Biochem. J. (1985) **227**, 719–725
Printed in Great Britain

Suicidal inactivation and labelling of ammonia mono-oxygenase by acetylene

Michael R. HYMAN and Paul M. WOOD*
Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, U.K.

(*Received 7 September 1984/3 December 1984; accepted 28 January 1985*)

Acetylene brings about a progressive inactivation of ammonia mono-oxygenase, the ammonia-oxidizing enzyme in *Nitrosomonas europaea*. High NH_4^+ ion concentrations were protective. The inactivation followed first-order kinetics, with a rate constant of $1.5\,min^{-1}$ at saturating concentrations of acetylene. If acetylene was added in the absence of O_2, the cells remained active until O_2 was re-introduced. A protective effect was also demonstrated with thiourea, a reversible non-competitive inhibitor of ammonia oxidation. Incubation of cells with [^{14}C]acetylene was found to cause labelling of a single membrane polypeptide. This ran on dodecyl sulphate/polyacrylamide-gel electrophoresis with an M_r value of 28000. It is concluded that acetylene is a suicide substrate for the mono-oxygenase. The labelling experiment provides the first identification of a constituent polypeptide of ammonia mono-oxygenase.

The nitrifying bacterium *Nitrosomonas europaea* gains energy from the oxidation of ammonia, and the name ammonia mono-oxygenase has been given to the enzyme responsible for the initial hydroxylation reaction (Hyman & Wood, 1983):

$$NH_3 + 2[H] + O_2 \rightarrow NH_2OH + H_2O$$

It has recently become clear that this enzyme can also bring about a range of organic oxidations, including alkane and arene hydroxylations and conversion of alkenes into epoxides (Drozd, 1980; Hyman & Wood, 1984). With other broad-specificity oxygenases, it has frequently been found that the attempted oxidation of certain chemicals results in inactivation of the enzyme, either on the first or on multiple turnovers. Many such suicide substrates are known for cytochrome *P*-450 (Ortiz de Montellano & Correia, 1983). For example, incubation of hepatic cytochrome *P*-450 with acetylene in the presence of NADPH and O_2 leads to production of an inactive green pigment, which has been shown to contain *N*-alkylated haem (Kunze *et al.*, 1983). Suicide substrates provide a highly specific means of labelling an enzyme's active site, as well as information on the mechanism of the catalytic process.

Hynes & Knowles (1978, 1982) have shown that acetylene is a potent inhibitor of ammonia oxidation in *Nitrosomonas europaea*. The oxidation of hydroxylamine was not impaired. Their experiments demonstrated that the inhibition was not readily reversible. Full recovery of cells exposed to acetylene took at least 10 days and appeared to require the synthesis of fresh protein. They reported acetylene inhibition to be non-competitive with respect to ammonia, with a very low K_i value of $0.25\,\mu M$. In conclusion they stated 'the mode of action of acetylene on this enzyme is not known'.

Many of the experiments described below make use of O_2-uptake rates, measured with an oxygen electrode. It should be noted that the cells show a low rate of respiration with no added reductant or with the mono-oxygenase totally inhibited (Hyman & Wood, 1983). The rate of O_2 uptake with NH_4^+ present is normally much higher than this endogenous rate, and consequently provides a good measure of the rate of ammonia oxidation. The stable product is nitrite, with no accumulation of hydroxylamine unless its oxidation is prevented (Dua *et al.*, 1979).

Experimental

Cell growth and usage

Cell growth and harvesting were as described in Hyman & Wood (1983), except for the addition of $1\,\mu M$-$CuSO_4$ to the growth medium. The washed cells were resuspended in 50mM-sodium phosphate buffer, pH 7.7, containing 2mM-$MgCl_2$, at about 0.2g wet wt./ml, stored at 0°C and used within 24h. The same medium was used in the experiments, unless otherwise specified. Protein was assayed by

* To whom all correspondence should be addressed.

the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Chemicals

Acetylene (commercial grade; British Oxygen Co., Bristol, U.K.) was passed through a wash bottle containing 10% (w/v) $Cu_2SO_4,5H_2O$ solution. A saturated solution was prepared by bubbling the scrubbed gas through a fine syringe needle into a test tube containing buffer at 0°C, the solubility at 1 atm pressure being 77 mM (Washburn, 1928). A more-dilute stock solution was prepared by adding a measured volume of this solution to a 10 ml flask filled with medium at 0°C and sealed with a Subaseal stopper (Gallenkamp, London, U.K.). Such solutions were stored at 0°C and used within 1 h of preparation.

