Mechanism of ammonia oxidation in Nitrosomonas europaea: role of cytochrome *c*-554

by

Dominic C.Y. Tsang

A thesis

submitted to

The Faculty of Graduate Studies and Research

The University of Manitoba

In partial Fulfillment

of the Requirement for the Degree

Master of Science

1982

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To my family

ACKNOWLEDGEMENTS

The author expresses his deepest gratitude to Dr. Isamu Suzuki for his guidance, advice and enlightenment throughout the course of this investigation and in the preparation of the manuscript.

Sincere thanks are offered to Mr. S.C. Kwok for his invaluable technical assistance and to Ms. Sharon McKinley for the typing of this thesis.

ABSTRACT

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Mechanism of ammonia oxidation was studied in the reconstituted system of *Nitrosomonas* membrane fraction plus the *Nitrosomonas* cytochrome *c*-554. The cytochrome *c*-554 was reduced by hydroxylamine, hydrazine and ammonia and the reduced cytochrome was oxidized upon the addition of ammonia or carbon monoxide. The oxidation of carbon monoxide in the presence of hydroxylamine or hydrazine was studied as a possible assay method for ammonia hydroxylase where hydroxylamine or hydrazine was supplying the reducing power required for the hydroxylation of carbon monoxide. The stoichiometry of the reaction, Km values for substrates and effects of pH and inhibitors were investigated. It is concluded that carbon monoxide, a competitive inhibitor for ammonia oxidation, is an alternate substrate for ammonia hydroxylase using the reduced cytochrome *c*-554 as the reducing power.

The possible involvement of peroxonitrite as an intermediate in ammonia oxidation was studied.

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LIST OF ABBREVIATIONS

DNase - deoxyribonuclease

EDTA - ethylene diamine tetraacetic acid

tris - tris(hdyroxymethyl)aminomethane

BSA - bovine serum albumin

DEAE - diethylaminoethyl

SDA - sodium dodecyl sulfate

INTRODUCTION

INTRODUCTION

The mechanism of ammonia oxidation has long been a subject of intensive research. The study on the mechanism is highlighted by the preparation of active cell-free extracts capable of ammonia oxidation (Suzuki *et al*, 1970). Later studies by these workers established the conditions necessary for the preparation of active cell-free extracts and showed that reduction of cytochromes present in the extract by ammonia was a necessary precondition before the initiation of oxygen consumption (Suzuki *et al*, 1981). They also reconstituted the ammonia oxidizing system using *Nitrosomonas* cytochrome *c*-554 and a *Nitrosomonas* membrane fraction.

The hydroxylation of ammonia to hydroxylamine by *Nitrosomonas* cells was conclusively demonstrated by Dua *et al* (1979) and Hollocher *et al* (1981) using $^{15}NH_4Cl$ and $^{18}O_2$ or $H_2{}^{18}O$.

Hughes (1970a) proposed a scheme for the oxidation of hydroxylamine to nitrite, suggesting the involvement of peroxonitrite ($ONOO^-$) as an intermediate in the process. Suzuki *et al* (1981) extended the idea to suggest that peroxonitrite was used as an agent for the hydroxylation of ammonia in the steady state of ammonia oxidation by *Nitrosomonas europaea*.

It is the purpose of this thesis to study the role of cytochrome *c*-554 in the oxidation of ammonia by the reconstituted *Nitrosomonas* membrane fraction plus *Nitrosomonas* cytochrome *c*-554 system. The possible involvement of peroxonitrite in the oxidation of ammonia is also studied.

HISTORICAL

HISTORICAL

The process of nitrification, the oxidation of ammonia to nitrate, is an integral process in the nitrogen cycle. The reaction was once considered purely chemical in nature. Its biological involvement was suggested by Pasteur (1862) and established by Winogradsky (1891) when he isolated Nitrosomonas and Nitrobacter in pure cultures from soil using an inorganic medium. Nitrosomonas oxidises ammonia to nitrite, while Nitrobacter oxidises nitrite to nitrate, thus constituting the two-stepped process of nitrification.

Nitrosomonas, as described by Winogradsky, was small, gram negative, oval in shape and motile with a single polar flagellum. The organism is chemoautotrophic as it derives all its energy for growth and cell synthesis from the oxidation of ammonia to nitrite and obtains its carbon source from carbon dioxide.

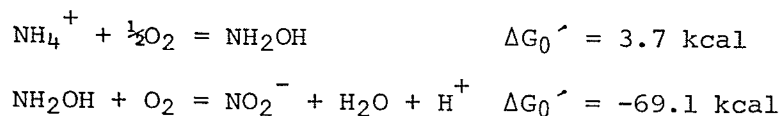
Despite its importance in the process of nitrification, little work was done successfully on Nitrosomonas in the years after its isolation, due partly to its long mean generation time and partly to its low molar growth yield. Major biochemical discoveries were made in the last thirty years.

The overall equation for ammonia oxidation is: $NH_3 + 1\frac{1}{2}O_2 \rightarrow NO_2^-$ $H_2O + H^+$.

In 1953, Hofman and Lees, using hydrazine as a selective inhibitor, observed the accumulation of hydroxylamine during ammonia oxidation by intact cells of Nitrosomonas europaea. Hydroxylamine was oxidised stoichiometrically to nitrite by washed cell suspensions of N. europaea (Lees, 1952). The oxidation of ammonia to hydroxylamine was

inhibited by allylthiourea (Hofman and Lees, 1953) and thiourea (Anderson, 1964). These authors suggested that the enzyme contained copper.

Lees (1954) proposed that the metabolism of ammonia to nitrite proceeded via hydroxylamine and an unknown intermediate in a series of three two-electron steps in which the energy and reducing power released could be efficiently utilised by the cell. Since hyponitrite, $H_2N_2O_2$, was not metabolised to nitrite, he suggested that the hypothetical intermediate was nitroxyl (NOH). Since the free energy released by the oxidation of ammonia to hydroxylamine was small, the metabolism of hydroxylamine to nitrite provided the main free-energy requirement of the cell. The free energy changes at pH 7 ($\Delta G_0'$) for the oxidation of ammonia and hydroxylamine (Anderson 1964) are as follows:



$$NH_4^+ + \tfrac{1}{2}O_2 = NH_2OH \qquad \Delta G_0' = 3.7 \text{ kcal}$$

$$NH_2OH + O_2 = NO_2^- + H_2O + H^+ \qquad \Delta G_0' = -69.1 \text{ kcal}$$

Lees (1954) noted the small free energy released by the oxidation of ammonia to hydroxylamine and suggested that since the reaction involved copper and molecular oxygen, the enzyme might be a type of oxidase.

The oxidation of hydroxylamine to nitrite by cell-free extract occurred in the presence of electron acceptors and air. Molecular oxygen was essential for nitrite formation. Anaerobically, hydroxylamine disappeared and negligible amounts of nitrite were formed (Falcone et al, 1963; Anderson, 1964; Hooper and Nason, 1965; Aleem and Lees, 1963). Nitric oxide and nitrous oxide were produced in amounts equivalent to the hydroxylamine added (Anderson 1964). These results suggested that the metabolism of hydroxylamine was a

two-stepped process. In stage one hydroxylamine was dehydrogenated to form nitroxyl which decomposed to nitrous oxide. The latter could be further dehydrogenated to nitric oxide. Stage two was the conversion of nitric oxide into nitrite by an enzyme system requiring oxygen (Anderson 1964). Such an idea was supported by Hooper and Terry (1979) using purified hydroxylamine oxidoreductase.

Extensive efforts were made to purify the enzyme system for hydroxylamine oxidation. The enzyme hydroxylamine oxidoreductase, or hydroxylamine-cytochrome c reductase, or hydroxylamine oxidase, which catalyzed the oxidation of hydroxylamine has been extensively purified and studied (Falcone et al, 1963; Aleem and Lees, 1963; Hooper and Nason, 1965; Rees, 1968; Ritchie and Nicholas, 1974; Hooper et al, 1978; Yamanaka et al, 1979; Erickson and Hooper, 1972). Rees (1968) and recently Yamanaka et al (1979) purified the enzyme to an electrophoretically homogeneous preparation with a molecular weight of 200,000 and 175,000 respectively. As determined by isoelectric focusing, the enzyme had an isoelectric pH value of 5.3. Preliminary experiments indicated that the protein consisted of subunits of smaller molecular weight although the protein was unusually resistant to dissociation by sodium dodecyl sulfate and mercaptoethanol (Hooper et al 1978). Although inhibitor studies suggested the involvement of flavin, it could not be detected. The enzyme had a high content of cytochrome. Approximately 30% of the dithionite-reducible cytochrome absorbancy in the wavelength range between 540 and 570 nm was reducible by either hydrazine or hydroxylamine. Physicochemical studies of the enzyme showed that the enzyme did not possess protoheme or a cytochrome b subunit although the α peak of the enzyme at 553 nm had a

shoulder at 559 nm. The enzyme was considered a c-type cytochrome with absorption peaks at 418 (γ peak), 521 (β peak), 553 (α peak) and 460 nm (Hooper et al, 1978; Yamanaka et al, 1979).

The absorbancy at 460 nm was due to the cytochrome p460 which was unique to the ammonia oxidizing nitrifying bacteria. From a comparison of recovery of enzyme activity, c-type cytochrome, and p460 in the homogeneous fraction of the enzyme, it is noted that 40% of the total cellular c-type cytochrome and essentially 100% of the total p460 was associated with hydroxylamine oxidoreductase (Hooper et al, 1978). The absorption maximum at 460 nm of hydroxylamine oxidoreductase did not appear in the presence of carbon monoxide, nitrite, hydroxylamine or hydrazine (Hooper, 1978; Nicholas, 1974; Yamanaka, 1979). The addition of either of these compounds, to the oxidised enzyme precluded the appearance of the reduced 460 nm absorbing band on subsequent addition of dithionite (Ritchie and Nicholas, 1974; Hooper, 1978; Yamanaka, 1979). Carbon monoxide at 5% which resulted in a complete shift of the 460 nm peak of the enzyme, did not inhibit the oxidation of hydroxylamine to nitrite (Hooper and Terry, 1973). It was thus considered unlikely that cytochrome p460 had a direct role in the oxidation of hydroxylamine to nitrite. That it has no direct role is also argued on the basis that no natural reductant has yet been found for it. Hooper and Terry (1977), however, found that treatment of the enzyme with H_2O_2 caused a simultaneous loss of absorbancy due to p460, substrate-reducibility of cytochromes, and hydroxylamine oxidoreductase activity, thus suggesting a role of p460 in the action of the enzyme.

The enzyme hydroxylamine oxidoreductase showed NH_2OH-cytochrome c

reductase and NH_2NH_2-cytochrome c reductase activities (Hooper and Nason 1965). The Km values for hydroxylamine and hydrazine as estimated were 3.6 μM and 4 μM. The enzyme showed specificity for the kind of cytochrome used as the electron acceptor: in general it reacted rapidly with eucaryotic cytochrome c while it reacted slowly or did not react at all with many bacterial cytochrome c (Yamanaka et al 1979). In the presence of either cytochrome c or phenazine methosulfate as electron acceptors, the enzyme catalyzed the removal of at least two electrons from hydroxylamine, on the basis of the number of moles of cytochrome reduced per mole of hydroxylamine oxidised (Falcone et al, 1963; Hooper and Nason, 1965). The direct product of hydroxylamine oxidation was not known but was presumably a substance with nitrogen in an oxidation state between that of hydroxylamine (-1) and nitrite (+3). The production of nitrous oxide under anaerobic conditions suggested nitroxyl (NOH) as the intermediate. Unlike whole cells which oxidised hydroxylamine to nitrite stoichiometrically, the nitrite yield by the highly purified enzyme system was less than 50% even aerobically. Other products formed included nitrate (HNO_3), nitric oxide (NO) and nitrous oxide (N_2O) (Hooper and Terry, 1979).

The compounds nitric oxide and nitrous oxide were also produced using the partially purified enzyme complex of nitrite reductase (Hooper, 1968) which reduced nitrite using hydroxylamine as electron donor aerobically or chemically reduced pyrocyanine as electron donor anaerobically.

The enzyme peroxidase was purified from Nitrosomonas (Anderson, et al, 1968), but no evidence was obtained to support its possible

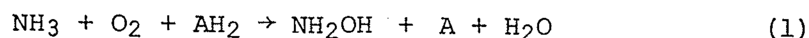
involvement in ammonia oxidation.

The early years of the nineteen seventies marked the purification of a number of cytochromes from N. europaea. Such cytochromes include cytochrome c-552, cytochrome c-554 (Yamanaka and Shinra, 1974), cytochrome c-553 (Tronson et al, 1973, cytochrome a_1 (Erickson et al, 1972), and cytochrome p-460 (Erickson and Hooper, 1972). Cytochrome c-552 is reduced by hydroxylamine (NH_2OH) in the presence of catalytic quantities of hydroxylamine oxidoreductase and cytochrome c-554, and reduced cytochrome c-552 is oxidised by cytochrome a_1 in air. Thus the flow of electron from NH_2OH is: $NH_2OH \rightarrow$ hydroxylamine oxido-reductase $\rightarrow$ c-554 $\rightarrow$ c-552 $\rightarrow$ a_1 $\rightarrow$ O_2 (Yamanaka and Shinra, 1974).

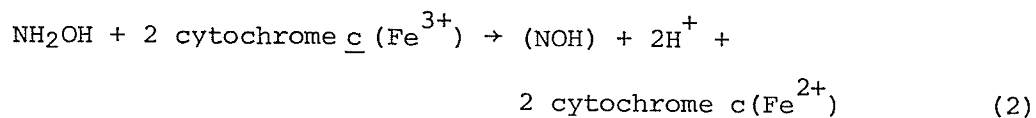
Despite all the successes in purifying and characterizing the enzymes and cytochromes in N. europaea, the challenging question of the mechanism of ammonia oxidation is still not clear. Part of the reason is the difficulty in getting active ammonia-oxidising cell-free extracts. The first active cell-free extract was obtained by Suzuki and Kwok (1970) using French pressure cell and in the presence of bovine serum albumin, Mg^{2+} or spermine. Such cell-free extracts stoichiometrically oxidised ammonia at a rate as high at 10%-20% of that of intact cells. Conditions for the preparation of such active system were elaborated further by these workers (Suzuki et al, 1981). The relative effectiveness of the activators (BSA, Mg^{2+} or spermine) on the membrane system was influenced by the concentration of phosphate. BSA was most effective in 0.1 M potassium phosphate and spermine at a lower phosphate concentration. BSA had an additional effect of stabilizing the ammonia-oxidizing activity of extracts. The presence of BSA appeared to keep the partial reduction

of cytochromes in the extracts upon addition of ammonia. Using such active systems, it was shown that the ammonia oxidising activity was optimal at pH 7.7 and 25°C. Electron microscopic studies of active extracts showed that there was an association of membranes in a structure resembling chromatophores in photosynthetic bacteria, such as association was absent in inactive extracts (Dular, 1975). Similar studies showed that inactive spheroplasts became active when incubated with Mg^{2+}, accompanied by an increase in membranous folds and invaginations (Suzuki and Kwok, 1969). Such studies indicated the importance of a structural membrane in ammonia oxidation. Ammonia oxidation by the active extract was competitively inhibited by carbon monoxide, methane, ethylene and methanol (Suzuki *et al*, 1976; Suzuki *et al*, 1981).

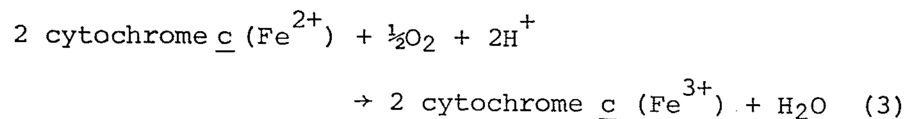
In 1974, Suzuki, on the basis of published information and unpublished work from his laboratory, proposed the following scheme of ammonia oxidation:



$$NH_3 + O_2 + AH_2 \rightarrow NH_2OH + A + H_2O \quad (1)$$

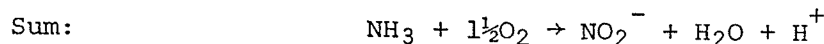


$$NH_2OH + 2 \text{ cytochrome } \underline{c}\,(Fe^{3+}) \rightarrow (NOH) + 2H^+ + 2 \text{ cytochrome } \underline{c}\,(Fe^{2+}) \quad (2)$$



$$2 \text{ cytochrome } \underline{c}\,(Fe^{2+}) + \tfrac{1}{2}O_2 + 2H^+ \rightarrow 2 \text{ cytochrome } \underline{c}\,(Fe^{3+}) + H_2O \quad (3)$$

$$NOH + A + H_2O \rightarrow NO_2^- + AH_2 + H^+ \quad (4)$$



Sum: $$NH_3 + 1\tfrac{1}{2}O_2 \rightarrow NO_2^- + H_2O + H^+$$

In his scheme, the oxidation of ammonia, as catalysed by the hypothetical ammonia hydroxylase, is tightly coupled to the oxidation of hydroxylamine. In the absence of ammonia, hydroxylamine oxidation is coupled to the electron transport chain with uptake of oxygen and

evolution of nitrous oxide (N_2O) due to decomposition of nitroxyl (NOH).

Rees and Nason (1966), on the basis of ^{18}O isotope studies, demonstrated the incorporation of atmospheric oxygen into one of the two oxygen atoms of nitrite during the oxidation of ammonia by *N. europaea*. This finding opened the possibility of the involvement of an oxygenase in ammonia oxidation. It was suggested by Suzuki and Kwok (1969) that if the oxidation of ammonia to hydroxylamine was catalysed by an oxygenase, the reaction was very likely to be a mixed-function type, requiring a reducing equivalent for the reduction of the remaining half of the oxygen molecule. This reducing equivalent could be supplied only during the oxidation of hydroxylamine to nitrite, since this organism derived all of the energy and reducing power for growth from the oxidation of ammonia to nitrite. Hooper (1969) reported the elimination of an initial lag phase of ammonia oxidation by resting cells by the addition of a small quantity of hydroxylamine. Suzuki and Kwok (1969) reported the oxidation of ammonia by spheroplasts of *N. europaea* when primed with hydroxylamine or when preincubated with Mg^{2+}.

