein/ml., was decanted. More nitrogenase activity could be obtained from the pellet after further treatment with the Soniprobe, so residues were retained for further extraction. The pH value of the supernatant, initially about 6·0, was adjusted to 7·4 with 2N-KOH. The material was heated under N_2 in a water bath at 60° for 7min. and then cooled in ice before centrifuging again at 36000*g* for 30min. The clear brown supernatant was decanted and its pH value adjusted to 7·4, if necessary. Heat-treated nitrogenase (8–16mg. of protein/ml.) was stable for at least a week at 4° under N_2 and was usually stored in this way; it was not appreciably damaged by repeated freezing and thawing or by freeze-drying.

Assay of nitrogenase activity by hydrogen evolution. Assays of the dithionite- and ATP-dependent evolution of H_2 (Bulen *et al.* 1965*a*) were carried out at 30° in Warburg flasks. Each flask contained 75μmoles of tris–HCl buffer, pH7·4, 5μmoles of ATP, 5μmoles of $MgCl_2$, 0·5mg. of creatine kinase (EC2.7.3.2, specific activity 15–50units/mg.), extract containing 3–12mg. of protein and water to 2·4ml. in the main compartment. The centre well contained 0·1ml. of 40% KOH. Approx. 3mg. of sodium dithionite and 10mg. of creatine phosphate as solids were placed in the side arm. Vessels were gassed at 30° with argon or N_2 for 5min. and, after temperature equilibration, the reaction was started by tipping in the dithionite and creatine phosphate. Evolution of gas was measured for 20–60min.

Assay of nitrogen fixation. Warburg flasks were set up as for evolution of H_2. At the end of the reaction time 1ml. samples were removed and the NH_3 content was determined by micro-diffusion and titration (Conway, 1962). The difference in diffusible NH_3 between the control under argon and that under N_2 was due to nitrogen fixed (Mortenson, 1962; Bulen *et al.* 1965*a*).

Assay of dithionite-dependent adenosine triphosphatase. An assay procedure similar to that of Hardy & Knight (1966*a*) was used. Warburg flasks containing 5μmoles of ATP, 5μmoles of $MgCl_2$, 30μmoles of tris–HCl buffer, pH7·4, extract containing about 3mg. of protein and water to 1ml. in the main compartment and about 1mg. of solid sodium dithionite in the side arm were gassed with argon or N_2 and, after temperature equilibration at 30°, the reaction was started by tipping in the dithionite. After 10min., 0·2ml. of reaction mixture was withdrawn and analysed for P_i by the method of Taussky & Shoor (1953). A blank was run without dithionite.

Assay of reduction of various compounds. Reduction of isocyanides, acetylenes or the cyanide ion was studied in conditions similar to those used for nitrogen fixation. To obtain gas samples, double-side-armed Warburg flasks were used with one side arm of each flask sealed with a gas-tight serum cap and the other sealed with the normal type of gassing stopper. Flasks were attached to two-way taps and gassed in the usual way; KOH was not added to the centre well, and substrate was placed in the main compartment. After temperature equilibration at 30°, the reaction was started by tipping in solid dithionite and creatine phosphate from the side arm. After 10–40min., 0·3ml. of 30% (w/v) trichloroacetic acid was injected through the serum cap, mixed with the contents of the main compartment to stop the reaction, and 1ml. or 2ml. gas samples were withdrawn through the serum cap into a syringe for analysis by gas–liquid chromatography. Simultaneously water was allowed to pass into the flask through the two-way tap to maintain the internal pressure at about 1atm. Gas samples were analysed for hydrocarbons in a Pye 104 gas-chromatography instrument fitted with a flame-ionization-detector head. Samples were injected into a 4ft. Porapak R column, 4mm. int. diam., at either 75° or 45° with N_2 as carrier at a flow rate of 75ml./hr. The retention times of C_1, C_2 and C_3 hydrocarbons were determined by injecting standard samples of these gases. The instrument was calibrated quantitatively for methane, ethylene and ethane by injecting measured volumes of these gases accurately diluted with argon. With a fixed volume of sample, the concentrations of methane, ethylene or ethane were proportional to peak height.