Other chemicals were research-grade products of BDH Chemicals (Poole, Dorset, U.K.), unless otherwise stated.

Oxygen measurement

O_2 measurements were made with an oxygen electrode at 30°C as described in Hyman & Wood (1983). The O_2 solubility in air-saturated medium was taken to be 230 μM (Truesdale & Downing, 1954).

Labelling with [^{14}C]acetylene

[U-^{14}C]Acetylene (99% pure, 115 Ci/mol) was purchased from Amersham International (Amersham, Bucks., U.K.) and stored at −20°C. The gas was supplied in sealed ampoules, each containing 1 mCi. Each ampoule terminated in a conical section designed for connection to a vacuum line. This was fitted into a ground-glass socket at the mouth of a 100 ml Quickfit conical flask. The flask contained 100 ml of medium, and also a clean steel rod. NH_4Cl and cells were added to respective concentrations of 1 mM and 0.74 mg of protein/ml. For a control experiment, thiourea was added to a final concentration of 20 μM. The system was equilibrated on a shaking water bath at 30°C. The acetylene was then released by inverting the flask and ampoule, allowing the rod to break the glass seal of the ampoule. The flask and ampoule were then shaken thoroughly, to ensure equilibration of the acetylene with the aqueous phase. The system was left for a 15 min incubation. The cells were then sedimented by centrifugation (25000*g* for 10 min) and resuspended in 10 ml of medium, plus 20 μM-thiourea. After four washes in this medium the cells were resuspended in 10 ml of 50 mM-sodium phosphate buffer, pH 7.0, and broken by three cycles of freezing and thawing as described by Miller & Wood (1982). The resulting homogenate was separated into crude soluble and membrane fractions by centrifugation (25000*g* for 30 min). The crude membrane pellet was then resuspended in 10 ml of 50 mM-sodium phosphate buffer, pH 7.0, with the aid of an ultrasonic cleaning bath (Townson and Mercer, Runcorn, Cheshire, U.K.) and gentle homogenization. After centrifugation (25000*g* for 30 min), this process was repeated three times with fresh buffer. All the resulting supernatants were pooled, and the total represented the soluble protein fraction. The final pellet was resuspended in 10 ml of 50 mM-sodium phosphate buffer, pH 7.0, and represented the membrane fraction.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Samples of soluble and membrane fractions were dispensed into Eppendorf-type tubes and solubilized in a buffer containing 1% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol and 62.5 mM-Tris/HCl, pH 6.8. The tubes were placed in water at 95°C for 2 min. Non-solubilized material was sedimented by centrifugation in a mini-centrifuge (12000*g* for 10 min). Electrophoresis was conducted at 4°C in 15%-acrylamide slab gels in accordance with the method of Laemmli (1970). M_r markers were as supplied by Sigma Chemical Co. (Poole, Dorset, U.K.) and consisted of bovine serum albumin (M_r 66000), egg albumin (M_r 45000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), carbonic anhydrase (M_r 29000), trypsin inhibitor (M_r 20100) and α-lactalbumin (M_r 14200). The gels were fixed in methanol/acetic acid/water (45:9:46, by vol.) and stained with Coomassie Brilliant Blue. For the detection of radiolabelled polypeptides the gels were treated for fluorography as described by Bonner & Laskey (1974). Fluorographs were produced with the use of Kodak Kodirex Kt X-ray film and a 4-day exposure time.

Results

Progressive nature of the inhibition and production by ammonia

In the experiments of Hynes & Knowles (1978) inhibition by acetylene took 10–15 min to reach its full extent. They added acetylene to the gaseous phase above a suspension of cells, and commented that the time lag could simply reflect the rate of equilibration of acetylene with the aqueous phase. This possibility was avoided in the present experiments by adding acetylene in aqueous solution to cells in an oxygen-electrode chamber. At pH 7.7 the NH_4^+ concentration giving half the maximal rate of oxidation (i.e. the K_m value) is about 1 mM (Suzuki *et al.*, 1974). Consequently, rates of O_2 uptake with 50 mM-, 30 mM- and 10 mM-NH_4^+ were essentially the same. The traces in

Figs. 1(*b*), 1(*c*) and 1(*d*) show profiles for O_2 uptake in the presence of these concentrations of NH_4^+, plus 25 μM-acetylene. The profiles demonstrate the progressive nature of the inhibition.