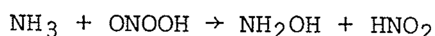
The hydroxylation of ammonia to hydroxylamine was conclusively demonstrated by analysing the oxime formed specifically from hydroxylamine using highly enriched $^{15}NH_4Cl$ and $^{18}O_2$ or $H_2{}^{18}O$ (Dua *et al*, 1979; Hollocher, 1981). In these experiments, hydrazine used as inhibitor of hydroxylamine oxidation presumably supplied electrons required for ammonia hydroxylation as a substrate for hydroxylamine oxidoreductase.

As discussed earlier, nitrite was not the only product of

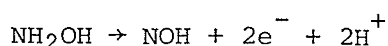
ammonia oxidation. Besides nitrous oxide and nitric oxide, Anderson (1964) first identified nitrate as an additional product of the oxidation of hydroxylamine in the presence of oxygen, methylene blue and extracts of *Nitrosomonas*. Hooper *et al* (1977) found that although nitrate was not produced in the oxidation of ammonia or hydroxylamine by intact cells of *Nitrosomonas*, a partially purified hydroxylamine oxidoreductase enzyme system catalysed the aerobic oxidation of hydroxylamine to nitrite and nitrate in roughly equimolar quantities. In the enzyme preparation the nitrate was not formed from nitrite. Diethyldithiocarbamate inhibited nitrate but not nitrite formation and was itself oxidised to bis(diethyl-dithiocarbamoryl) disulphide.

Suzuki *et al* (1981), suggested that the formation of nitrate from hydroxylamine possibly supported the model of hydroxylamine oxidation involving peroxonitrite ($ONOO^-$) advanced by Hughes and Nicklin (1970a) based on studies of hydroxylamine autooxidation in alkaline solution (Hughes and Nicklin 1970b). In their model the nitroxyl ion (NO^-) produced from hydroxylamine, forms peroxonitrite in combination with oxygen: $NO^- + O_2 \rightarrow ONOO^-$. Copper ion catalyses the reaction of peroxonitrite with hydroxylamine: $ONOO^- + NH_2OH + OH^- \rightarrow NO_2^- + NO^- + 2H_2O$. The overall reaction is the oxidation of hydroxylamine to nitrite: $NH_2OH + O_2 + OH^- \rightarrow NO_2^- + 2H_2O$. Peroxonitrite, when protonated, is unstable and isomerizes to nitrate: $ONOO^- + H^+ \rightarrow ONOOH \rightarrow HNO_3$ (Hughes and Nicklin, 1968; Benton and Moore, 1970). If peroxonitrite is indeed the intermediate of biological hydroxylamine oxidation the production of nitrate as well as nitrite is understandable. The effect of diethyldithiocarbamate may then be explained as the reduction of peroxonitrite.

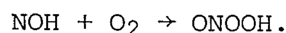
Peroxonitrite, although unstable at nonalkaline conditions, is an attractive candidate as an intermediate of ammonia oxidation because of its ability to hydroxylate benzene to phenols (Halfpenny and Robinson, 1952) and to transfer oxygen atoms to nucleophiles such as cyanide: $ONOO^- + CN^- \rightarrow NO_2^- + CN^-$ (Hughes et al, 1971). If ammonia can be hydroxylated by peroxonitrite, then the following hypothetical reactions can be visualized:



$$NH_3 + ONOOH \rightarrow NH_2OH + HNO_2$$



$$NH_2OH \rightarrow NOH + 2e^- + 2H^+$$

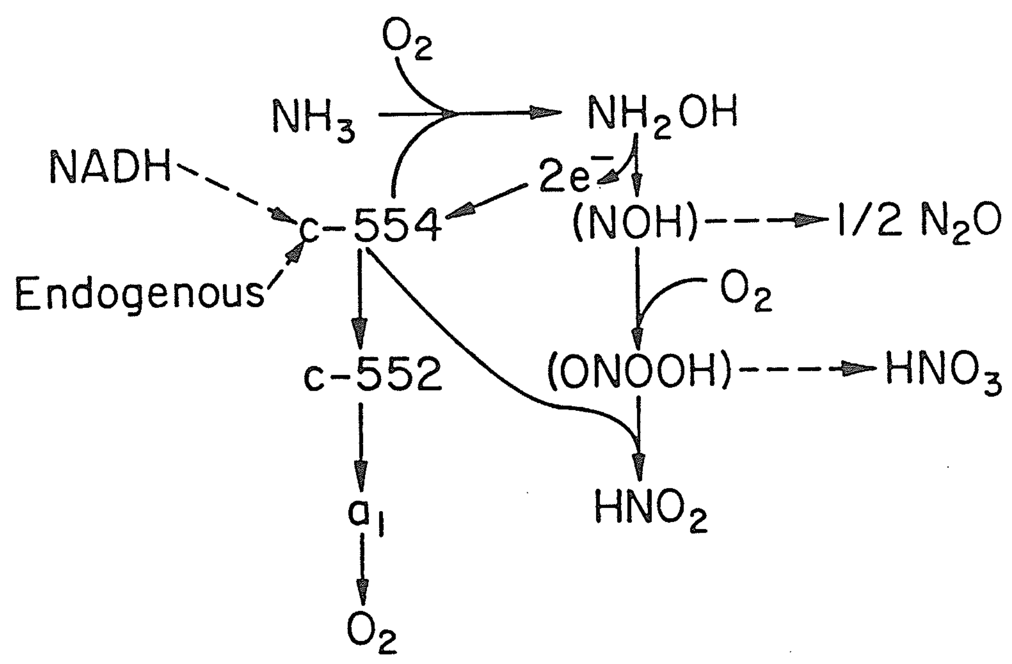


$$NOH + O_2 \rightarrow ONOOH.$$

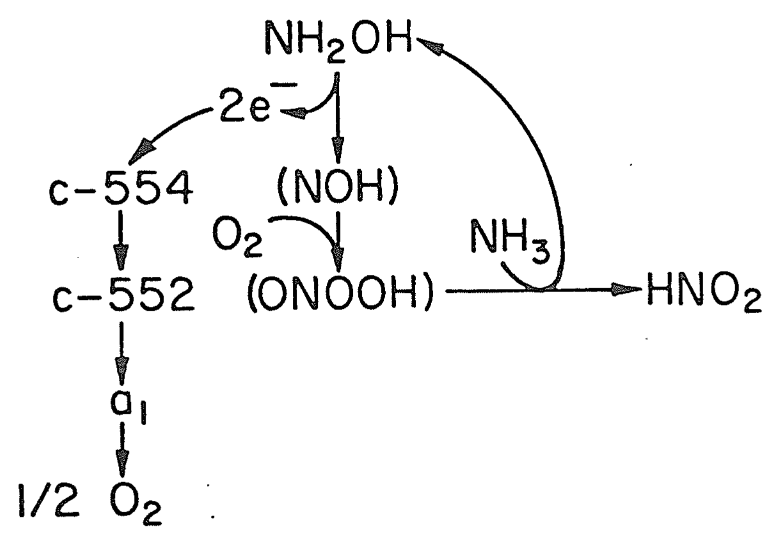
If the first and last reactions are tightly coupled we have $NH_3 + NOH + O_2 \rightarrow NH_2OH + HNO_2$, a reaction compatible with the mechanism proposed earlier (Suzuki, 1974). The hypothetical scheme of ammonia oxidation involving peroxonitrite is shown in Figs. i and ii. Ammonia is initially hydroxylated by oxygen plus electrons derived from endogenous metabolism or added NADH, hydroxylamine, or hydrazine. Once the hydroxylation is initiated, electrons from hydroxylamine oxidation became available for further hydroxylation of ammonia (Fig. i). At the steady state of ammonia oxidation the oxidation of nitroxyl (NOH) is coupled to ammonia hydroxylation with peroxonitrite as possible intermediate (Fig. ii). The only electrons going through the electron transport system to molecular oxygen for possible energy generation are the two electrons derived from hydroxylamine.

The resolution of the cell-free ammonia-oxidizing system of N. europaea was reported by Suzuki and Kwok (1981) by the passage of extracts through a sepharose 6B column. The membrane or particulate fraction prepared had cytochrome a_1 and other cytochromes, a strong

i



ii



cytochrome oxidase activity and very little NH_2OH-cytochrome *c* oxido-reductase activity. It retained activity for the ammonia-stimulated NADH oxidation (Suzuki *et al*, 1976), but did not oxidise ammonia. The ammonia oxidising activity was restored when a sepharose fraction containing hydroxylamine oxidoreductase and a fraction containing a *c*-type cytochrome (spectrally similar to *c*-552 but not replaceable with the purified cytochrome *c*-552) were combined with the membrane fraction. Ammonia-oxidising activity was also restored when cyto-chrome *c*-554 was added to the membrane fraction.

MATERIALS AND METHODS

MATERIALS AND METHODS

MATERIALS

All chemicals and reagents used were of analytical grades. Bovine serum albumin (fraction V), deoxyribose nuclease I, 2,2'-bipyridyl, disodium EDTA, and 8-hydroxyquinoline were obtained from Sigma Chemical Co., St. Louis, Missouri. Magnesium sulfate, ammonium sulfate, monobasic potassium phosphate, dibasic potassium phosphate, and diethyldithiocarbamate were obtained from Fisher Scientific Company, Fairlawn, N.J., U.S.A. Calcium chloride, ferric chloride, N-1-Naphthyl-ethylenediamine dihydrochloride, and sulfanilic acid were obtained from Matheson, Coleman & Bell manufacturing chemists, Norwood, Ohio; East Rutherford, N.J., U.S.A. Potassium carbonate, hydrazine sulfate, hydroxylamine hydrochloride, and Triton X-100 were obtained from J.T. Baker Chemical Co., Phi-lipsburg, N.J., U.S.A. Sepharose 6B was obtained from Pharmacia, Uppsala, Sweden. Carbon monoxide, methane, and acetylene were obtained from Union Carbide Canada Limited.

Organism and growth of organism

A culture of Nitrosomonas europaea (Schmidt strain) was supplied by Dr. A.B. Hooper, University of Minnesota. The organism was cultivated at 28°C in a modified ATCC medium No. 221: 3.0 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 150 mg $MgSO_4 \cdot 7H_2O$, 12 mg $CaCl_2 \cdot 2H_2O$, 0.4 mg chelated iron, and distilled water to 1.0 L. Chelated iron was prepared by mixing solutions of 2.0 mg $FeCl_3 \cdot 6H_2O$ 10 mL^{-1} with 2.4 mg disodium EDTA 10 mL H_2O^{-1}. The chelated iron solution and a solution of magnesium and calcium salts were autoclaved separately from the rest of the medium components. Batch cultures of 25 L were grown in 30 L

glass carboys under forced aeration through spargers after inoculation with an actively growing *N. europaea* culture (two, 500 mL in 1 L flask, 4 days on a rotary shaker at 120 rpm). During growth of the organism the pH of the medium was maintained at 7.6 by the addition of a 50% (w/v) potassium carbonate solution by means of a radiometer pH-stat unit.

After 4 days of growth when 75% ammonia nitrogen was used up, the cells were harvested by Sharples centrifugation at 50,000 rpm. Ammonia nitrogen in the medium was determined by the method of Crowther and Large (1956). The cells (1 g wet weight•10 L medium^{-1}) were washed three times in 0.1 M potassium phosphate buffer (pH 7.5) and finally suspended in the same buffer (20 mg wet cells or 3.2 mg protein•mL^{-1}). The cell suspension so obtained was stored at 4°C and was used within 3 days.

METHODS

Preparation of cell-free extracts

Ammonia oxidizing extracts were prepared by addition of bovine serum albumin (Sigma, fraction V, 20 mg•mL^{-1}) and DNase 1 (Sigma, 100 μg•mL^{-1}) to the cell suspension (30 mg wet cells or 4.8 mg protein•mL^{-1}) in 0.1 M potassium phosphate buffer (pH 7.5) before the passage through a French pressure cell. All the procedures were carried out at 4°C. Cells were disrupted by passing this cell suspension through a French pressure cell at 18,000 psi (124 Mpa). Intact cells were removed by centrifugation at 2,000 x g for 20 min and the resulting cell-free extract was used as the source of ammonia-oxidizing system. Complete removal of cells by this centrifugation procedure was confirmed by the microscopic examination of extracts under a

phase contrast or electron microscope.

Preparation of the membrane fraction

The *N. europaea* membrane fraction (fraction 1) was obtained by passage of 5 mL cell-free extract at room temperature through a column (2 cm x 30 cm) of Sepharose 6B (Pharmacia) equilibrated with 0.1 M potassium phosphate buffer (pH 7.5). The same buffer was used for elution. Fractions of 6 mL were collected starting with the turbid membrane fraction (fraction 1) which was eluted at the void volume. Elution rate was 1 $mL \cdot min^{-1}$. A slower elution rate resulted in inactive fractions.

Preparations and determinations of cytochrome c-554, cytochrome c-552, and hydroxylamine cytochrome c reductase

The methods used were essentially the same as those described by Yamanaka and Shinra (1974) with slight modifications.

Cells of *N. europaea* that were stored frozen were defrosted and suspended (100 mg wet $cells \cdot ml^{-1}$) in 10 mM Tris-HCl buffer, pH 8.5. The suspension was treated with a sonic oscillator at 4°C (10 $kc \cdot sec^{-1}$, Raytheon) for 20 min. The sonicate was treated with 2 mg each of phospholipase C (Sigma) and lipase D (Sigma) for 3 hrs at 28°C. To the suspension was added $(NH_4)_2SO_4$ to 10% saturation, and the pH of the solution obtained was adjusted to 7.5 by the addition of 5% ammonium hydroxide solution. The suspension was allowed to stand overnight at 4°C with gentle stirring. The dark brown coloured suspension was then centrifuged at 13,000 x g (10,000 rpm) for 30 min to remove the cell debris. The supernatant thus obtained was 90% saturated with $(NH_4)_2SO_4$, stirred gently at 25°C for 20 min and centrifuged at 30,000 x g for 30 min. The supernatant was stored at 4°C (supernatant I).

The pellet was suspended in 10 mM Tris-HCl (pH 8.5) containing 80% saturated ammonium sulphate. The suspension was stirred gently for 20 min and centrifuged at 30,000 x g for 30 min. The supernatant (supernatant II) was saved and the pellet was suspended in 10 mM Tris-HCl (pH 8.5) containing 70% saturated ammonium sulfate. The suspension was stirred gently for 20 min and centrifuged at 30,000 g for 30 min to obtain the precipitate and supernatant fractions. The supernatant (supernatant III) obtained was combined with supernatant I and supernatant II and was dialysed for 2 days against running tap water and then for 1 or 2 days against 10 mM ammonium phosphate buffer, pH 6.5. The precipitate fraction was suspended in a minimal volume of 10 mM Tris-HCl buffer, pH 8.5, and the solution obtained was dialysed for 2 days against the same buffer with occasional changes of the buffer. The precipitate fraction contained cytochrome c-552 and hydroxylamine-cytochrome c reductase, whereas cytochrome c-554 was found in the combined supernatant fraction.

Purification of Cytochrome c-554. The dialysed supernatant fraction in the preceding section containing cytochrome c-554 was loaded on an Amberlite CG-50 column (1 x 9 cm) previously equilibrated with 10 mM ammonium phosphate buffer, pH 6.5. The void volume of the column was 6 ml. Cytochrome c-554 was adsorbed on the column. After the column had been washed with about 10 ml of 0.3 M ammonium phosphate buffer, pH 6.5, cytochrome c-554 adsorbed on the column was eluted with 30 ml of 0.5 M ammonium phosphate buffer, pH 7.0, collected in 2 ml fractions. The eluate thus obtained contained cytochrome c-554 and was further purified by rechromatography on an Amberlite CG-50 column (1 x 9 cm) in the same way as described above, and the eluate

thus obtained with 0.5 M phosphate buffer, pH 7.0, was used as a purified preparation of cytochrome c-554 after dialysis against 0.1 M potassium phosphate buffer, pH 7.5. The concentration of the cytochrome was determined from the absorption at the α peak using the millimolar extinction coefficient value of 24.6 (Yamanaka and Shinra, 1974).

Purification of cytochrome c-552 and hydroxylamine cytochrome c reductase. The dialysed precipitate fraction described in the first section was loaded on a DEAE-cellulose column (1 x 12 cm) previously equilibrated with 10 mM Tris-HCl buffer, pH 8.5. The void volume of the column was 7 ml. Cytochrome *c*-552 and hydroxylamine cytochrome *c* reductase were adsorbed on the column. After washing the column with the loading buffer cytochrome c-552 was eluted with 10 mM Tris-HCl buffer, pH 8.5, containing 50 mM NaCl, while the reductase was eluted with 10 mM Tris-HCl buffer, pH 8.5 containing 0.15 M NaCl. The column was washed with 10 mM Tris-HCl buffer, pH 8.5, containing 0.10 M NaCl between the two elution steps. The last eluate was dark brown in colour and was used as a purified preparation of hydroxylamine-cytochrome *c* reductase. The eluate obtained above containing cytochrome *c*-552 was again dialysed against 10 mM Tris-HCl buffer, pH 8.5, and rechromatographed on a DEAE-cellulose column (1 x 12) in the same way. The eluate obtained was used as a purified preparation of cytochrome *c*-552. The concentration of the cytochrome was determined from the absorption at the α peak using the millimolar extinction coefficient of 30.6 (Yamanaka and Shinra, 1974).

Preparation of peroxonitrite

Stable solutions of sodium peroxonitrite were prepared by adding acidified hydrogen peroxide solution to sodium nitrite solution and immediately adding alkali to stabilize the peroxonitrite anion (Hughes and Nicklin, 1968). The timing of these operations was critical. To obtain the best yields, all three solutions: 12.5 c.c. of aqueous solution containing 0.5 g of sodium nitrite of analytical reagent purity; 12.5 c.c. of aqueous solution containing 0.75 c.c. of 35 percent hydrogen peroxide and 0.2 c.c. of 96 percent sulphuric acid; and 25 c.c. of aqueous solution containing 1.25 g of sodium hydroxide of analytical reagent purity, were cooled in ice-water. Glass-distilled water was used in preparing all three solutions. With the nitrite solution being well stirred, the hydrogen peroxide solution was poured quickly into the nitrite solution, followed immediately by the alkali. The resulting bright yellow solution was then treated with manganese (IV) oxide for about 15 min to destroy the excess of peroxide and filtered. Normally yields of 40% were obtained.