Detection of amines as reduction products of isocyanides. The amines from 1ml. samples of reaction mixture were micro-distilled into about 0·5ml. of 6·7mN-H_2SO_4 (Conway, 1962) and determined quantitatively by titration. For qualitative determinations, solutions were concentrated to about 50μl. with a stream of air at room temperature and then spotted on to Whatman no. 4 chromatography paper. Methylamine, ethylamine and dimethylamine (5μmoles of each) were included as markers, and the paper was run for 5hr. as an ascending chromatogram in butan-1-ol–acetic acid–water (4:1:5, by vol.) (Lederer & Lederer, 1957). The paper was then dried and sprayed with 0·25% ninhydrin in butan-1-ol and heated at 80° for 10min. Amines were apparent as pink spots.

Determination of protein. Protein concentrations were determined by the biuret method, as modified by Gornall, Bardawill & David (1949).

Mass spectrometry. Aqueous samples were analysed for ^{15}N in an A.E.I. MS 3 instrument, after conversion into N_2, by Miss S. Hill, and hydrocarbon samples were analysed for CD_4, C_2D_4 and C_2D_6 in an A.E.I. MS 9 instrument by Mr F. Bloss.

Chemicals. Biochemicals used in this work were obtained from Sigma (London) Chemical Co., London, S.W. 6. A neutralized 100mM solution of ATP was prepared and distributed in 1ml. amounts in tubes; these were stored at −20° and thawed as required. Creatine kinase (10mg./ml. of 25mM-tris–HCl buffer, pH7·4) was stored in a similar way.

Methyl isocyanide, ethyl isocyanide and phenyl isocyanide were prepared by Dr R. Richards by the procedure of Pritchard & Shaw (1966); vinyl isocyanide was prepared by methods of Matheson & Baily (1967). Methyl isocyanide and vinyl isocyanide were stored at −70° in a solid-CO_2 cabinet, and ethyl isocyanide and phenyl isocyanide were stored at −20°. Solutions of these compounds in water were prepared as required and discarded after 4hr. Acetylene (3%, v/v, in argon) was obtained from Hilger and Watts Ltd., London, N.W. 1. Methylacetylene was obtained from Cambrian Chemicals Ltd., London, S.E. 16. Helium was obtained from Fisons Scientific Apparatus Ltd., Loughborough, Leics. D_2O (99·7%) was obtained from Anderman and Co. Ltd., London, S.E. 1. Other chemicals used in this work were the best grade commercially available.

RESULTS

General properties of nitrogenase preparations. The general properties of the heat-treated extracts were very similar to those described for crude nitrogenase preparations from *A. vinelandii* (Bulen *et al.* 1965*a*). The optimum pH for both hydrogen evolution and nitrogen fixation was 7·4; fixation of nitrogen was confirmed by using $^{15}N_2$. The rate of hydrogen evolution or nitrogen fixation was proportional to the enzyme concentration between the limits 3–14mg. of protein, and both activities were linear with time for 30–60min. All nitrogenase activity was sedimented after 3hr. at 200000*g*. The reactions were inhibited by buffer concentrations greater than 35mM or if more than 10μmoles of ATP/vessel was used. No hydrogen evolution or nitrogen fixation was observed if ATP or dithionite was omitted from the system, nor did extracts of urea-grown cells catalyse either reaction; this information, with evidence for partial purification by gradient centrifugation, has been reported (Kelly, 1966).

Effect of cyanide, isocyanides and acetylenes on hydrogen evolution. The ATP- and dithionite-dependent hydrogen evolution catalysed by nitrogenase preparations from *A. chroococcum* was determined in the presence of a number of compounds. The results given in Fig. 1 show that 10% carbon monoxide had no effect on hydrogen evolution under argon, but that the cyanide ion, methyl isocyanide or ethyl isocyanide inhibited gas evolution. Acetylene (3% in argon) also inhibited gas evolution, but a saturated solution of phenyl isocyanide or 5mM-methyl cyanide had no effect on hydrogen evolution, nor did methylamine or formaldehyde at 5mM. Formaldehyde and methylamine were tested because they arise by spontaneous hydrolysis of methyl isocyanide. Since inhibition of hydrogen evolution could occur either because the compound was an alternative substrate to nitrogen or because it was an ordinary inhibitor, evidence for reduction of these compounds was sought.