It can also be seen that the rate of inhibition fell as the NH_4^+ concentration was increased. This protective effect of high NH_4^+ concentrations implies a competition between NH_4^+ and acetylene for the active site of the mono-oxygenase. Contrary to this, Hynes & Knowles (1982) reported acetylene to act as a non-competitive inhibitor. They studied the effects of low concentrations of acetylene (0.04, 0.2, 0.38 and 1.9 μM) on the amount of ammonia consumed during a much longer incubation, 2 h in length. It is likely that the bulk of the acetylene was consumed at a relatively early stage, in which case the concentration of active enzyme present for most of the time course would be independent of the ammonia concentration.

Inhibition follows first-order kinetics

The simplest kinetic model for an irreversible inhibitor that competes with a substrate for an active site on an enzyme is as follows (Dixon & Webb, 1979):

$$E + I \underset{}{\overset{K_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E\text{-}I$$

It is assumed that the formation of an enzyme–inhibitor complex (EI) can occur reversibly, but that the complex can also give rise to an irreversible complex (E–I), at a rate given by k_2. An important test of suicidal inactivation is that the rate of inactivation should follow first-order kinetics. In the case of ammonia mono-oxygenase, the loss of activity that occurs in the presence of acetylene can be followed easily by monitoring the rate of O_2 uptake in an oxygen-electrode incubation. A series of experiments was conducted where whole cells were incubated in an oxygen electrode with 2 mM-NH_4^+ and various concentrations of acetylene (0–5 μM). Fig. 2 shows logarithmic plots of the rate of O_2 uptake as a function of time. The straight lines produced imply a first-order rate of inactivation of the mono-oxygenase.

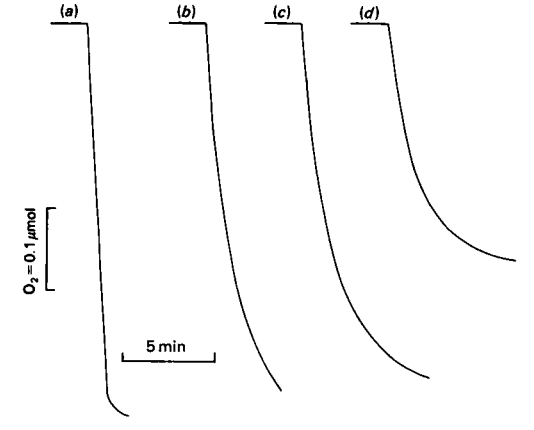

Fig. 1. *Progressive inhibition of ammonia mono-oxygenase by acetylene*

The Figure shows traces from four experiments conducted in an oxygen electrode. A 2 ml portion of medium was placed in the electrode chamber at 30°C. NH_4Cl was added to an initial concentration of (*a*) 50 mM, (*b*) 50 mM, (*c*) 30 mM and (*d*) 10 mM. Traces (*b*), (*c*) and (*d*) were conducted in the presence of 25 μM-acetylene. In each case O_2 uptake was initiated by the addition of whole cells to a final concentration of 0.18 mg of protein/ml.

Inactivation rate at saturating acetylene concentrations

The slope of each line in Fig. 2 can be quantified as a pseudo-first-order rate constant for loss of activity, $k_{inact.}$:

$$k_{inact.} \cdot t = -\ln(\text{fractional activity remaining at time } t)$$

If the dissociation constant of the enzyme–ammonia complex is denoted by K_s, the following relationship links $k_{inact.}$ to the rate of inactivation at saturating concentrations of acetylene, k_2 (Dixon & Webb, 1979):

$$\frac{1}{k_{inact.}} = \frac{1}{k_2} + \frac{K_i}{k_2} \cdot \left(1 + \frac{[NH_4^+]}{K_s}\right) \cdot \frac{1}{[C_2H_2]}$$

Fig. 3 shows a plot of $1/k_{inact.}$ versus $1/[C_2H_2]$, which gives a good straight line. The intercept at $1/[C_2H_2] = 0$ corresponds to $k_2 = 1.54\,min^{-1}$.