Peroxonitrite solutions with higher yields may be prepared by the careful autoxidation of hydroxylamine in alkali (Hughes *et al*, 1971). Oxygen was bubbled for three hours via a fine sinter at room temperature into a solution of 1 M NaOH (24 ml), 0.1 M ethylenediaminetetraacetic acid (EDTA) (0.5 ml), 0.1 M $NH_2OH \cdot HCl$ (5 ml) and glass distilled water (19.5 ml). The yellow colour of peroxonitrite was visible after 30 min and maximum conversion (50%) occurred after three hours.

Spectrophotometric studies on the reduction and oxidation of cytochrome c-554

Absorption change at 420 nm (ΔA_{420} nm) or 430 nm (ΔA_{430}nm) were

followed in a Shimadzu MPS-50 L spectrophotometer (1 cm light path). In experiments where cytochromes and fraction 1 were used in the sample cell, equal amounts of cytochromes and inactivated fraction 1 were added to the reference cell. Inactivated fraction 1 was obtained by heating the membranous fraction in a boiling water bath for 2 minutes. When reagents were added to the sample cell equal volumes of phosphate buffer were added to the reference cell. Both cells were well mixed after each addition. The addition of reagents was made in microlitre quantities with Hamilton microlitre syringes. Gases were added to all assays as saturated solutions in 0.1 M potassium phosphate buffer (pH 7.5) at 25°C and atmospheric pressure.

Anaerobic experiments were done in either 3 ml suba sealed cuvettes or 4 ml glass stoppered cuvettes with a side arm. When the suba sealed cuvettes were used, oxygen was removed by gas exchange with nitrogen for 20 minutes using hypodermic needles, and the reagents were added with microliter syringes through the suba seal. When the glass-stoppered cuvettes were used, oxygen was removed by evacuation, and reagents to be added were placed in the side arm.

Assay of ammonia oxidizing activity

The oxidation of ammonia was routinely followed by oxygen uptake in a Gilson oxygraph (Clark oxygen electrode) to determine the ammonia oxidizing activities of either the whole cells, the cell-free extracts, or the membrane fractions of *N. europaea*. The activities were confirmed by the colorimetric determination of nitrite formed (Bratton and Marshall, 1939) to agree with the stoichiometry expected of the equation: $NH_3 + 1\frac{1}{2}O_2 \rightarrow HNO_2 + H_2O$ (Suzuki and Kwok, 1970; Suzuki *et al*, 1976) the standard reaction mixture, in a total volume of 1.2 mL, contained 1.7

mM $(NH_4)_2SO_4$, 0.1 M potassium phosphate buffer, and either 24 μl whole cell suspension (3.2 mg protein·mL^{-1}), or 0.2 ml cell-free extract (4.8 mg protein·mL^{-1}), or 0.1 ml fraction 1 (1.4 mg protein·mL^{-1}) plus cytochrome c-554 (3.4 nmol). The addition of ammonia, and other reagents was made in microlitre quantities with Hamilton micro-litre syringes. All experiments were carried out at 25°C. The activity was expressed in nanomols oxygen consumed per minute after correction for endogenous respiration measured with the addition of 2,2'-bipyridyl. Bipyridyl at 0.3 mM inhibited ammonia oxidation completely but had no effect on endogenous respiration.

Assay of carbon monoxide oxidizing activity

The oxidation of carbon monoxide by the fraction 1 (*N. europaea* membrane fraction) plus cytochrome c-554 system in the presence of either hydroxylamine or hydrazine was followed by oxygen uptake in a Gilson oxygraph (Clark oxygen electrode). The standard reaction mixture contained 0.1 mL of fraction 1 (*N. europaea*) 3.4 nmoles of cytochrome c-554, 100 nmoles of $NH_2 \cdot NH_2 \cdot H_2SO_4$, 100 nmoles of carbon monoxide, and 0.1 M potassium phosphate buffer (pH 7.5) in a total volume of 1.2 mL. Reaction was started by the addition of fraction 1. The addition of reagents was made in microlitre quantities with Hamilton microlitre syringes. All experiments were carried out at 25°C. The activity was expressed in nanomols oxygen consumed per minute. A saturated solution of carbon monoxide was prepared by bubbling carbon monoxide for ten minutes in a suba-seal rubber stoppered bottle containing 10 ml potassium phosphate buffer (pH 7.5). The saturated solution was stored at 4°C. Hydrazine and hydroxylamine solutions were prepared fresh.

Determination of ammonia

Ammonia was determined by the method of Crowther and Large (1956). The following reagents were prepared. (A) Sodium phenoxide prepared by dissolving 62.5 g phenol in a smallest amount of ethanol, followed by the addition of 2 ml methanol and 18.5 ml acetone and finally made up to 100 ml with ethanol. The solution was stored at 4°C. (B) 27% NaOH (w/v). (C) Sodium hypochlorite solution (0.9% active chlorine). Reagent (D) was prepared by mixing 20 ml of reagent (A) with 20 ml of reagent (B) and diluting to 100 ml with distilled water.

To 1 ml sample was added 0.4 ml reagent (D) and 0.3 ml reagent (C). After 20 minutes at room temperature the mixture was diluted to 5 ml with distilled water. The blue colour developed was measured in a Klett Summerson colorimeter with a No. 66 filter.

A standard curve was prepared from the above procedure using known concentration of ammonium sulfate.

Determination of hydroxylamine

Hydroxylamine was determined by the method of Frear & Burrell (1955), using 8-quinolinol. The following reagents were prepared. (A) 8-Quinolinol solution was made by dissolving 1.0 gram of 8-quinolinol (Eastman Kodak Co.) in 100 ml of absolute alcohol. The solution was tightly stopped. (B) 1.0 M sodium carbonate. (C) 12% (w/v) Trichloroacetic acid solution.

To one ml of the sample was added 1.0 ml of 0.05 M potassium phosphate buffer (pH 6.8), 1.0 ml water, 0.2 ml of trichloroacetic acid solution, and 1.0 ml of 8-quinolinol solution. The reaction mixture was swirled gently. Next, 1.0 ml of the 1.0 M sodium carbonate solution was added, shaken vigorously, and stoppered before

placing in a boiling water bath for 1 minute to develop the green colour. On removal from the water bath, the mixture was cooled for 15 minutes. The green colour developed was measured in a Klett Summerson colorimeter with a No. 66 filter.

A standard curve was prepared using the above procedure with fresh hydroxylamine standard solutions. Beer's law was obeyed over the range of 0 to 5 x 10^{-2} millimole of hydroxylamine per ml of solution.

Determination of peroxonitrite

The concentration of peroxonitrite was determined from the absorption at 302 nm with a millimolar extinction coefficient of 1.67 using the Shimadzu MPS-50L spectrophotometer (1 cm light path) (Hughes and Nicklin, 1968).

Decomposition of peroxonitrite was followed by the absorption change at 302 nm using the same spectrophotometer.

Determination of nitrite

Nitrite was determined by the method of Bratton and Marshall (1939). The reagents consisted of 0.12% N-(1-naphthyl)-ethylene diamine dihydrochloride in distilled water and 1% sulfanilic acid in 20% HCl.

To one ml of sample were added 1 ml of sulfanilic acid and 1 ml of N-(1-naphthyl)-ethylene diamine dihydrochloride solution. The samples were incubated at room temperature for 20 minutes to insure maximum colour development. The volume of each reaction sample was then made up to 10 ml with distilled water. The colour intensity was measured in a Klett Summerson colorimeter with a No. 54 filter.

A standard curve was prepared from the above procedure using

known concentrations of sodium nitrite.

Determination of protein

Protein was determined by the colorimetric method of Lowry *et al* (1951). Crystalline bovine serum albumin was used as the standard protein.

Measurement of carbon monoxide

Carbon monoxide was measured by T.C. Kuchnicki, using a hydrogen sensitized Clark oxygen electrode (T.C. Kuchnicki, M.Sc. thesis 1982). A 1.2 ml water-jacketed oxygen electrode chamber, with the electrode inserted horizontally, was used as the reaction vessel. Reagents were added through a capillary bore stopper. The electrode (YSI 4004, Yellowspring) that had been sensitized (T.C. Kuchnicki, M.Sc. thesis 1982) was coupled to the electrometer constructed by K. Carter, 1978 (T.C. Kuchnicki, M.Sc. thesis 1982). A high sensitivity membrane (YSI 5776) was used. The polarographic potential used was 600 mv. The system was standardized before and after the experiment.

RESULTS

RESULTS

Properties of the membrane fraction

As described in Materials and Methods, fraction 1 (*N. europaea* membrane fraction) was obtained by chromatography of *N. europaea* extracts in Sepharose 6B. Profiles of protein concentration and absorbance at 410 nm (1 cm light path) were determined (Fig. 1).

The membrane fraction showed a cytochrome oxidase activity, a hydroxylamine-cytochrome *c* reductase activity (measured with horse heart cytochrome *c*) stimulated by cytochrome *c*-554 as reported previously (Suzuki and Kwok, 1981), and an ammonia oxidizing activity in the presence of cytochrome *c*-554 (Suzuki and Kwok, 1981).

As described in later sections the membrane fraction, in addition, showed the following activities: ammonia or NH_2OH-dependent reduction of cytochrome *c*-554, oxidation of reduced cytochrome *c*-554 with ammonia, carbon monoxide *etc*. and oxidation of carbon monoxide coupled to NH_2OH or NH_2NH_2 oxidation in the presence of cytochrome *c*-554.

All these activities were relatively stable for 3-4 days when stored at 4°C, but were destroyed by heating the fraction for 2 min in a boiling water bath.

Ammonia oxidation and the kinetics of oxidation and reduction of cytochrome c-554

The membrane fraction of *N. europaea* reduced cytochrome *c*-554 partially with ammonia, hydroxylamine or hydrazine (Fig. 2). The cytochrome was also slightly reduced by the endogenous substrate present in the membrane fraction. The cytochrome reduced with hydroxylamine or hydrazine showed a characteristic and asymmetric Soret band with a 430 nm shoulder in addition to its 421 nm peak (Yamanaka and

Shinra, 1974). The patterns of reduction of the two peaks by ammonia were different (Fig. 3). Although the reduction with hydroxylamine or hydrazine was rapid, data not shown, that with ammonia was much slower with a lag period (Fig. 4). The lag period was shorter with higher ammonia concentration but longer with addition of hydroxylamine-cytochrome c reductase, cytochrome c-552 or horse heart cytochrome c (data not given). Under the same conditions, there was a lag period in the uptake of oxygen during ammonia oxidation. The duration of such a lag period varied similarly with the concentration of ammonia and with the addition of various other cytochromes. The rate of reduction of the cytochrome also increased with ammonia concentration.

The reduction of cytochrome c-554 by ammonia (50 mM), was biphasic with time: stage 1 and stage 2. The cytochrome was initially reduced rapidly for 30-45 sec (stage 1) and was subsequently reduced more slowly (stage 2) until it reached an equilibrium level. At this level, addition of hydroxylamine could not further reduce the cytochrome. Changes in the amount of the membrane fraction in the system changed the rate of reduction in stage 1, but did not affect that of stage 2 (Table I).

The reduction of cytochrome c-554 was not affected by superoxide dismutase or catalase, suggesting that superoxide anion was not responsible for the reduction of the cytochrome by ammonia under aerobic condition. It was only under aerobic condition that cytochrome c-554 was reduced by ammonia in the presence of the membrane fraction.

The cytochrome c-554 partially reduced by hydroxylamine in the presence of the membrane fraction remained reduced until disappearance of hydroxylamine by oxidation (Fig. 5). Under the experimental

conditions used in Fig. 5 the cytochrome was reduced with 2 nmoles hydroxylamine to the same level as with 10 nmoles hydroxylamine, but was re-oxidized within one minute. With 10 nmoles hydroxylamine it remained reduced for a few minutes before the reoxidation started. The addition of ammonia to this hydroxylamine-reduced cytochrome _c_-554 partially oxidized the cytochrome instantly (Fig. 5) presumably through the hydroxylation of ammonia to hydroxylamine. The complete reoxidation of cytochrome _c_-554 was delayed perhaps due to the availability of hydroxylamine formed from ammonia (see the curve with 0.1 mM NH_3 in Fig. 5). The above pattern of reduction and oxidation were observed at either the 420 nm peak or the 430 shoulder of cytochrome _c_-554. With a high concentration of ammonia (50 mM) the initial rapid oxidation of cytochrome c-554 at either 420 nm or 430 nm was more extensive. The 420 nm peak was slowly reduced back to the original level of reduction before ammonia addition, while the 430 nm shoulder remained reduced at a level about 50% of that before ammonia addition (Fig. 6).

Under the same experimental conditions, the addition of ammonia started the oxygen uptake instantly. The uptake of oxygen during ammonia oxidation was associated with a steady state of reduction of cytochrome _c_-554. Similar results were obtained when hydrazine was used in place of hydroxylamine.

Horse heart cytochrome _c_ reduced by hydroxylamine under similar conditions did not react with ammonia. Cytochrome _c_-554 reduced by hydroxylamine with a partially purified hydroxylamine-cytochrome _c_ reductase (Yamanaka and Shinra, 1974) did not react with ammonia in the absence of the membrane fraction, i.e. no instant partial oxidation

of the cytochrome upon ammonia addition and no effect by ammonia on the autooxidation of cytochrome c-554. The autooxidation of cytochrome c-554 was too fast when the cytochrome was reduced with a limiting amount of sodium dithionite to demonstrate its interaction with ammonia.

Effect of carbon monoxide, methane, and methanol on ammonia oxidation and cytochrome c-554 oxidation.

Ammonia oxidation by *N. europaea* is competitively inhibited by carbon monoxide, methane, methanol and ethylene (Suzuki *et al*, 1976; Suzuki and Oh, 1981). Carbon monoxide at 10 μM had no effect on the ammonia-cytochrome c interaction with 50 mM ammonia, but increased the extent of instant partial oxidation of the cytochrome with 1 mM ammonia (Fig. 7). Carbon monoxide at 20 μM inhibited the re-reduction of cytochrome, presumably competing with the ammonia hydroxylation reaction leading to hydroxylamine production. In fact carbon monoxide could replace ammonia in the reoxidation of reduced cytochrome c-554 in the presence of the membrane fraction (Fig. 8a). The competitiveness of carbon monoxide as an alternate substrate for the hydroxylase could also be shown when cytochrome c-554 was reduced by different concentrations of ammonia and the effect of carbon monoxide studied. At high ammonia concentration (50 mM), carbon monoxide at a concentration of 10 μM or 20 μM could not oxidize the cytochrome. At low ammonia concentration (1 mM), cytochrome c-554 was oxidized. Both the extent and the rate of oxidation increased with the concentration of carbon monoxide added. In contrast to experiments with ammonia (Fig. 5) in the oxidation of cytochrome c-554 reduced by hydroxylamine, however, there was no subsequent reduction of the cytochrome with CO

(Fig. 8), presumably due to lack of hydroxylamine production. The complete oxidation of the cytochrome was achieved faster with increasing concentration of carbon monoxide. The extent of initial rapid oxidation of reduced cytochrome c-554 depended on the concentration of carbon monoxide (Fig. 8a), similarly to the effect of ammonia concentration (Fig. 5), the results compatible with a theory that reduced cytochrome c-554 was used as electron donor for hydroxylation of ammonia or carbon monoxide. Methane and methanol showed a similar response to carbon monoxide.

Hydrazine which can replace hydroxylamine in hydroxylamine-cytochrome c reductase of N. europaea (Hooper and Nason, 1965) partially reduced cytochrome c-554 in the presence of the cytochrome similarly to the hydroxylamine-reduced one (Fig. 8b). Methane and methanol also oxidized the cytochrome.

The oxidation of cytochrome c-554 by CO occurred only under aerobic conditions. Under anaerobic conditions, the extent of oxidation increased with the amount of oxygen added to the system (Fig. 9). Complete oxidation of the cytochrome occurred only when the system was opened to air.

Effect of acetylene and 2,2'-bipyridyl on cytochrome c-554 oxidation

Acetylene which is not a competitive inhibitor with respect to ammonia (Suzuki and Oh, 1981; Hynes and Knowles, 1982) did not oxidize cytochrome c-554 partially reduced with hydroxylamine or hydrazine, but inhibited the initial rapid oxidation of the cytochrome by ammonia and the ensuing slow reduction (Fig. 10). Thus acetylene unlike carbon monoxide inhibits the hydroxylation of ammonia not by competition as an alternate substrate, but by inhibiting the ammonia hydroxylase

activity. As shown in Fig. 11 acetylene inhibited the hydroxylation of carbon monoxide as well. 2,2'-Bipyridyl, another inhibitor of ammonia oxidation, showed a similar inhibition.

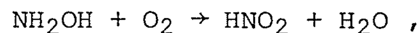
Oxidation of carbon monoxide by the membrane fraction

If hydroxylamine or hydrazine donates electrons for the hydroxylation through cytochrome c-554 and hydroxylase, the hydroxylation of carbon monoxide should consume oxygen according to the equation:

$$CO + O_2 + AH_2 \rightarrow CO_2 + A + H_2O,$$

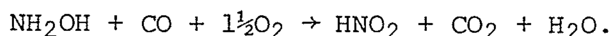
where AH_2 is hydroxylamine or hydrazine. Fig. 12 shows an increased oxygen consumption rate upon addition of carbon monoxide to the membrane fraction plus cytochrome c-554 oxidizing hydrazine. The rate returned to the original slow rate after approximately 1 mol of oxygen consumption per mol of carbon monoxide. In these experiments the hydrazine concentration was higher than the concentration of carbon monoxide. When the experiments were carried out in the presence of excess carbon monoxide the results in Table II were obtained. Carbon monoxide alone was not oxidized, but with hydroxylamine or hydrazine present it increased the rate and total quantity of oxygen consumption. Cytochrome c-552 stimulated hydroxylamine oxidation as reported earlier (Suzuki and Kwok, 1981) or hydrazine oxidation, but was not required for its oxidation in the presence of carbon monoxide. In fact the cytochrome c-552 reduced the total oxygen consumed, presumably by draining the electrons required for carbon monoxide hydroxylation through the cytochrome oxidase (Yamanka and Shinra, 1974). The amount of nitrite formed from hydroxylamine was not affected by the presence of carbon monoxide. The total oxygen consumed agreed with the stoichiometry expected of

the following equation:



$$NH_2OH + O_2 \rightarrow HNO_2 + H_2O \ ,$$

or

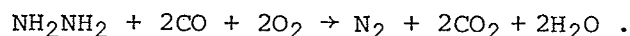


$$NH_2OH + CO + 1\tfrac{1}{2}O_2 \rightarrow HNO_2 + CO_2 + H_2O.$$

In the case of hydrazine there was no nitrite formation, but the total oxygen agreed with the equation:

$$NH_2NH_2 + O_2 \rightarrow N_2 + 2H_2O \ ,$$

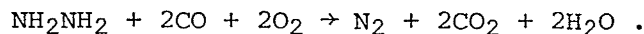
or



$$NH_2NH_2 + 2CO + 2O_2 \rightarrow N_2 + 2CO_2 + 2H_2O \ .$$

These equations have to be considered as hypothetical until the stoichiometry of all the substrates and products can be established.