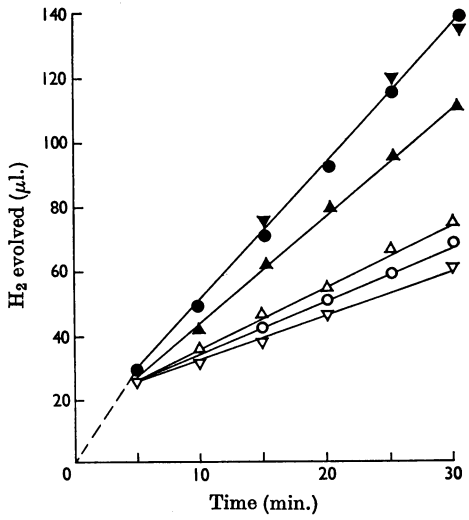

Fig. 1. Effect of various compounds on reductant-dependent hydrogen evolution from *A. chroococcum* extracts. Hydrogen evolution from an extract of *A. chroococcum* under argon was measured manometrically after dithionite had been tipped, in the presence of an ATP-generating system of creatine phosphate+creatine kinase. Manometer vessels contained 6mg. of protein and soluble substrates were at 5mM. For other experimental details see the Methods and Materials section. ●, Control; ▼, 10% (v/v) CO in atmosphere; ▲, ethyl isocyanide; △, methyl isocyanide; ○, nitrogen; ▽, KCN.

Reduction products of various substrates. (*a*) Hydrocarbons. Chromatographic analysis of gas samples, after incubation of substrates with the complete system, showed that methane was the major product from both cyanide and the isocyanides. As reported by Kelly *et al.* (1967), methyl isocyanide was reduced more rapidly than cyanide, and ethyl isocyanide was reduced only slowly. The rate of reduction of vinyl isocyanide was comparable with that of methyl isocyanide. All these substrates yielded ethane and ethylene as minor products, the smallest proportion of C_2 products coming from the cyanide ion. In the light of the report by Hardy & Jackson (1967) of C_3 products formed from comparable substrates, the products from methyl isocyanide were re-examined after freezing them out in liquid nitrogen, and propane was detected. Methylamine, methyl cyanide and ethyl cyanide (5mM) did not yield methane in our test conditions; Hardy & Knight (1967) have since reported that they are slowly reduced. Acetylene was reduced to ethylene, as reported by other workers; methylacetylene gave propylene. None of these products was observed if the ATP-generating system, dithionite or extract was omitted, nor did extracts of urea-grown cells produce methane, ethylene or ethane from substrates.

(*b*) Amines. Samples (1ml.) of reaction mixture were analysed for amines after reduction of methyl isocyanide or ethyl isocyanide as described. The amine product from methyl isocyanide was methylamine, the amine from ethyl isocyanide was ethylamine, and the amount formed, when measured by titration, corresponded approximately to the amount of hydrocarbon formed. The amine product from vinyl isocyanide was not examined.

Reduction of methyl isocyanide by undamaged cells. Suspensions of *A. chroococcum* from nitrogen-free medium, when harvested and resuspended in spent

medium (4–10mg. dry wt. of bacteria/ml.), formed small amounts of methane after incubation for 1hr. with 1 μmole of methyl isocyanide/ml. Other work in this Laboratory has shown that *Beijerinckia indica* (N.C.I.B. 8597), *Derxia gummosa* (N.C.I.B. 9064), *Clostridium pasteurianum* (strain W.5) and a nitrogen-fixing strain of *Desulfovibrio desulfuricans* (N.C.I.B. 8338) also formed methane from methyl isocyanide under similar conditions. Root nodules of *Trifolium subterraneum* L. (strain Bacchus Marsh), infected with *Rhizobium trifolium* (strain 212), formed methane from methyl isocyanide after 3hr. at 30° under argon with or without 0·05atm. of oxygen. Methane was not formed by non-nitrogen-fixing bacteria such as *Escherichia coli* or *Desulfovibrio vulgaris* (N.C.I.B. 8303).