The rate of inactivation in the absence of ammonia was also investigated. Cells were incubated with 5 μM-acetylene, either with no added reductant or with hydroxylamine added to 30 μM. After a set length of time, 10 mM-NH_4^+ was added and the resultant O_2 uptake rate was determined. With such a high NH_4^+ concentration further inactivation could be ignored. The kinetics of inactivation corresponded to $k_{inact.} = 0.9\,min^{-1}$, both in the absence and in the presence of added reductant. This is somewhat lower than the value of k_2 derived above, implying that the acetylene concentration was not saturating. Our earlier experiments have shown that organic substrates for mono-oxygenase are hydroxylated much faster if reductant is added (Hyman & Wood, 1984). Since reductant had no effect on the rate of inactivation, one must conclude that the rate of supply of reducing power is not a limiting factor. This implies a low $V_{max.}$ or inactivation at the first turnover.

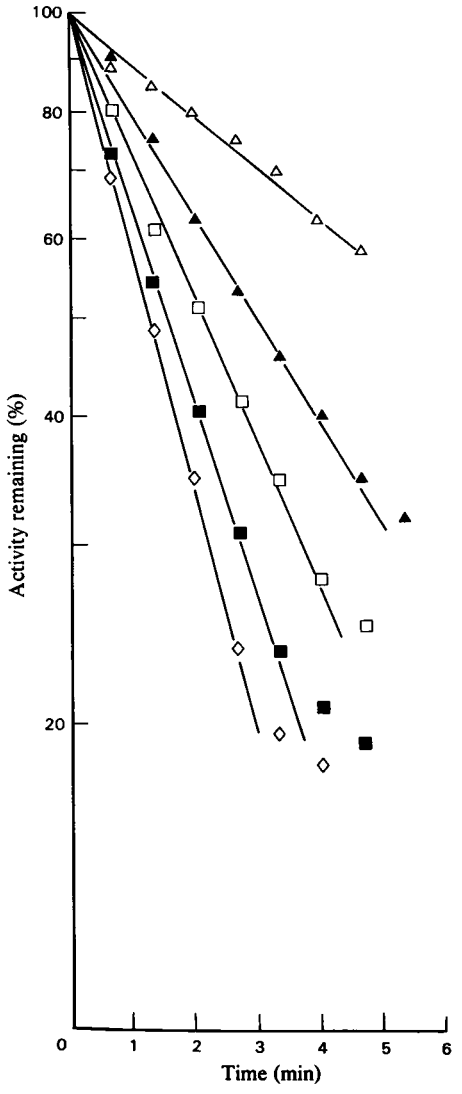

Fig. 2. *First-order inactivation of ammonia oxidation by ammonia mono-oxygenase*

A 2ml portion of medium was placed in an oxygen-electrode chamber at 30°C. The medium was supplemented with 2mM-NH_4^+ and acetylene at the following concentrations 1μM (△), 2μM (▲), 3μM (□), 4μM (■) and 5μM (◇). The experiments were initiated by the addition of cells to a final concentration of 0.14mg of protein/ml. The ordinate is a logarithmic scale of percentage activity remaining, relative to the rate without acetylene. The points were obtained by drawing tangents to traces similar similar to those in Fig. 1.

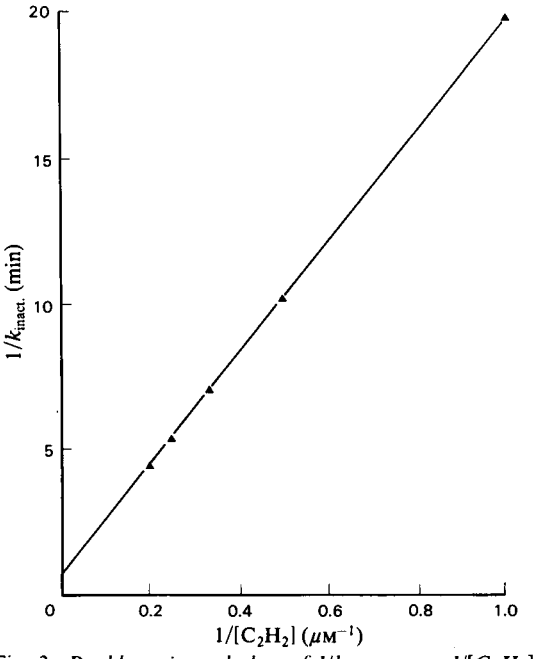

Fig. 3. *Double-reciprocal plots of $1/k_{inact.}$ versus $1/[C_2H_2]$ for ammonia mono-oxygenase*