Experiments were done to show the disappearance of carbon monoxide under similar condition where limited amount of carbon monoxide was oxidised with a stoichiometric uptake of oxygen in the presence of excess hydrazine. The pattern of disappearance of carbon monoxide as measured showed a striking similarity to the pattern of consumption of oxygen (Fig. 13). The amount of oxygen consumed in the complete oxidation of the carbon monoxide present supported the equation:



$$NH_2NH_2 + 2CO + 2O_2 \rightarrow N_2 + 2CO_2 + 2H_2O \ .$$

In the presence of excess carbon monoxide, hydrazine, and cytochrome *c*-554, the activity of carbon monoxide oxidation (rate of oxygen consumption) linearly increased with the increasing amount of the membrane fraction (Table 3).

The oxidation of carbon monoxide under similar condition was not affected by the hydroxyl radical scavenger, mannitol (10 mM).

Effect of pH on the oxidation of carbon monoxide

The carbon monoxide-oxidizing activity of fraction 1 (*N. europaea* membrane fraction) varied with pH, exhibiting a normal pH dependency curve (Fig. 14). With 200 nmol hydrazine and 100 nmol carbon monoxide per 1.2 ml, the optimal pH range for the reaction was pH 7.0-8.4. Fig. 14 showed also the change in the rate of oxidation of hydrazine with pH. Below pH 8.5 the rate of oxidation of hydrazine was relatively small. Beyond pH 8.5 the rate of oxidation increased sharply.

Determination of Km values of carbon monoxide, hydroxylamine, hydrazine, and cytochrome c-554.

When a fixed amount of hydrazine was oxidized by the membrane fraction plus cytochrome *c*-554 system in the presence of a small amount of carbon monoxide the initial fast oxygen uptake rate sharply slowed down to the rate of hydrazine oxidation presumably when carbon monoxide was exhausted (Fig. 12). The total oxygen consumption to the inflection point corresponded to the amount of carbon monoxide added (Table 4). It was possible to estimate the apparent Km value for carbon monoxide from the rate of initial fast oxygen consumption as 16 μM by the double reciprocal rate-concentration plot (Fig. 15). It was assumed here that hydrazine was preferentially oxidized through the hydroxylation of carbon monoxide until carbon monoxide was nearly exhausted when hydrazine was oxidized at a slower rate through the cytochrome oxidase and the autooxidation of cytochrome *c*-554.

In the presence of excess carbon monoxide (100 nmol/1.2 ml) with varied and limiting concentrations of hydroxylamine or hydrazine (essentially the same condition as in Table 2) the apparent Km value

was obtained as 2-3 μM NH_2OH or NH_2NH_2 by the double reciprocal rate concentration plot (Fig. 16).

Under similar conditions the apparent Km value for cytochrome *c*-554 was estimated as 1.2 μM from the initial velocities with 0.1 ml fraction 1 and varied concentrations of the cytochrome (Fig. 17).

Determination of the Km value for ammonia in the presence of hydrazine was complicated by the fact that upon hydroxylation ammonia, unlike carbon monoxide, becomes hydroxylamine, an electron donor for further hydroxylation. The apparent Km value for ammonia with 100 nmol hydrazine in 1.2 ml was estimated as 0.1 mM (NH_4^+ + NH_3) without subtracting the rate of hydrazine oxidation in the absence of ammonia. When the hydrazine rate was subtracted the apparent Km value for ammonia became 1.3 mM (NH_4^+ + NH_3) or 24 μM NH_3, the same value obtained with a cell-free extract of *N. europaea* for ammonia oxidation without hydrazine at pH 7.5 (Suzuki *et al*, 1974).

Preliminary studies on the inhibitors of carbon monoxide oxidation

The oxidation of carbon monoxide by the fraction 1 plus cytochrome *c*-554 system in the presence of either hydroxylamine or hydrazine was inhibited by metal chelators. 2,2'-Bipyridyl, (1 mM) diethyldithio-carbamate (50 μM) and KCN (1 mM) inhibited the activity by over 90%. At lower concentrations, the inhibitors, 2,2'-bipyridyl (50 μM), diethyldithiocarbamate (2 μM), and KCN (50 μM) showed a time-dependent inhibition, after which a further addition of the fraction 1 restarted the oxidation. Hydroxylamine oxidation was not inhibited by 2,2'-bipyridyl (1 mM).

Preliminary studies on the effect of detergents, phospholipase A, phospholipase D, and sonication on the activities of fraction 1

The detergents: Triton X-100, SDS, and sodium cholate were tested for their effect on the carbon monoxide-oxidizing activity or ammonia-oxidizing activity of fraction 1 at different concentrations. Triton X-100 at 0.05% (v/v) showed 85% inhibition. Cholate at 0.1% (v/v) showed 22% inhibition with the fraction partially solubilized. SDS at 0.01% (w/v) showed a time dependent inhibition of ammonia oxidizing activity and solubilized the fraction.

Treatment of the membranous fraction 1 with phospholipase D (25 μg/ml) for 1 hr at 27°C did not affect either the ammonia-or carbon monoxide-oxidizing activity (O_2 uptake). Sonication (20 kc per sec, 10 min) of the phospholipase D treated membrane fraction had no effect. With the phospholipase D treatment, about 5% of the carbon monoxide oxidizing activity was found in the supernatant, the rest remained in the pellet. Treatment of fraction 1 with phospholipase A (25 μg/ml) for 30 min at room temperature destroyed the ammonia-dependent reduction of cytochrome c-554.

Preliminary studies on the properties of peroxonitrite

Peroxonitrite anion ($ONOO^-$), as measured by the absorption at 302 nm, was stable at alkaline pH (pH 10-12). Cu^{2+} increased the rate of decomposition of $ONOO^-$. When peroxonitrite was in excess, hydroxylamine decomposed peroxonitrite instantly at a fast rate but slowed down to a steady rate of decomposition. The process of decomposition could be repeated with additions of NH_2OH. With an equivalent amount of hydrazine, however, the decomposition of peroxonitrite was more extensive. The rate of decomposition of

$ONOO^-$ increased with the ionic strength of the alkaline solution. KCl at 0.4 M increased 15 times the rate of decomposition.

Under alkaline condition, hemes of horse heart cytochrome c and hydroxylamine cytochrome c reductase were stable, but were oxidised and decomposed by $ONOO^-$. Under similar condition, dithiothreitol reacted stoichiometrically with $ONOO^-$. The extent of decomposition of peroxonitrite was proportional to the amount of dithiothreitol. The rate of decomposition of $ONOO^-$ depended on the concentration of either of the two reagents. Diethyldithiocarbamate did not react with $ONOO^-$ under similar condition. In the presence of Cu^{2+} (15 μM), ammonia (0.4 M) accelerated the decomposition of $ONOO^-$. Potassium cyanide also accelerated the decomposition (0.2 ∿ 0.4 mM).

At pH 7.5, $ONOO^-$ was very unstable. The rate of decomposition was too fast for experiments to be done at this pH. Organic solvents like methanol, dioxane did not help stabilize peroxonitrite.

Preliminary studies on the effect of $Ni(CN)_4^=$

Reduction of cytochrome c-552 with hydroxylamine in 0.1 M potassium phosphate, pH 7.5, in the presence of catalytic amount of cytochrome c-554 and hydroxylamine cytochrome c reductase was inhibited when hydroxylamine was in excess (Table 5). Such inhibition was released by $[Ni(CN)_4^=]$ (1 mM) (Table 5) with a faster rate of reduction, and a complete reduction of the cytochrome in the presence of the complex ion. The complex $Ni(CN)_4^=$ is known to act as a nitroxyl trap with the formation of the complex $[Ni(CN)_4NO]^=$ Under similar conditions, the reduction of horse heart cytochrome c was not affected by $Ni(CN)_4^=$.

Fig. 1. Chromatography of *N. europaea* extracts in Sepharose 6B. Fraction 1 was eluted at the void volume. Protein concentration and absorbance at 410 nm (1-cm light path) were determined.

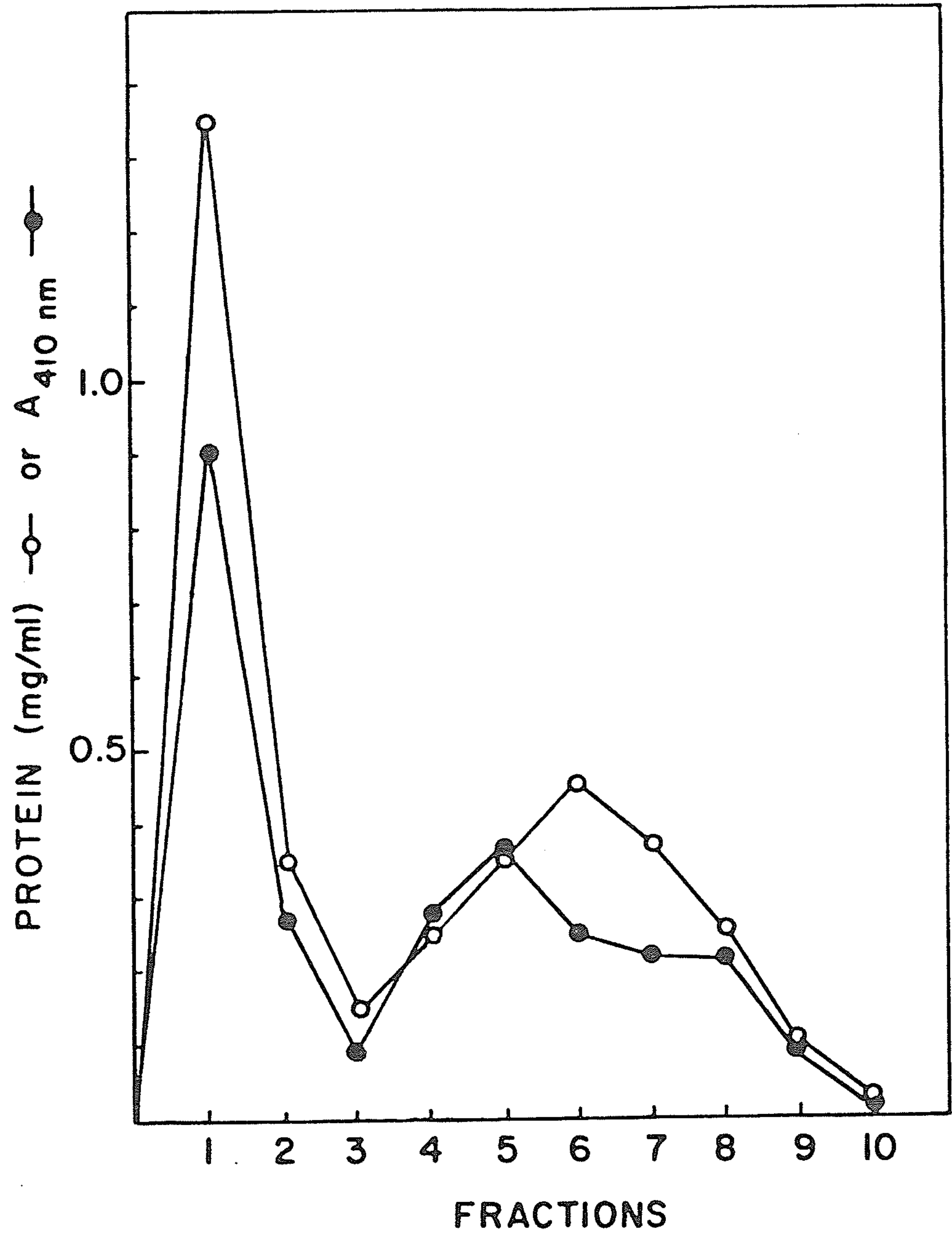
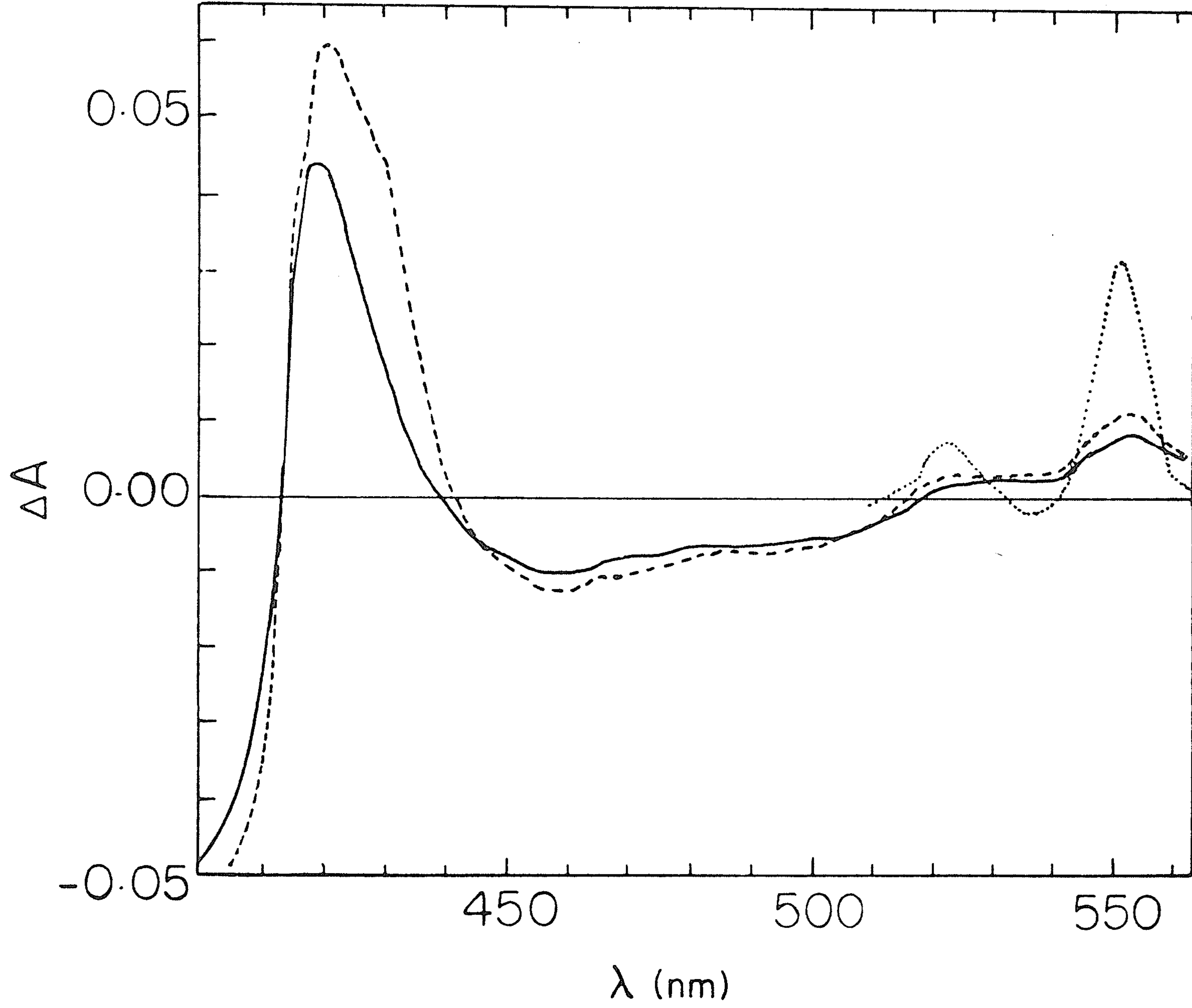

PROTEIN (mg/ml) —o— or $A_{410\ nm}$ —●—
1.0
0.5
1
2
3
4
5
6
7
8
9
10
FRACTIONS

Fig. 2. Difference spectra of _Nitrosomonas_ cytochrome _c_-554. Reference and sample cuvettes (1-cm light path) each contained 0.1 mL of fraction 1 (_N. europaea_ membrane fraction) and 2.5 nmoles cytochrome _c_-554 in 0.1 M potassium phosphate buffer (pH 7.5) in a total volume of 1 mL. Ammonium chloride, 50 mM, was added to the sample cuvette and the ammonia-reduced minus oxidized difference spectrum was recorded (——). $NH_2OH \cdot HCl$ (10 μM) and $NH_2NH_2 \cdot H_2SO_4$ (10 μM) were added similarly to two fresh samples and the corresponding reduced minus oxidized difference spectra were recorded (---). The two spectra reduced by NH_2OH and NH_2NH_2 were identical. The dithionite-reduced minus oxidized difference spectrum is shown as a reference (···). A Shimadzu MPS-50L spectrophotometer was used.



ΔA
0.05
0.00
-0.05
450
500
550
λ (nm)

Fig. 3. Reduction of cytochrome *c*-554 at 420 nm peak and 430 nm shoulder by ammonia.

Reaction mixture contained 0.1 mL of fraction 1 (*N. europaea* membrane fraction), 4.1 nmoles cytochrome *c*-554, and 0.1 M potassium phosphate buffer (pH 7.5) in a total volume of 1.0 mL. Ammonia (50 mM) was added to the reaction mixture where indicated. Absorption change at 420 nm (——) and 430 nm (---) were followed as described in Materials and Methods.