General properties of the isocyanide-reducing system. The optimum pH for reduction of methyl isocyanide to methane was about 7·4. The rate of reduction was linear with time up to 30min. and proportional to the amount of enzyme within the limits 3–14mg. of protein. Reduction to methane was inhibited by a buffer concentration greater than 35mM or ATP at more than 10 μmoles/flask. All isocyanide-reducing activity was sedimented by centrifuging at 200000*g* for 3hr.

Effect of preincubation with methyl isocyanide. Although the reduction of isocyanide was linear with time, irreversible binding of this substrate to the nitrogenase complex might occur, thus inhibiting nitrogen fixation. Three flasks were set up: flask (*a*) with all components for nitrogen fixation was gassed with nitrogen; flask (*b*) with all components for nitrogen fixation + 5mM-isocyanide was gassed with argon; flask (*c*) with all components except the ATP-generating system and dithionite was gassed with argon. After temperature equilibration the reactions were started; at the end of 30min. flask contents were removed and centrifuged separately at 200000*g*. The three pellets were resuspended in 25mM-tris–hydrochloric acid buffer, pH7·4, and each resuspended sample was assayed for hydrogen evolution, nitrogen fixation and methyl isocyanide reduction. No significant differences in hydrogen evolution, nitrogen fixation or isocyanide reduction were detected among the three samples. Any isocyanide that remained bound thus did not affect the nitrogenase activity.

Effect of methyl isocyanide on adenosine triphosphatase activity. Dithionite-dependent adenosine triphosphatase activity was determined under argon + 5mM-methyl isocyanide, under argon ± 5% carbon monoxide and under nitrogen. No significant differences in the activities were observed.

Effect of substrate concentration on rate of reduction. The general properties and requirements for isocyanide reduction were thus very similar to those of nitrogen fixation. These similarities do not necessarily prove that reduction of isocyanide occurs at the same site as nitrogen fixation; the kinetics of isocyanide reduction were therefore examined to obtain further evidence on the question. The effect of varying the concentrations of the cyanide ion, methyl isocyanide, ethyl isocyanide, acetylene or nitrogen was determined. Reaction rates were measured as the rates of evolution of methane or ethylene or formation of ammonia; K_m values for these substrates were determined graphically (Lineweaver & Burk, 1934). Fig. 2 shows such a plot for methyl isocyanide; inhibition of methane formation was observed at high substrate concentrations. The K_m values were: methyl isocyanide, $1{\cdot}8 \times 10^{-4}$M; ethyl isocyanide, $2{\cdot}5 \times 10^{-2}$M; cyanide

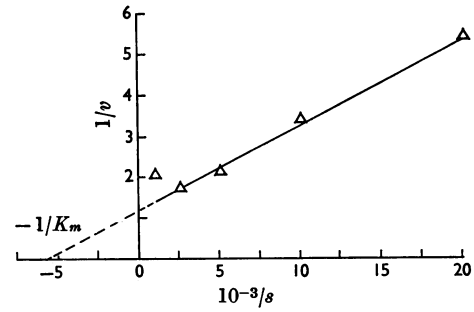

Fig. 2. Lineweaver–Burk plot of the rate of methane production against methyl isocyanide concentration for *A. chroococcum* extracts. Reaction flasks contained 6mg. of protein, an ATP-generating system of creatine phosphate + creatine kinase and dithionite. Methane formation was measured chromatographically at intervals after solid creatine phosphate + dithionite had been tipped; for other experimental details see the Methods and Materials section. Initial velocity, *v*, is expressed in μmoles/hr.; substrate concentration, *s*, is expressed in moles/l. $K_m = 1{\cdot}8 \times 10^{-4}$M.

Table 1. *Effect of methyl isocyanide concentration on the proportions of methane, ethylene and ethane produced*

For assay conditions see the Methods and Materials section. Methane, ethylene and ethane were measured chromatographically. Ratios are expressed with the methane concentration adjusted to 1000.

Concn. of isocyanide (mM)	Methane	Ethylene	Ethane
0·5	1000	0·46	1·1
1·0	1000	0·60	2·0
1·0	1000	3·4	11·0
10·0	1000	6·9	20·6
20·0	1000	13·4	49·0

ion, $1{\cdot}4 \times 10^{-3}$M; acetylene, $1{\cdot}2 \times 10^{-2}$M; nitrogen, $1{\cdot}2 \times 10^{-4}$M.