The Figure shows data from the experiment in Fig. 2 replotted in the form of $1/k_{inact.}$ versus $1/[C_2H_2]$. The values of $k_{inact.}$ are derived from the slopes in Fig. 2, as described in the text.

Requirement of O_2 for inactivation

Our previous investigations with organic substrates such as ethylene have shown that substrate oxidation does not occur under anaerobic conditions (Hyman & Wood, 1984). If acetylene acts as a suicide substrate for ammonia mono-oxygenase, it would therefore be expected that anaerobic conditions would prevent inactivation of the enzyme. This possibility has been investigated in an experiment utilizing two oxygen electrodes. A suspension of cells (6.7mg of protein/ml) was placed in an oxygen-electrode chamber and allowed to become anaerobic. Samples were withdrawn and added to aerated medium plus a high concentration of NH_4^+, in a second oxygen-electrode chamber. It was found that a decline in activity of about 10% occurred as the incubation medium became anaerobic, but thereafter activity was constant. After 120min the lid was removed from the incubation chamber, and sampling was continued after the cells had become aerobic. In a second experiment 90μM-acetylene was added during the anaerobic incubation. After an immediate small loss of activity (perhaps associated with traces of O_2 in the acetylene solution), the activity remained steady until O_2 was re-introduced. On re-aeration the cells rapidly became completely inactivated. Thus inhibition by acetylene requires the presence of O_2.

Protection by a reversible inhibitor

Thiourea is a potent and specific non-competitive inhibitor of ammonia oxidation in *N. europaea*

(Lees, 1952). Thiourea inhibition is also largely reversible by simple washing of exposed cells (Hooper & Terry, 1973). In contrast, the inhibition brought about by acetylene is irreversible (Hynes & Knowles, 1982). Table 1 shows results from an investigation into the effect of thiourea on inactivation by acetylene. Flask A served as a control. Flask B was treated with thiourea (20 μM). After multiple washing, over 50% of its original activity was recovered. Flask C was treated with acetylene. After washing only 3.3% of the original O_2-uptake activity remained. Finally, flask D was treated with acetylene after thiourea had been present for several minutes. When washed as for the other flasks, the activity was almost as high as for thiourea treatment alone. It should be noted that thiourea was included in the first washing step, to ensure that the enzyme remained inactive until all acetylene had been removed. Omission of this precaution led to a much lower final activity. The results demonstrate convincingly that thiourea protects against inactivation by acetylene.

Labelling of a specific polypeptide

Cells were incubated with [^{14}C]acetylene, either in the presence or in the absence of thiourea. They were washed thoroughly, broken by freezing and thawing and separated into soluble and membrane fractions. These fractions were treated with sodium dodecyl sulphate, and loaded on to separate tracks of a sodium dodecyl sulphate/polyacrylamide gel. After electrophoresis the gel was stained for protein and photographed (see Fig. 4). The gel was then treated for fluorography. As shown in Fig. 4, a single membrane protein was strongly radioactive, and labelling was greatly diminished by the presence of thiourea. The labelled polypeptide ran with an apparent M_r of 28000. No labelling could be detected in the supernatant.

Phenylacetylene

Ammonia mono-oxygenase is known to be capable of hydroxylating simple aromatic compounds such as benzene (Drozd, 1980). The behaviour of phenylacetylene was therefore tested. It caused progressive inactivation of ammonia oxidation, in a manner similar to that of acetylene (see Fig. 5). A parallel experiment with an NH_4^+-ion-selective electrode confirmed that NH_4^+ oxidation was being inhibited. The inhibition could not be reversed by washing.