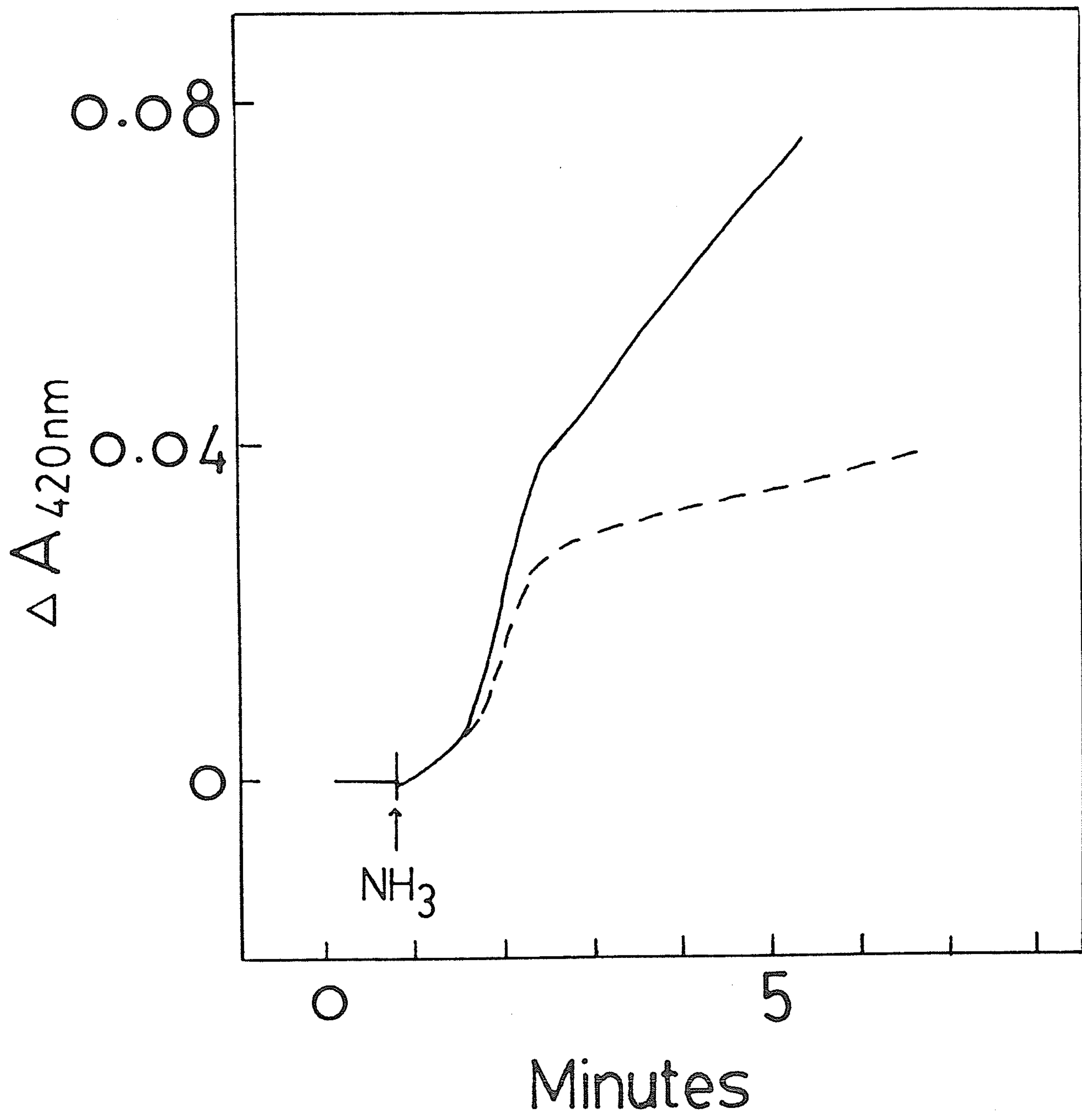
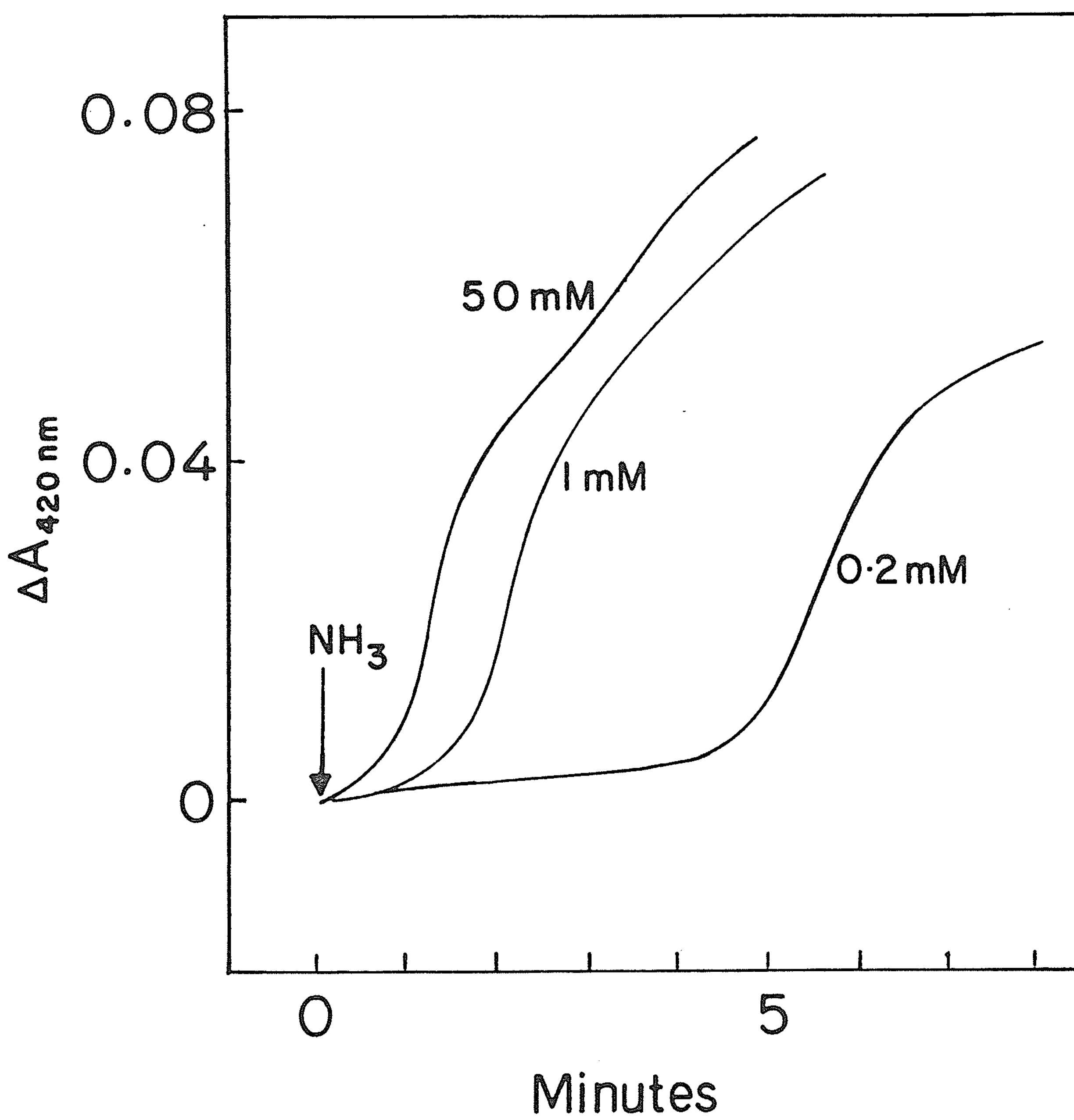
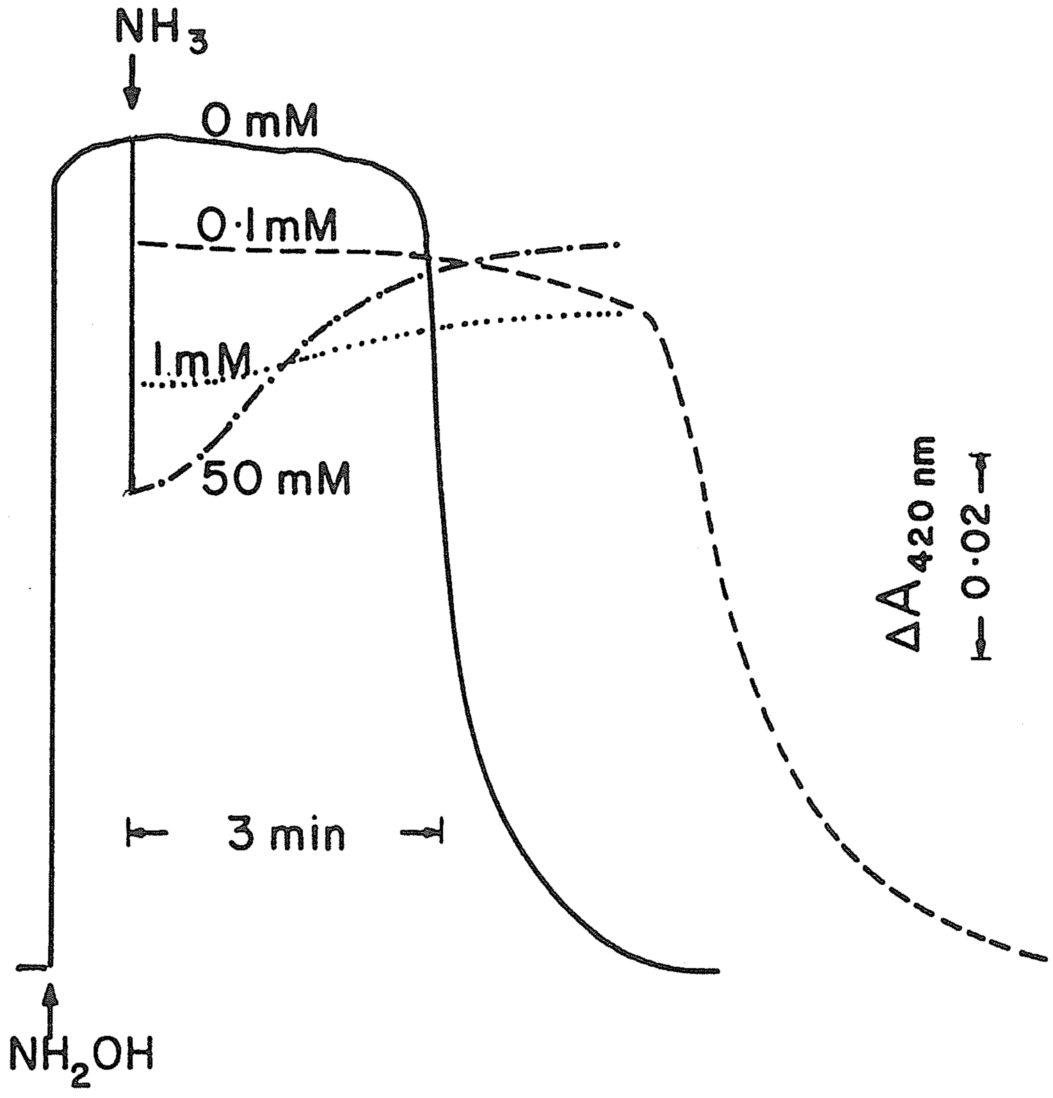

0.08
0.04
0
ΔA_{420nm}
NH_3
0
5
Minutes

Fig. 4. Effect of ammonia concentration on the lag of the reduction of cytochrome *c*-554 by ammonia. Reaction mixtures contained 0.1 mL of fraction 1 (*N. europaea* membrane fraction), 3.3 nmoles cytochrome *c*-554 and 0.1 M potassium phosphate buffer (pH 7.5), in a total volume of 1.0 mL. Different concentrations of ammonia were added as indicated. Absorption change at 420 nm ($\Delta A_{420\ nm}$) was followed in a Shimadzu MPS-50L spectrophotometer (1-cm light path) as described in Materials and Methods.



0.08
0.04
0
$\Delta A_{420\,nm}$
NH3
50 mM
1 mM
0·2 mM
0
5
Minutes

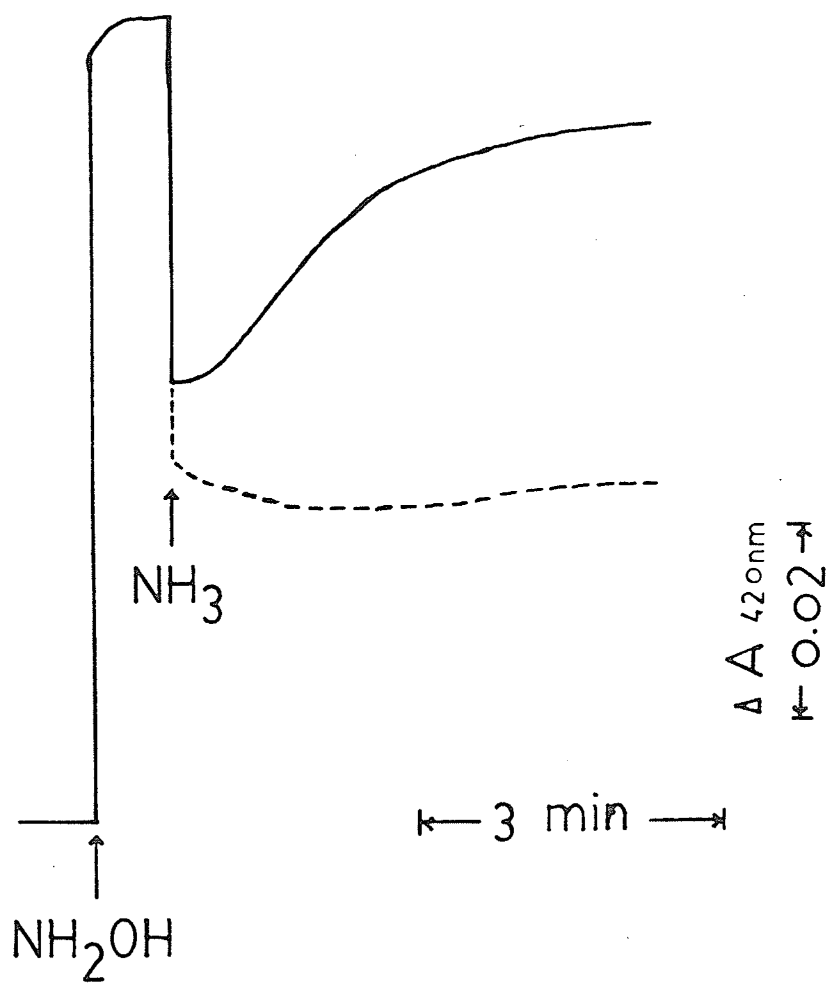
Fig. 5. Effect of different concentrations of ammonia on the oxidation of cytochrome *c*-554 reduced by hydroxylamine. Reaction mixtures contained 0.1 mL of fraction 1 (*N. europaea* membrane fraction), 3.4 nmoles cytochrome *c*-554, and 0.1 M potassium phosphate buffer (pH 7.5) in a total volume of 1.0 mL. Ten nmoles $NH_2OH \cdot HCl$ were added to the reaction mixture, followed by ammonia of different concentrations as indicated. Absorption change at 420 nm was followed as described in Materials and Methods.



NH$_3$
0 mM
0·1mM
1mM
50 mM
3 min
NH$_2$OH
ΔA$_{420\ nm}$
0·02

Fig. 6. Effect of ammonia on the oxidation of cytochrome c-554 at 420 nm and 430 nm.

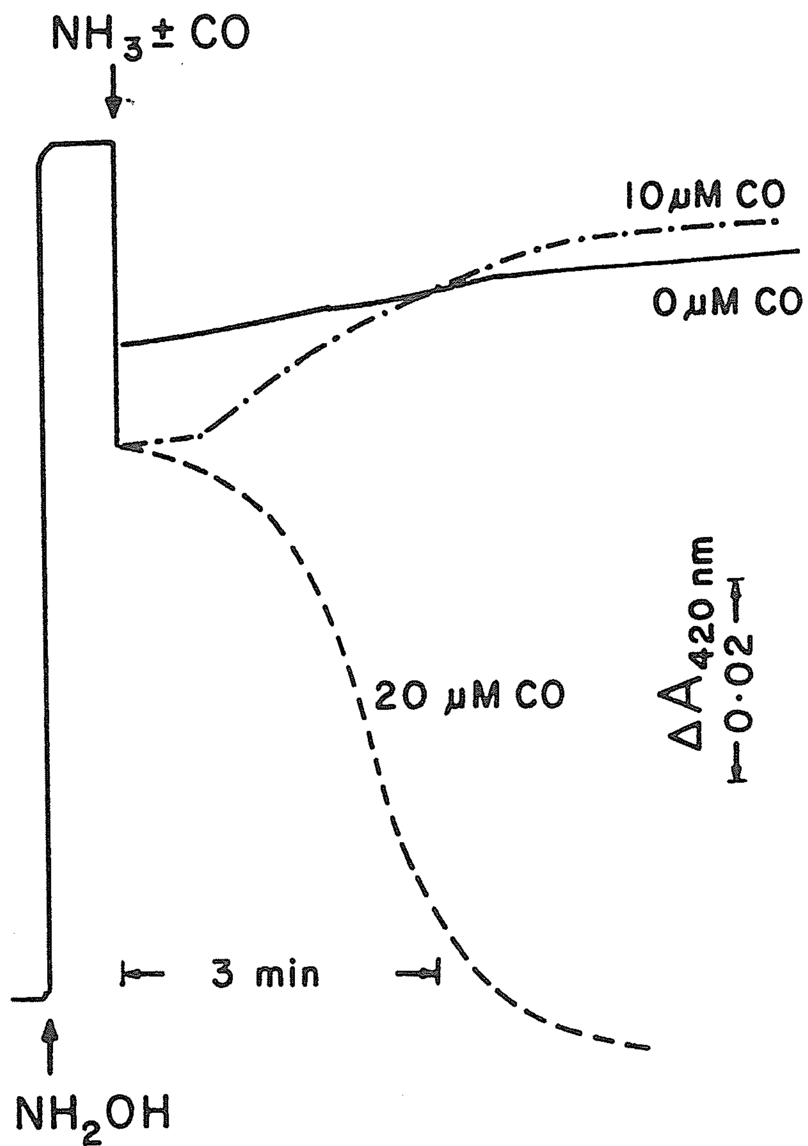
Reaction mixture contained 0.1 mL of fraction 1 (*N. europaea* membrane fraction), 3.4 nmoles cytochrome c-554, and 0.1 M potassium phosphate buffer (pH 7.5) in a total volume of 1.0 mL. Ammonia (50 mM) and hydroxylamine (10 μM) were added to the reaction mixture where indicated. Absorption change at 420 nm (——) and 430 nm (---) were followed as described in Materials and Methods.