Effect of methyl isocyanide concentration on the relative amounts of methane, ethylene and ethane. If all the ethylene and ethane formed came from interactions of isocyanide molecules bound to the active site, then the amounts formed should have been independent of substrate concentrations above a saturation level of $2 \times K_m$ for methyl isocyanide, but, if a free isocyanide molecule reacted with a bound one, the amounts of by-products would be related to total isocyanide concentration. The relative amounts of methane, ethylene and ethane were determined over a range of isocyanide concentrations with the results given in Table 1. The proportion of ethylene and ethane formed increased with increasing concentration of isocyanide, though the ratio of ethylene to ethane remained about 1:3.

Inhibition of isocyanide reduction by carbon monoxide, hydrogen or nitrogen. Reduction of methyl isocyanide at various concentrations was determined under argon or under nitrogen or hydrogen. Nitrogen or hydrogen gas inhibited the reduction, and the following K_i values were determined by Lineweaver & Burk (1934) plots: nitrogen, $4{\cdot}3 \times 10^{-4}$M; hydrogen, $1{\cdot}3 \times 10^{-3}$M. Carbon monoxide, which is a powerful inhibitor of nitrogen fixation (Bulen *et al.* 1965*b*), inhibited methane production from 1mM-isocyanide by 90% and from 5mM-isocyanide by 15% at 5% (v/v) in argon. In contrast, carbon monoxide stimulated ethylene production: twice as much ethylene appeared in these conditions as was formed without carbon monoxide. Further kinetic experiments showed that carbon monoxide was a competitive inhibitor of methane formation with K_i $6{\cdot}8 \times 10^{-5}$M.

Reduction of cyanide and isocyanides in D_2O. If all the methane arises from the isocyanide carbon, reduction in 100% D_2O should give only CD_4, and this result was obtained (Kelly *et al.* 1967). Similarly, the ethylene and ethane formed from isocyanides should also be entirely deuterated if they arise solely from the isocyanide carbon, but the amounts of ethylene and ethane formed, with the procedure described by Kelly *et al.* (1967), were too small for direct analysis in the MS 9 instrument. By carrying out the reaction on a larger scale, freezing the products in liquid nitrogen and evacuating for 30sec., the residual gases obtained by warming the flask to $-70°$ contained five times the concentration of methane and about 50 times the concentration of ethylene and ethane. The selective enrichment obtained was due to differences in the b.p. of methane, ethylene and ethane ($-184°$, $-103°$ and $-88{\cdot}3°$). Although a complete quantitative analysis of the gas samples was not practicable, only deuterated hydrocarbons were observed on mass-spectrographic analysis.

DISCUSSION

The very similar general properties of isocyanide reduction and nitrogen fixation by the nitrogenase preparation and, more particularly, the competitive inhibition of isocyanide reduction by nitrogen itself make it very probable that reduction of isocyanides takes place at the same site on the nitrogenase complex as does reduction of nitrogen itself. The fact that methyl isocyanide and methylacetylene are substrates indicates that the reduction mechanism does not necessarily require the substrate to be bound at two points. The nitrogen molecule could bind endways on to a metal atom at the active site, and chemical complexes containing nitrogen bound in this way have been prepared (Allen & Senoff, 1965; Collman & Kang, 1966). Yamamoto, Kitazume, Pu & Ikeda (1967) reported a cobalt complex able to bind gaseous nitrogen end-on, yielding a stable complex with molecular nitrogen. Work on the reduction products of model platinum isocyanides by R. Richards in this Laboratory has shown that C_2 and C_3 products can be obtained in addition to methane with isocyanides bound to a single metal atom. The production of ethylene and ethane from methyl isocyanide can be explained in various ways (Kelly *et al.* 1967). The observation that the amounts of ethylene plus ethane formed continued to increase after the enzyme was saturated with isocyanide according to the K_m suggests that free isocyanide molecules play some part in formation of the secondary hydrocarbon products, as has been suggested by Hardy & Jackson (1967).

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