Discussion

The designation of an enzyme inactivation as suicidal can only be made if specific criteria are satisfied (Abeles & Maycock, 1976). One is that the inactivation should be irreversible, as was established for the present system by Hynes & Knowles (1982). Another is that activity should decline exponentially with time, as shown in Fig. 2. This implies that the rate of inactivation is proportional to the concentration of active enzyme, as expected if the inactivation process occurs before the release of an activated product into the surrounding medium. Thirdly, the inhibition should be competitive with respect to the normal substrate, with high substrate concentrations providing protection. This was demonstrated in Fig. 1. Fourthly, the enzyme must be catalytically competent for inactivation to occur. With ammonia mono-oxygenase studied in whole cells, it is not possible to cut off the supply of reducing power effectively. However, inactivation has been shown to require the presence of O_2 and is prevented by a reversible non-competitive inhibitor (Table 1). These observations are consistent with attempted oxidation of acetylene before inactivation of the enzyme. We

Table 1. *Protection of ammonia mono-oxygenase by a reversible inhibitor*

A 10ml portion of medium was placed in each of four 10ml flasks (A to D), plus cells at 1.71mg of protein/ml and 1mM-NH_4^+. The flasks were closed with Subaseal stoppers and placed in a shaking water bath at 30°C. Samples (50 μl) were withdrawn from each flask and assayed for rate of O_2 uptake in the presence of 10mM-NH_4^+ as described for Fig. 5. After 10min, thiourea (20 μM) was added to flasks B and D, and acetylene (150 μM) was then added to flasks C and D. After incubation for 30min, the cells were sedimented by centrifugation (25000*g* for 10min). For flask A resuspension was in 10ml of medium, and for flasks B–D in 10ml of medium plus 20 μM-thiourea. The cells were then washed four times with medium plus 0.3mM-NH_4^+, and were finally resuspended in 10ml of medium alone. Samples (50 μl) were withdrawn and assayed as before.

Flask ...	A	B	C	D
Initial activity (μmol of O_2/h per mg of protein)	55.2	52.8	54.6	55.2
Additions ...	None	Thiourea	Acetylene	Thiourea, then acetylene
Final activity (μmol of O_2/h per mg of protein)	59.4	33.0	1.8	25.8

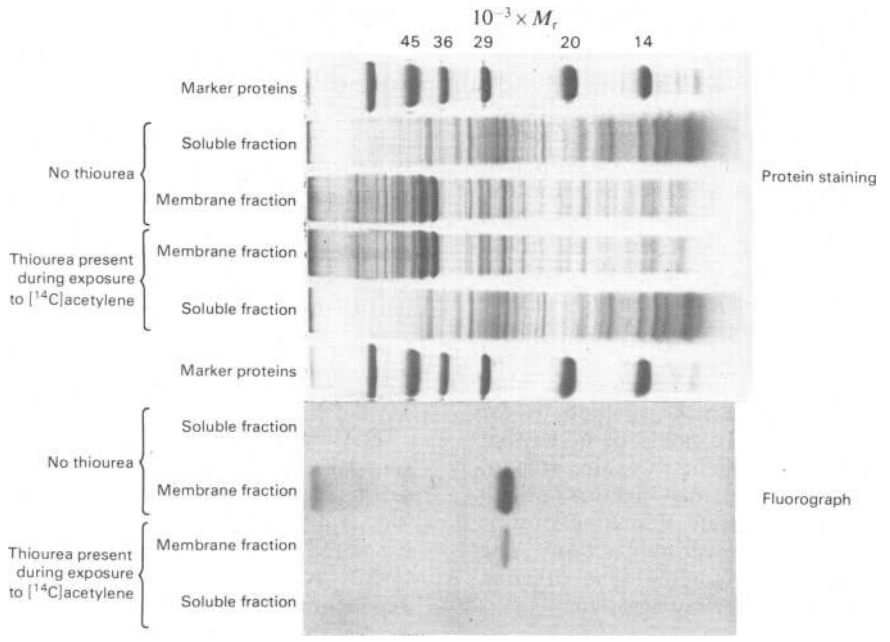

Fig. 4. *Labelling of a single N. europaea membrane polypeptide by exposure to* [^{14}*C*]*acetylene*
The upper part of the Figure shows a protein staining pattern obtained by dodecyl sulphate/polyacrylamide-gel electrophoresis. The first and sixth tracks were loaded with marker proteins. The second and third tracks were loaded respectively with the soluble and membrane fraction of cells incubated with [^{14}C]acetylene in the absence of thiourea. The fourth track was loaded with the membrane fraction and the fifth with the soluble fraction of cells exposed to [^{14}C]acetylene in the presence of thiourea. Each of these tracks was loaded with 50μg of protein. The lower part of the Figure shows a fluorograph of the four tracks loaded with material from *N. europaea*. For further information see the Experimental section.