NH3
NH2OH
3 min
ΔA 420nm
0.02

Fig. 7. Inhibitory effect of carbon monoxide on the re-reduction of cytochrome c-554 by ammonia.

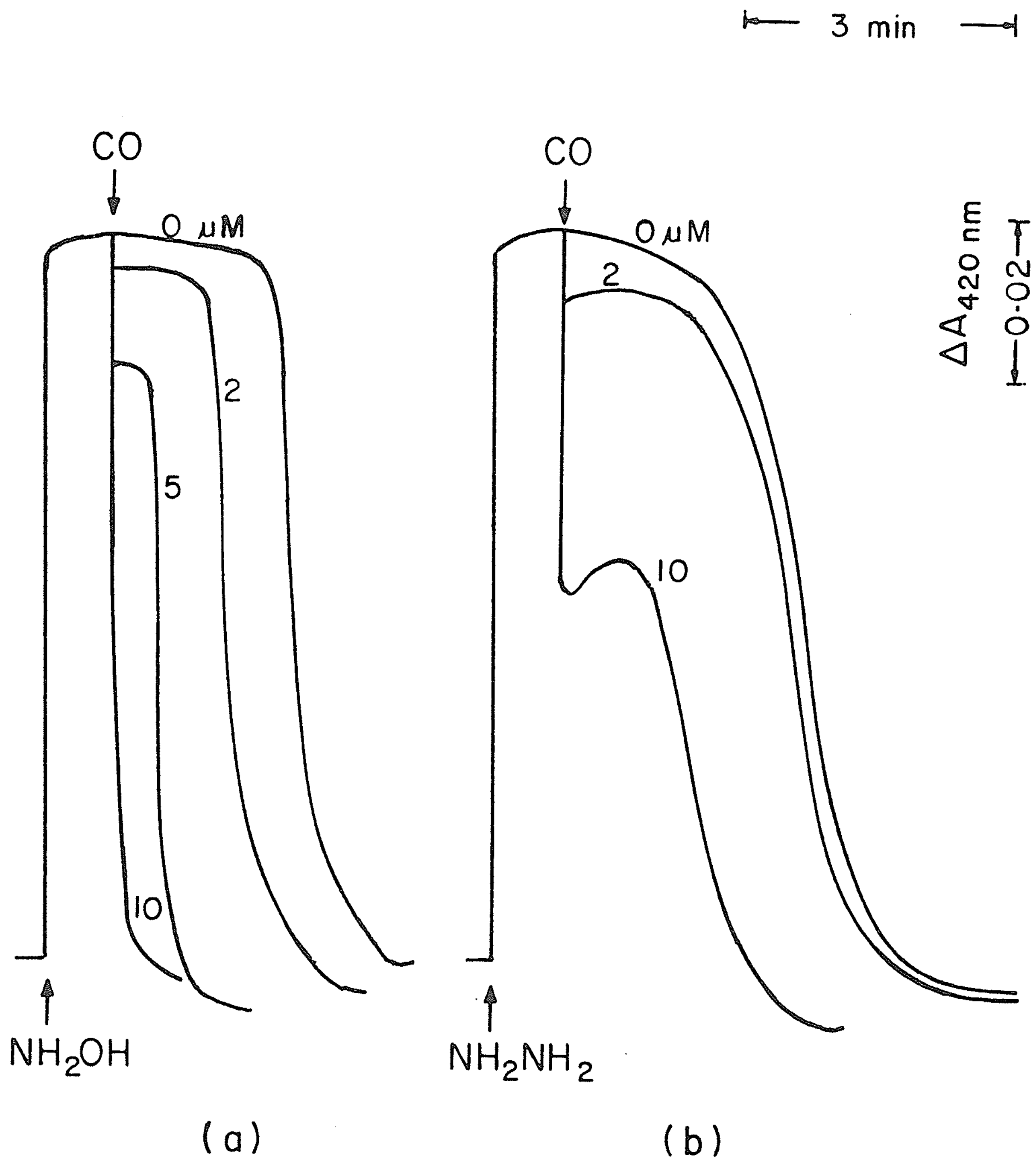
Reaction mixtures contained 0.1 mL of fraction 1 (*N. europaea* membrane fraction), 3.4 nmoles cytochrome *c*-554, and 0.1 M potassium phosphate buffer (pH 7.5) in a total volume of 1.0 mL. Ten nmoles of $NH_2OH \cdot HCl$ were added to the reaction mixture, followed by the addition of ammonia (1 mM) with or without carbon monoxide. The concentrations of carbon monoxide used in each case was indicated. Absorption change at 420 nm was followed as described in Materials and Methods.



$NH_3 \pm CO$
10 μM CO
0 μM CO
20 μM CO
$\Delta A_{420\,nm}$
0·02
3 min
NH_2OH

Fig. 8. Effect of different concentrations of carbon monoxide on the oxidation of cytochrome _c_-554 reduced by hydroxylamine (a) or hydrazine (b).

Reaction mixtures contained 0.1 mL of fraction 1 (_N. europaea_ membrane fraction), 3.4 nmoles cytochrome _c_-554, and 0.1 M potassium phosphate buffer (pH 7.5) in a total volume of 1.0 mL. Ten nmoles of $NH_2OH \cdot HCl$ (a) or $NH_2NH_2 \cdot H_2SO_4$ (b) were added to the reaction mixture, followed by the addition of different concentrtions of carbon monoxide as indicated. Absorption change at 420 nm was followed as described in Materials and Methods.



3 min
CO
0 μM
2
5
10
NH2OH
(a)
CO
0 μM
2
10
NH2NH2
(b)
ΔA420 nm
0·02

Fig. 9. Effect of oxygen on the oxidation of cytochrome *c*-554 by carbon monoxide.

The reaction mixture contained 0.3 mL of fraction 1 (*N. europaea* membrane fraction), 10.2 nmoles cytochrome *c*-554, and 0.1 M potassium phosphate buffer (pH 7.5) in a total volume of 3.0 mL. The mixture was stoppered with a Suba-Seal and gassed with nitrogen for 20 minutes. Thirty nmoles of $NH_2NH_2 \cdot H_2SO_4$ was added to the reaction mixture. Carbon monoxide (20 μM) and oxygen saturated potassium phosphate buffer (a, 10 μl; b, 20 μl) were added to the mixture where indicated. The system was opened to air as indicated by c. Absorption change at 420 nm was followed as described in Materials and Methods.

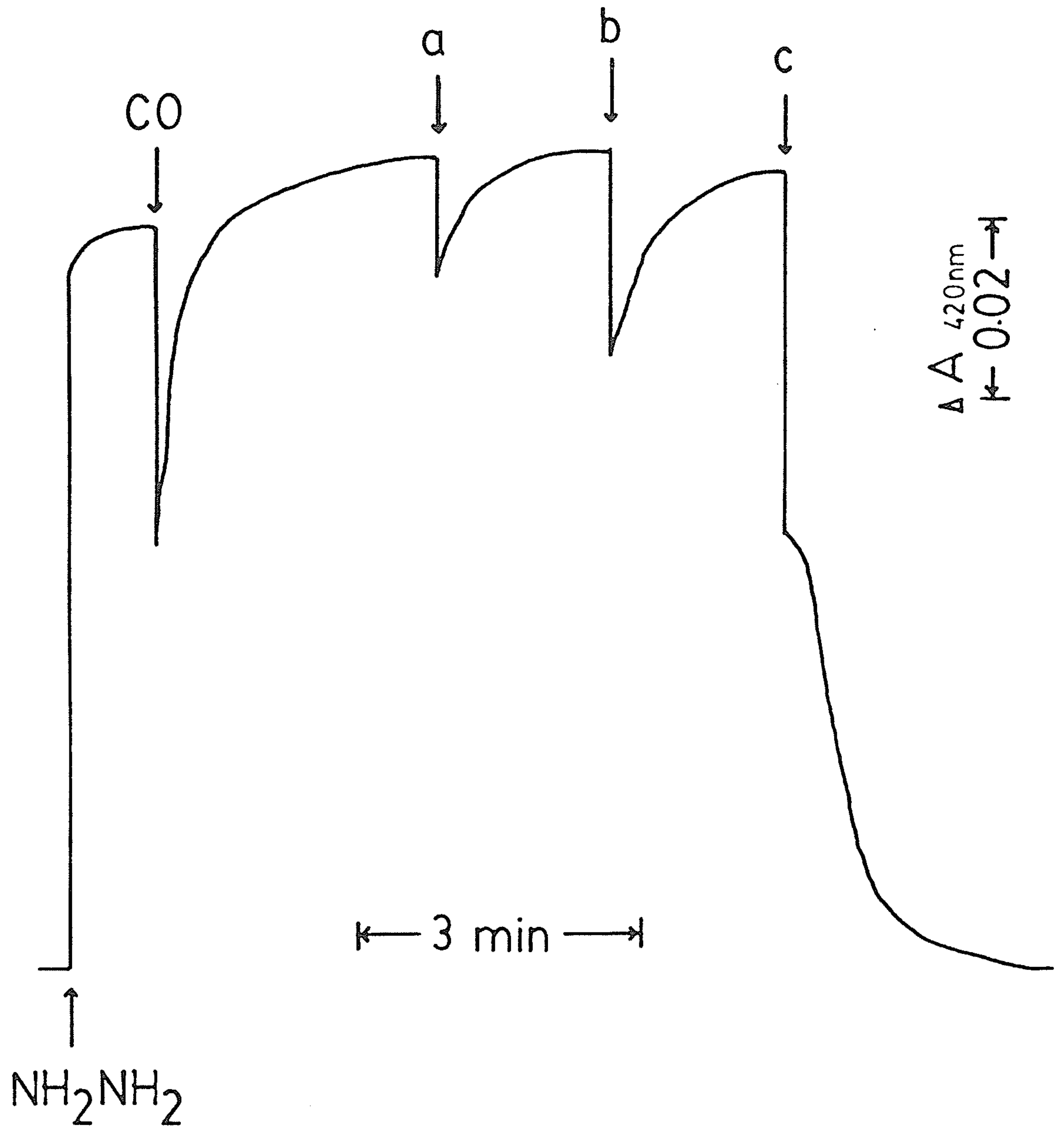
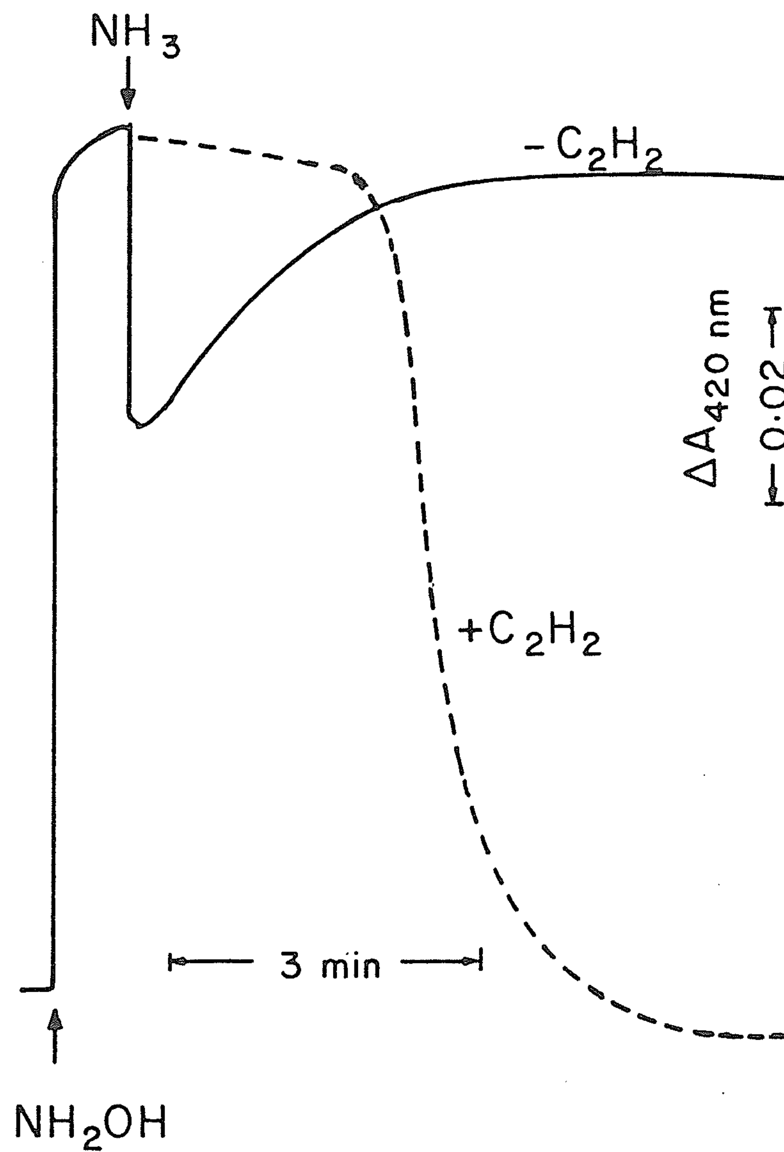

CO
a
b
c
ΔA_{420nm}
0.02
3 min
NH_2NH_2

Fig. 10. Inhibition of ammonia hydroxylase activity by acetylene. Reaction mixtures contained 0.1 mL of fraction 1 (*N. europaea* membrane fraction), 3.4 nmoles of cytochrome *c*-554, and 0.1 M potassium phosphate buffer (pH 7.5) in a total volume of 1.0 mL. Ten nmoles of $NH_2OH \cdot HCl$ were added to the reaction mixture, followed by the addition of ammonia (50 mM). Acetylene, when present, was added before NH_2OH at a concentration of 0.45 mM. Absorption change at 420 nm was followed as described in Materials and Methods.



NH3
-C2H2
ΔA420 nm
0.02
+C2H2
3 min
NH2OH

Fig. 11. Inhibition of carbon monoxide hydroxylase activity by acetylene.

Reaction mixtures contained 0.1 mL of fraction 1 (*N. europaea* membrane fraction), 2.0 nmoles of cytochrome *c*-554, and 0.1 M potassium phosphate buffer (pH 7.5) in a total volume of 1.0 mL. Ten nmoles of $NH_2OH \cdot HCl$ were added to the reaction mixture, followed by the addition of carbon monoxide (20 μM). Acetylene, when present, was added before NH_2OH at a concentration of 0.45 mM. Absorption change at 420 nm was followed as described in Materials and Methods.

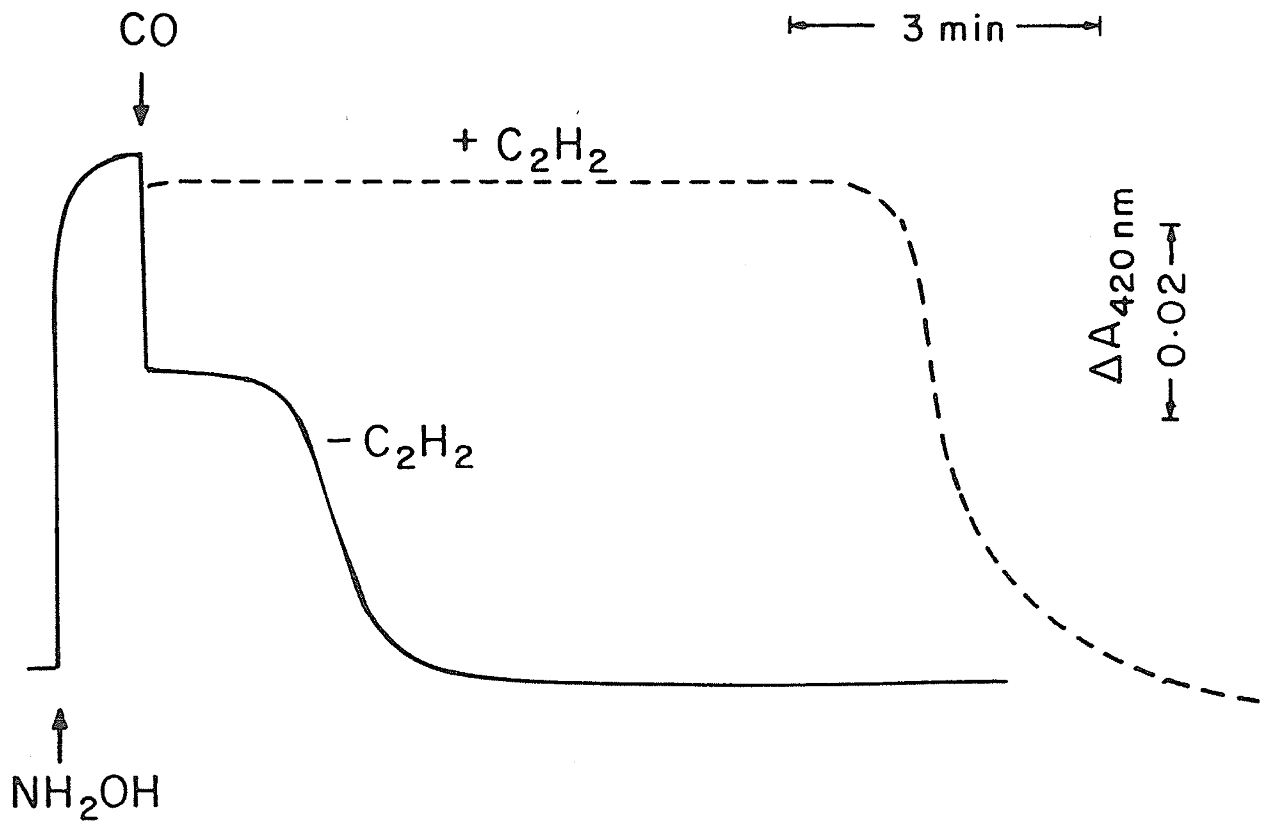

CO
+ C2H2
- C2H2
3 min
ΔA420 nm
0.02
NH2OH

Fig. 12. Oxidation of carbon monoxide by fraction 1 (*N. europaea* membrane fraction) in the presence of cytochrome *c*-554 and excess hydrazine.

The Oxygraph reaction mixture contained 0.1 mL of fraction 1 (*N. europaea* membrane fraction), 3.4 nmoles of cytochrome *c*-554, 100 nmoles of $NH_2NH \cdot H_2SO_4$, different amounts of carbon monoxide as indicated, and 0.1 M potassium phosphate buffer (pH 7.5) in a total volume of 1.2 mL. The reaction was started by the addition of fraction 1 as indicated by the arrow (↓). Oxygen consumption was followed in a Gilson Oxygraph as described in Materials and Methods.