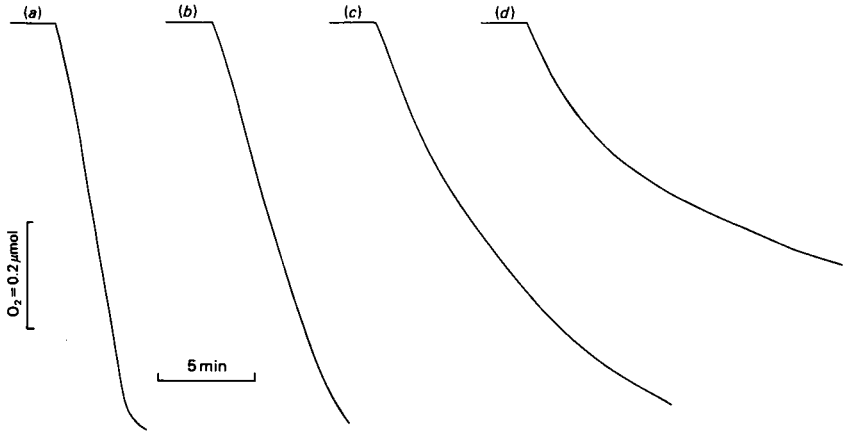

Fig. 5. *Progressive inhibition of ammonia mono-oxygenase by phenylacetylene*
The Figure shows results of oxygen-electrode experiments, with a medium consisting of 3ml of 15mM-Hepes/NaOH buffer, pH 7.7, containing 2mM-$MgCl_2$. After addition of NH_4^+ (1mM), O_2 uptake was initiated by adding cells to 1.32mg wet wt./ml. Phenylacetylene was present at the following concentrations: (*a*) none; (*b*) 3.3μM; (*c*) 6.6μM; (*d*) 9.9μM. It was added from a stock solution in dimethyl sulphoxide.

have also demonstrated that inactivation is associated with covalent attachment of carbon from acetylene to a specific polypeptide. Thiourea protected against labelling by acetylene, just as it protected against permanent inactivation.

Since oxidation of ethylene by ammonia mono-

oxygenase results in the formation of ethylene oxide (Hyman & Wood, 1984), it might be expected that acetylene oxidation would yield an unsaturated epoxide. As discussed by Ortiz de Montellano & Kunze (1980) in connection with cytochrome *P*-450 inactivation by acetylenes, such compounds (oxirenes) are much less stable than epoxides, because of strain and electronic effects, and indeed are only known by indirect evidence. Ammonia mono-oxygenase is inhibited by chelating agents for cuprous copper (e.g. thioureas) and has other properties strongly suggestive of a copper enzyme, as discussed by Shears & Wood (1985). The unsaturated epoxide, or its immediate decomposition product, may attack one or more of the amino acid side chains that bind the (putative) copper. This may be compared with the inactivation of the copper enzyme dopamine β-mono-oxygenase by derivatives of allylbenzene (Rajashekhar *et al.*, 1984).

The purification of ammonia mono-oxygenase has not yet been carried beyond the stage of a crude cell-free extract (Suzuki *et al.*, 1981). Thus the present experiments provide the first identification of a constitutent polypeptide, of apparent M_r 28000. In substrate specificity and inhibitor sensitivity the mono-oxygenase is very similar to the membrane form of methane mono-oxygenase, as discussed in Hyman & Wood (1983). Stanley *et al.* (1983) have shown for *Methylococcus capsulatus* (Bath) that synthesis of the membrane enzyme requires addition of copper to the growth medium, and coincides with the appearance of membrane proteins with apparent M_r values of 23000, 25000 and 45000 (Dalton *et al.*, 1984). It is not yet clear which of these polypeptides contains the active site. Since acetylene inhibits methane mono-oxygenase, in both membrane and soluble forms (Stirling & Dalton, 1979), labelling experiments should give interesting results.

The discovery of a suicide substrate for ammonia mono-oxygenase is also significant for a very different reason, which arises from the agricultural implications of nitrification (Stewart, 1983). When an ammonium salt is applied as a fertilizer, NH_4^+ cations are normally bound firmly to colloidal particles in the soil. Nitrifying bacteria convert these cations into anions, with nitrate as the final product. Being anionic, nitrate is far more mobile, and is consequently liable to loss by leaching. If O_2 becomes depleted in the local environment, nitrate N can also be lost by denitrification. Thus suicide substrates of ammonia mono-oxygenase with suitable properties in the field may find application as fertilizer additives. At least one acetylenic, acetylenedicarboxamide, has already been patented for this purpose (Chiba *et al.*, 1973).

We are grateful to the Science and Engineering Research Council for a research grant and a research studentship to M. R. H.

References

Abeles, R. H. & Maycock, A. L. (1976) *Acc. Chem. Res.* **9**, 313–319

Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88

Chiba, A., Toyoda, H. & Takahashi, M. (1973) *Jpn. Kokai Tokkyo Koho* **73**, 103351; *cited in Chem. Abstr.* **80**, 144800k

Dalton, H., Prior, S. D., Leak, D. J. & Stanley, S. H. (1984) *Microb. Growth C_1 Compounds, Proc. Int. Symp. 4th* 75–82

Dixon, M. & Webb, E. C. (1979) *The Enzymes*, 3rd edn., pp. 374–375, Longman, London

Drozd, J. W. (1980) in *Diversity of Bacterial Respiratory Systems*, vol. 2 (Knowles, C. J., ed.), pp. 87–111, CRC Press, Boca Raton

Dua, R. D., Bhandari, B. & Nicholas, D. J. D. (1979) *FEBS Lett.* **106**, 401–404

Hooper, A. B. & Terry, K R. (1973) *J. Bacteriol.* **115**, 480–485

Hyman, M. R. & Wood, P. M. (1983) *Biochem. J.* **212**, 31–37

Hyman, M. R. & Wood, P. M. (1984) *Arch. Microbiol.* **137**, 155–158

Hynes, R. K. & Knowles, R. (1978) *FEMS Microbiol. Lett.* **4**, 319–321

Hynes, R. K. & Knowles, R. (1982) *Can. J. Microbiol.* **28**, 334–340

Kunze, K. L., Mangold, B. L. K., Wheller, C., Beilon, H. S. & Ortiz de Montellano, P. R. (1983) *J. Biol. Chem.* **258**, 4202–4207

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Lees, H. (1952) *Biochem. J.* **52**, 134–139

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Miller, D. J. & Wood, P. M. (1982) *Biochem. J.* **207**, 511–517

Ortiz de Montellano, P. R. & Correia, M. A. (1983) *Rev. Pharmacol. Toxicol.* **23**, 481–503

Ortiz de Montellano, P. R. & Kunze, K. L. (1980) *J. Biol. Chem.* **255**, 5578–5585

Rajashekhar, B., Fitzpatrick, P. F., Colombo, G. & Villafranca, J. J. (1984) *J. Biol. Chem.* **259**, 6925–6930

Shears, J. H. & Wood, P. M. (1985) *Biochem. J.* **226**, 499–507

Stanley, S. H., Prior, S. D., Leak, D. J. & Dalton, H. (1983) *Biotechnol. Lett.* **5**, 487–492

Stewart, W. D. P. (chairman) (1983) *The Nitrogen Cycle of the United Kingdom: A Study Group Report*, The Royal Society, London

Stirling, D. I. & Dalton, H. (1979) *Eur. J. Biochem.* **96**, 205–212

Suzuki, I., Kwok, S. C. & Dular, U. (1974) *J. Bacteriol.* **115**, 556–558

Suzuki, I., Kwok, S. C., Dular, U. & Tsang, D. C. Y. (1981) *Can. J. Biochem.* **59**, 477–483

Truesdale, G. A. & Downing, A. L. (1954) *Nature (London)* **173**, 1236

Washburn, E. W. (ed.) (1928) *International Critical Tables*, vol. 3, p. 260, McGraw-Hill, New York