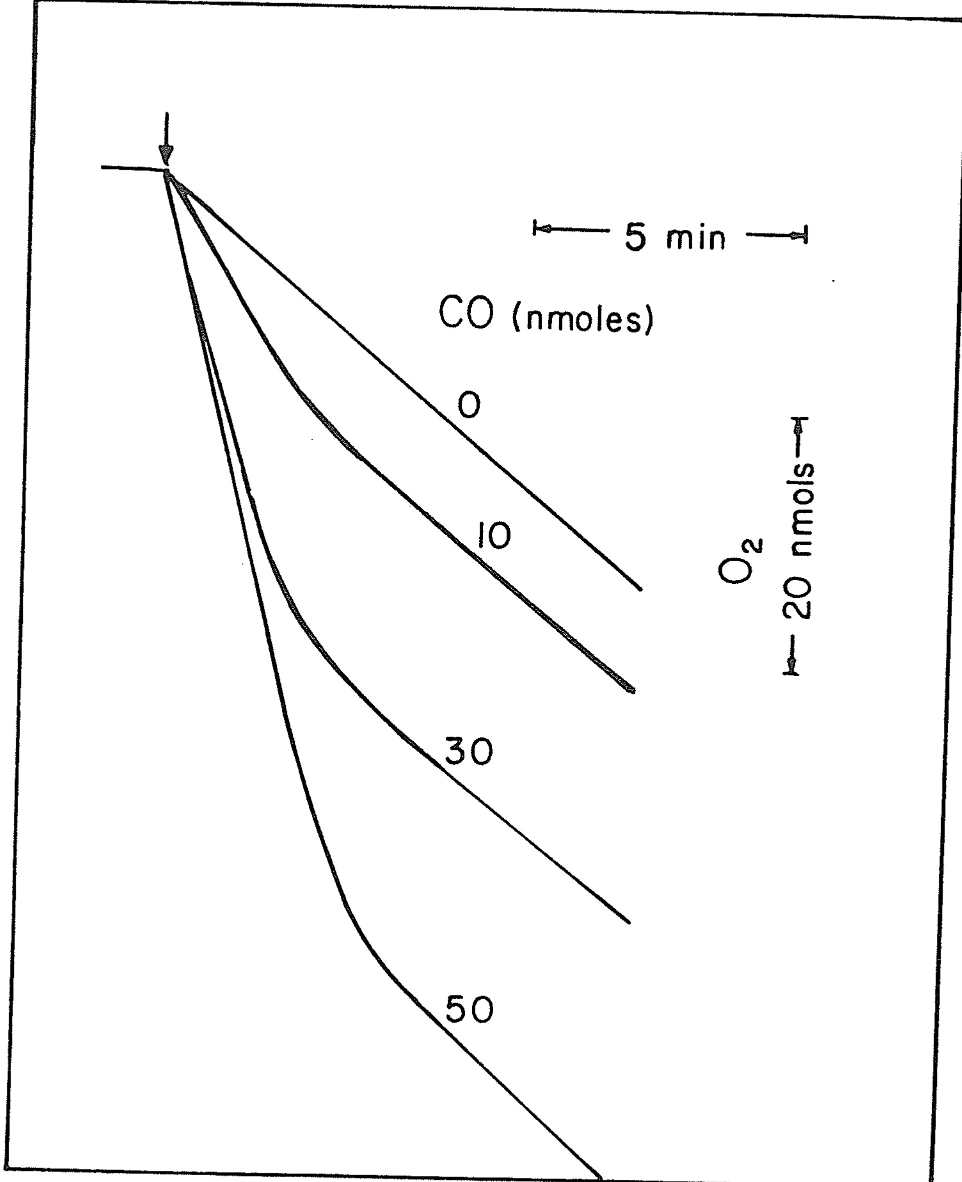
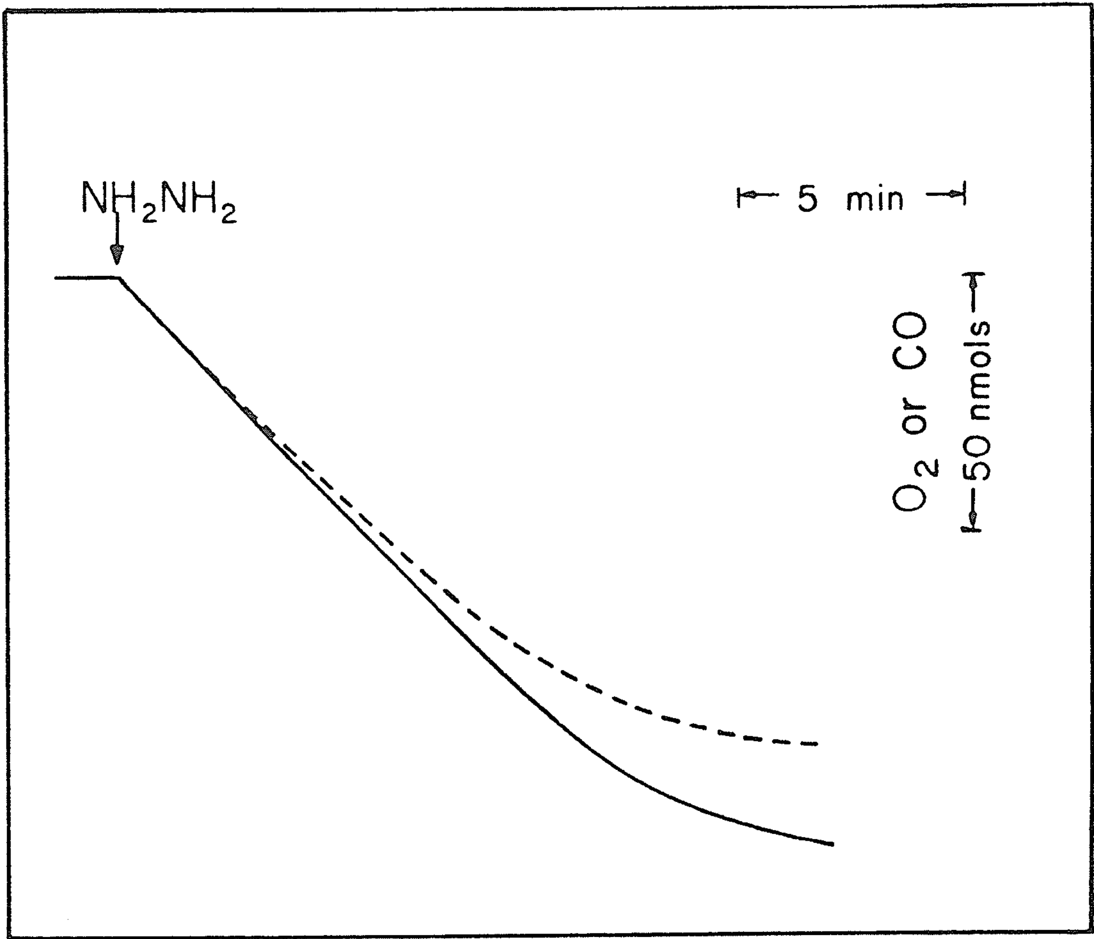

5 min
CO (nmoles)
0
10
30
50
O2
20 nmols

Fig. 13. Comparison of carbon monoxide disappearance and oxygen consumption during carbon monoxide oxidation.

The reaction mixture contained 0.1 ml fraction 1 (*N. europaea* membrane fraction), 3.4 nmol cytochrome *c*-554, 100 nmol carbon monoxide, 200 nmol $NH_2 \cdot NH_2 \cdot H_2SO_4$, and 0.1 M potassium phosphate buffer in a total volume of 1.2 mL. Reaction was started by the addition of hydrazine as indicated. Consumption of oxygen (——) or carbon monoxide (---) were followed as described in Materials and Methods.



NH_2NH_2
5 min
O_2 or CO
50 nmols

Fig. 14. Effect of pH on the carbon monoxide hydroxylase activity.

The rate of oxygen consumption was followed in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture contained 0.1 mL of fraction 1 (*N. europaea* membrane fraction), 3.4 nmoles cytochrome *c*-554, 200 nmoles of hydrazine, 100 nmoles of carbon monoxide, and 0.1 M potassium phosphate buffer of different pH's in a total volume of 1.2 mL. The activity of carbon monoxide hydroxylase was indicated by the solid line (——). The rate of hydrazine oxidation was indicated by the broken line (---).

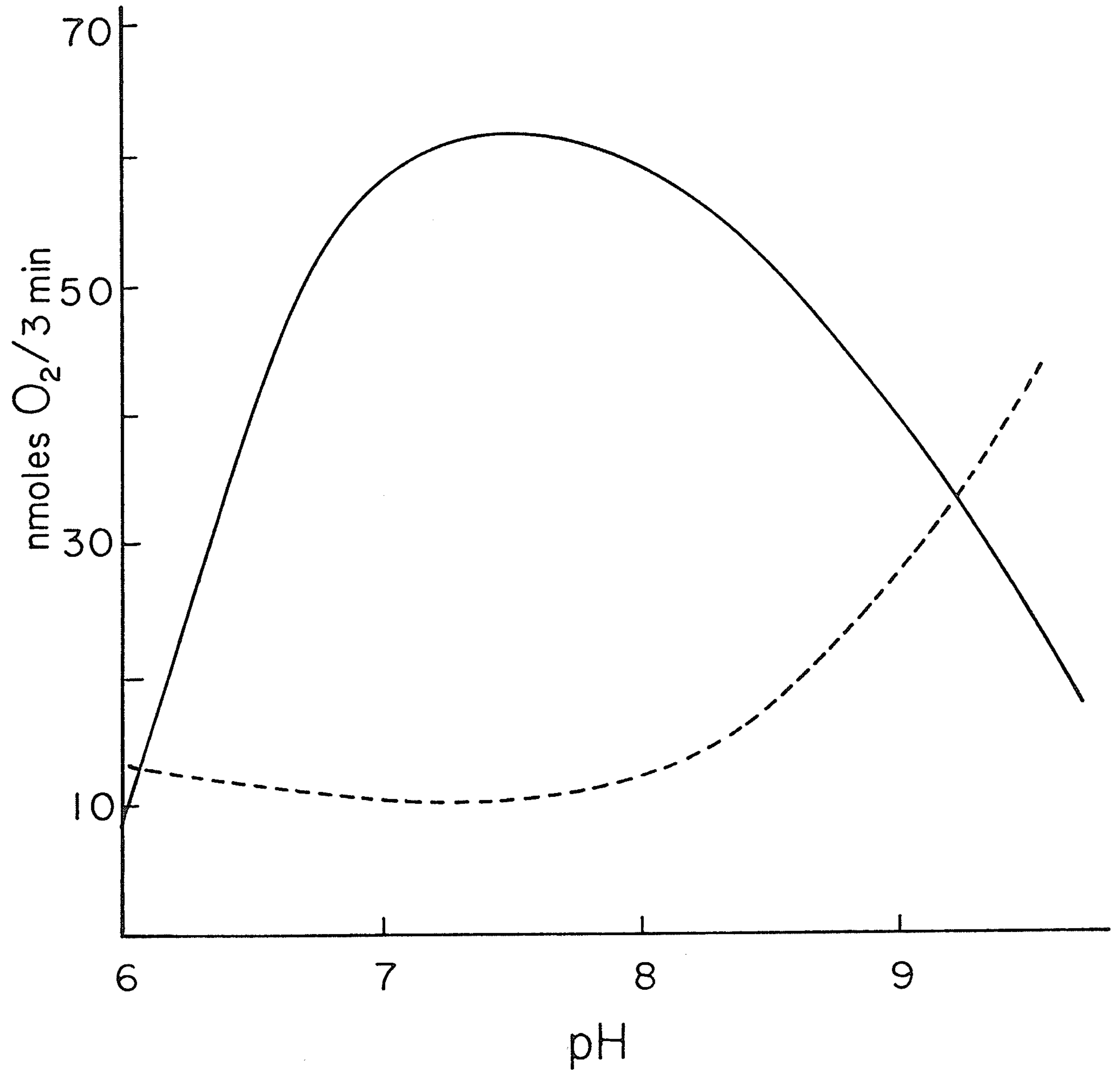
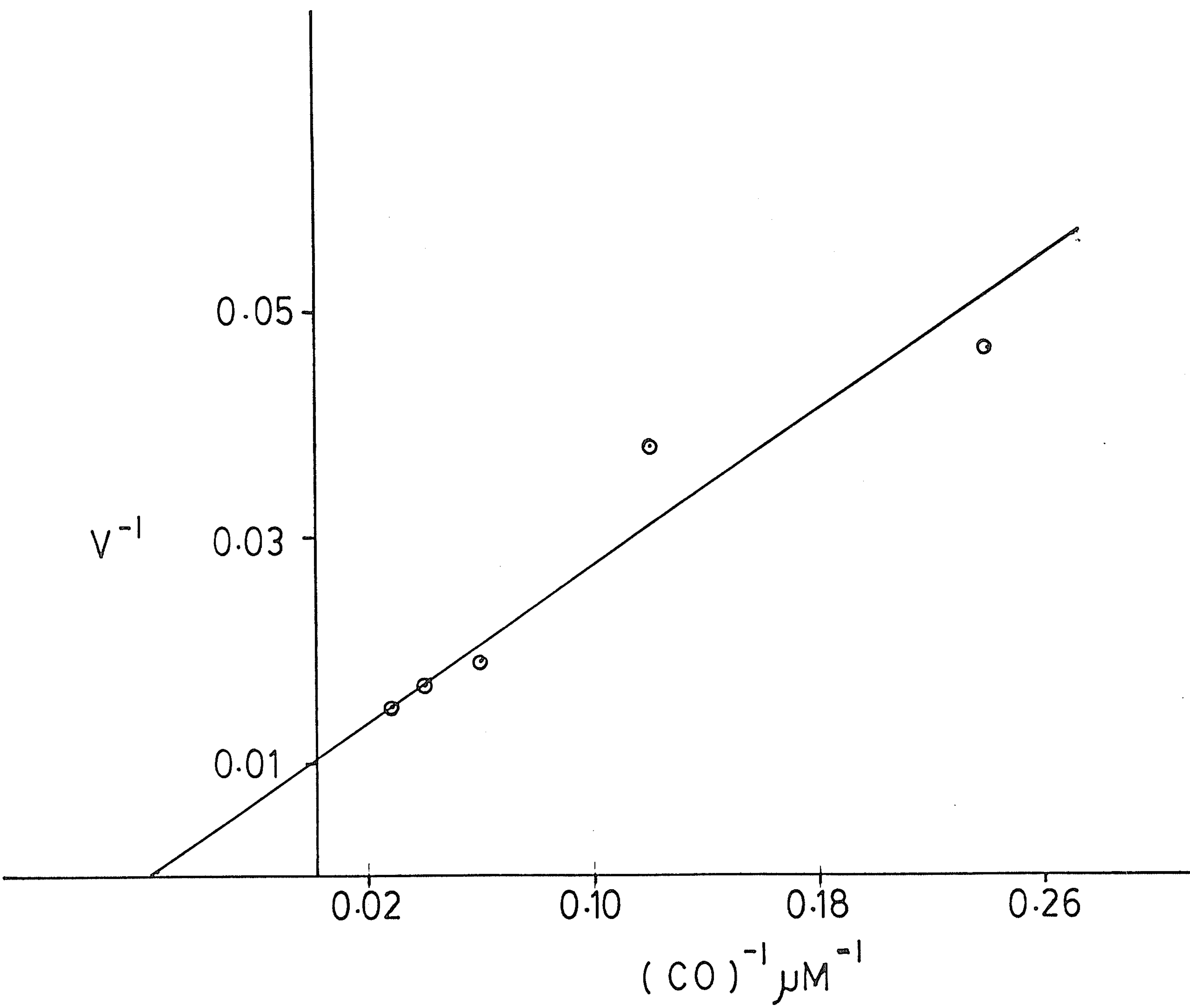

70
50
30
10
nmoles O_2/3 min
6
7
8
9
pH

Fig. 15. Effect of carbon monoxide concentration on the rate of carbon monoxide oxidation.

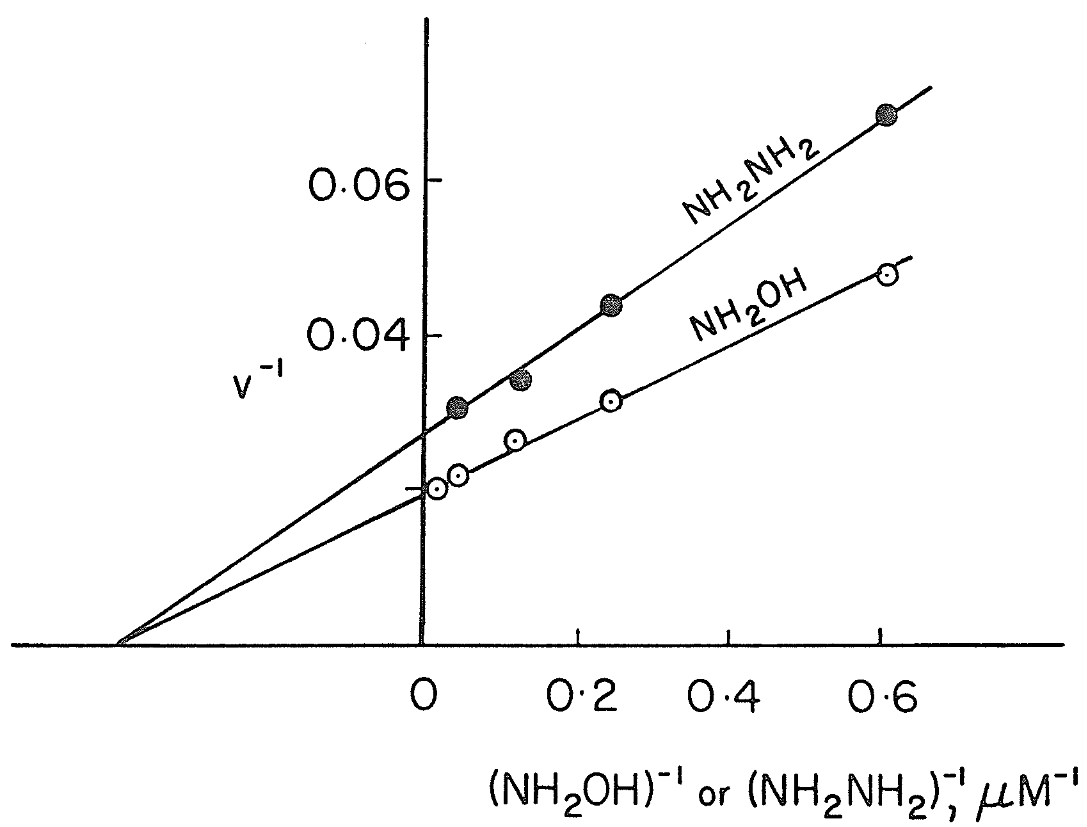
Reaction mixture contained 0.1 ml fraction 1 (*N. europaea* membrane fraction), 3.4 nmol cytochrome *c*-554, 100 nmol $NH_2 \cdot NH_2 \cdot H_2SO_4$, 0.1 M potassium phosphate buffer, and varied amount of carbon monoxide in a total volume of 1.2 mL. Reaction was started by the addition of fraction 1. Oyxgen consumption was followed in a Gilson Oxygraph as described in Materials and Methods.



V⁻¹
0·05
0·03
0·01
0·02
0·10
0·18
0·26
(CO)⁻¹ μM⁻¹

Fig. 16. Effect of hydroxylamine or hydrazine concentration on the rate of carbon monoxide oxidation.

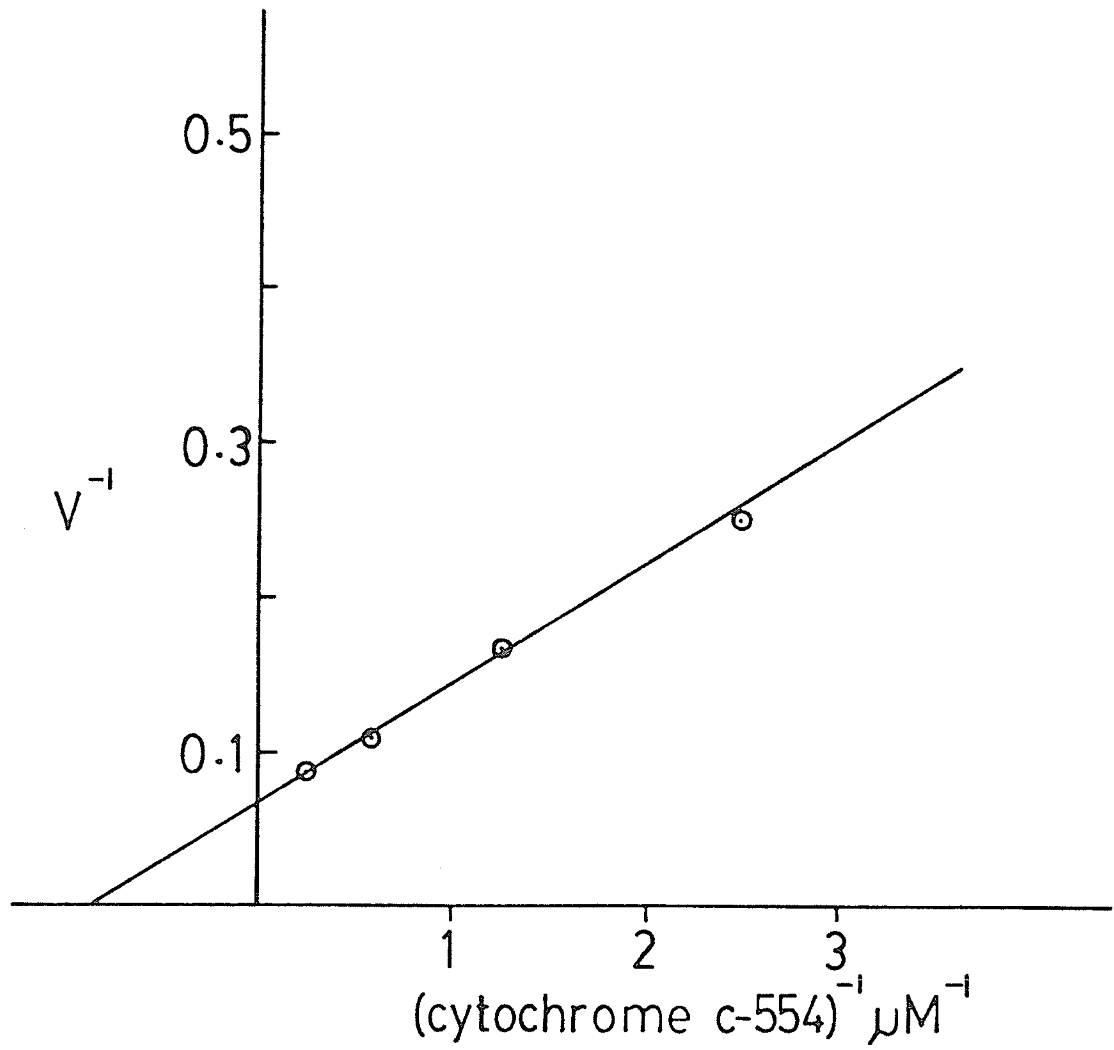
The rate of oxygen consumption (v:nmol O_2/min) was followed in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture contained 0.1 mL of fraction 1 (*N. europaea* membrane fraction), 3.4 nmoles of cytochrome *c*-554, 200 nmoles of carbon monoxide, varied and limiting concentrations of hydroxylamine or hydrazine, and 0.1 M potassium phosphate buffer (pH 7.5) in a total volume of 1.2 mL. The reaction was started by the addition of either hydroxylamine or hydrazine.



0·06
0·04
v^{-1}
NH_2NH_2
NH_2OH
0
0·2
0·4
0·6
$(NH_2OH)^{-1}$ or $(NH_2NH_2)^{-1}$, μM^{-1}

Fig. 17. Effect of the concentration of cytochrome *c*-554 on the rate of carbon monoxide oxidation.

Reaction mixture contained 0.1 ml fraction 1 (*N. europaea* membrane fraction), 200 nmol carbon monoxide, 50 nmol hydrazine, 0.1 M potassium phosphate buffer (pH 7.5), and varied amount of cytochrome *c*-554 in a total volume of 1.2 mL. Reaction was started by the addition of hydrazine. Oxygen consumption was followed in a Gilson Oxygraph as described in Materials and Methods.



0.5
0.3
0.1
V⁻¹
1
2
3
(cytochrome c-554)⁻¹ µM⁻¹

Table 1. Effect of the amount of the membrane fraction on the rate of reduction of cytochrome *c*-554 by ammonia.

Membrane (μl)	Rate of reduction (ΔA_{420}nm·3 min^{-1}) Stage 1	Stage 2
30	0.035	0.029
60	0.084	0.028
100	0.106	0.030

Note: The reaction mixture contained 3.4 nmoles cytochrome *c*-554, ammonia (50 mM), 0.1 M potassium phosphate buffer (pH 7.5), and different amount of fraction 1 (*N. europaea* membrane fraction) in a total volume of 1.0 mL. Absorption change at 420 nm was followed as described in Materials and Methods. Stage 1 lasted for about 30 to 45 seconds and was followed by stage 2.

Table 2. Oxidation of hydroxylamine or hydrazine by *N. europaea* membrane plus cytochrome *c*-554 in the presence of carbon monoxide.

System	Hydroxylamine (nmol)	Hydrazine (nmol)	Carbon monoxide (nmol)	Activity (nmol O_2 $\cdot min^{-1}$)	Total O_2 (nmol)
Complete	50	-	-	3	-
Complete + *c*-552	50	-	-	16	50
Complete	50	-	200	25	78
Complete + *c*-552	50	-	200	25	54
Complete	-	50	-	3	-
Complete + *c*-552	-	50	-	15	60
Complete	-	50	200	34	108
Complete + *c*-552	-	50	200	25	88

Note: The complete system contained 0.1 mL of fraction 1 (*N. europaea* membrane fraction), 3.4 nmol cytochrome *c*-554 and substrates as indicated in a total volume of 1.2 mL in 0.1 M potassium phosphate buffer (pH 7.5). The amount of cytochrome *c*-552, when present, was 4.9 nmol.

Table 3. Effect of the amount of fraction 1 on the rate of oxidation of carbon monoxide.

fraction 1 (µl)	Activity (nmol $O_2 \cdot min^{-1}$)
20	8.0
50	19.6
100	34.0

Reaction mixture contained 3.4 nmoles of cytochrome *c*-554, 200 nmoles of carbon monoxide, 50 nmoles of hydrazine, 0.1 M potassium phosphate buffer (pH 7.5), and varying amount of fraction 1 (*N. europaea* membrane fraction) in a total volume of 1.2 mL. Reaction was started by the addition of fraction 1. The rate of oxygen consumption was followed as described in Materials and Methods.

Table 4. Effect of carbon monoxide on hydrazine oxidation.

Carbon monoxide (nmol)	Activity (nmol $O_2 \cdot min^{-1}$)	Total O_2 (nmol)
0	2.7	-
10	5.2	13
20	10.7	21
30	12.0	29
50	13.3	48

Note: The reaction mixture contained 0.1 mL of fraction 1, 3.4 nmol cytochrome *c*-554, 100 nmol hydrazine and carbon monoxide as indicated in a total volume of 12. mL in 0.1 M potassium phosphate (pH 7.5).

Table 5. Effect of $Ni(CN)_4^{=}$ on the reduction of cytochrome c-552.

NH_2OH (nmoles)	$Ni(CN)_4^{=}$ (mM)	Activity ($\Delta A_{416} \cdot 3\ min^{-1}$)
2		0.152
2	1	0.255
500		0.024
500	1	0.21

Reaction mixture contained 19 nmoles cytochrome *c*-552, 0.256 nmoles cytochrome *c*-554, 0.04 nmoles hydroxylamine cytochrome *c* reductase, and 0.1 M potassium phosphate buffer, pH 7.5, in a total volume of 1.0 mL.

DISCUSSION

DISCUSSION

The results presented demonstrate further the important role played by the cytochrome *c*-554 of *Nitrosomonas europaea* isolated by Yamanaka and Shinra (1974) in the oxidation of ammonia. In the presence of the *Nitrosomonas* membrane fraction, the cytochrome *c*-554 reconstitutes the ammonia-oxidizing activity (Suzuki & Kwok, 1981) and is partially reduced by ammonia, hydroxylamine or hydrazine (Fig. 2). The membrane fraction contains, in addition to ammonia hydroxylase, a cytochrome oxidase (cytochrome a_1, Erickson et al, 1972) and a hydroxyl-lamine cytochrome *c* reductase activity (measured with horse heart cyto-chrome *c*) stimulated by cytochrome *c*-554 as reported previously (Suzuki & Kwok, 1981). It is now clear that the membrane fraction reduces the cytochrome *c*-554 with hydroxylamine or hydrazine very effectively.

Yamanaka and Shinra (1974) suggested that cytochrome *c*-554 may contain two molecules of heme *c* in the molecule, with absorption maxima at 421 nm and 430 nm. The suggestion is supported by the observation that there are differences in the pattern of reduction (Fig. 3) and oxidation (Fig. 6) of the two peaks by ammonia. The interesting observ-ation that the 430 nm heme stays partially oxidized while the 420 nm heme is fully re-reduced after the addition of ammonia (Fig. 6) suggests that if cytochrome *c*-554 is indeed the electron donor for the hypothetical ammonia hydroxylase (as discussed later), the 430 nm heme is very likely the heme involved directly in the donation of electrons.

The observation that reduction of cytochrome *c*-554 by ammonia occurs only under aerobic condition suggests that hydroxylamine, the product of the ammonia hydroxylation reaction, is responsible for the

reduction of the cytochrome. Endogenous substrate which can reduce cytochrome *c*-554 is probably used to initiate the hydroxylation reaction. The effect of additions of the various cytochromes on the lag period of both the reduction of cytochrome *c*-554 and oxygen uptake agrees with the idea that electrons that can be used for the hydroxylation of ammonia are drained through the cytochrome *c* oxidase.

The cytochrome *c*-554 partially reduced by hydroxylamine or hydrazine is instantly oxidized upon the addition of ammonia or carbon monoxide, the extent of oxidation depending on the concentration of ammonia or carbon monoxide (Figs. 5 and 8). The results are consistent with the interpretation that the addition of ammonia or carbon monoxide shifts the steady state equilibrium between the reduction and oxidation of cytochrome *c*-554. The oxidation of ammonia or carbon monoxide [NH_3 or CO + O_2 + 2 cyt. *c*-554 (Fe^{2+}) $\rightarrow$ NH_2OH or CO_2 + H_2O + 2 cyt. *c*-554 (Fe^{3+})] now competes with the reduction of cytochrome *c*-554 by hydroxylamine or hydrazine still present in the reaction mixtures. In the case of ammonia (Fig. 5) the initial rapid oxidation of cytochrome is followed by a slow reduction due to hydroxylamine produced from ammonia, while with carbon monoxide (Fig. 8) it is followed by a further oxidation leading to a complete oxidation of the cytochrome within a shorter period of time than in the control experiment without carbon monoxide, presumably due to the faster exhaustion of hydroxylamine.

Ammonia oxidation by cell-free extract of *Nitrosomonas europaea* was competitively inhibited by methane, carbon monoxide or methanol (Suzuki *et al*, 1976). It was suggested that the inhibition was due to the competition of methane or carbon monoxide for the ammonia

binding site of Nitrosomonas. The exact mechanism of the inhibition was not clear. The ammonia-oxidizing activity was consistently reconstituted by adding Nitrosomonas cytochrome c-554 to the membrane fraction. Using the reconstituted system, it is found that carbon monoxide competes with ammonia in the oxidation of cytochrome c-554 reduced by hydroxylamine or hydrazine (Fig. 7). At low ammonia concentration (1 mM), carbon monoxide (20 μM) inhibits the re-reduction of cytochrome c-554 by ammonia. Such an inhibition disappears at a high ammonia concentration (50 mM). If cytochrome c-554 is the electron donor for the ammonia hydroxylase, the mechanism of the inhibition of ammonia oxidation by carbon monoxide can be understood.

The effect of acetylene (Figs. 10 and 11) supports the result with intact cells of Nitrosomonas where the oxidation of ammonia but not of hydroxylamine was inhibited by acetylene (Hynes and Knowles, 1982). Acetylene; unlike carbon monoxide, methane, methanol, and ethylene; is not a competitive inhibitor with respect to ammonia (Suzuki et al, 1976; Suzuki et al, 1981; Hynes and Knowles, 1982) and is not a substrate for ammonia hydroxylase, but inhibits the hydroxylation of ammonia (Fig. 10) or carbon monoxide (Fig. 11).

While carbon monoxide alone was not oxidized by Nitrosomonas cells or extracts (Suzuki et al, 1976), it is found to be oxidized by the Nitrosomonas reconstituted system in the presence of hydroxylamine or hydrazine. The results in Fig. 12 and Tables 2 and 4 establish that the Nitrosomonas membrane fraction in the presence of the cytochrome c-554 oxidizes carbon monoxide with hydroxylamine or hydrazine as electron donor and the amount of oxygen consumed agrees with the hydroxylation of carbon monoxide. NADH cannot replace hydroxylamine

or hydrazine as electron donor. The rate of oxygen consumption in this system was considered as a measure of ammonia hydroxylase activity. The apparent Km values for CO (16 μM), NH_2OH (2-3 μM), NH_2NH_2 (2-3 μM) and cytochrome *c*-554 (1.2 μM) compared favourably with the K_i value for CO (3 μM) in the inhibition of ammonia oxidation (Suzuki *et al*, 1976) and the Km values for NH_2OH (3.6 μM) and NH_2NH_2 (4 μM) in hydroxylamine cytochrome *c* reductase (Hooper & Nason 1965) and for cytochrome *c*-554 (3.3 μM) in the reconstitution of ammonia-oxidizing activity (Suzuki & Kwok, 1981). Hydroxylamine and hydrazine oxidation by Nitrosomonas cells or extracts is also stimulated by carbon monoxide (results not shown).

Similarities and differences between the hydroxylation of methane by methylotrophs and the hydroxylation of ammonia by *N. europaea* were discussed on the basis of the inhibition by different substrates and certain chemical compounds (Wilkinson, 1975; Ferenci *et al*, 1975; Suzuki, 1975; Suzuki *et al*, 1981). Carbon monoxide, a competitive inhibitor of methane oxidation by *Methylomonas methanica* (Ferenci, 1974), was stoichiometrically oxidized by a particulate membrane fraction of the organism in the presence of NADH as an electron donor according to the equation: $CO + O_2 + NADH + H^+ \rightarrow CO_2 + NAD^+ + H_2O$. Evidence that the same monooxygenase was used for both oxidation of carbon monoxide and methane was discussed (Ferenci *et al*, 1975). Carbon monoxide was also a competitive inhibitor of ammonia oxidation in *Nitrosomonas*. As demonstrated, it is stoichiometrically oxidised in the presence of hydroxylamine or hydrazine as electron donors according to the equations:

$$NH_2OH + CO + 1\frac{1}{2}O_2 \rightarrow HNO_2 + CO_2 + H_2O$$

$$NH_2NH_2 + 2CO + 2O_2 \rightarrow N_2 + 2CO_2 + 2H_2O \ .$$

Both hydroxylamine and hydrazine were oxidized by *Nitrosomonas* hydroxylamine cytochrome *c* reductase (Hooper & Nason 1965). Hydroxylamine, a stable intermediate of ammonia oxidation (Lees, 1952), is probably the physiological electron donor. Although exogenous hydroxylamine is not required for the oxidation of ammonia, there seems to be an obligatory coupling between ammonia hydroxylation and hydroxylamine oxidation in *Nitrosomonas* (Suzuki *et al*, 1981).

The oxidation of carbon monoxide in the presence of hydroxylamine or hydrazine appeared to be catalysed by a combined action of ammonia hydroxylase and hydroxylamine cytochrome *c* reductase that are present in the membrane fraction of *N. europaea* with cytochrome *c*-554 as the electron carrier. In the absence of a purified preparation of the hydroxylase one cannot state unequivocally that carbon monoxide is oxidised by ammonia hydroxylase, but much of the evidence points to this being so. Both oxidations used hydroxylamine as electron donor and required molecular oxygen and cytochrome *c*-554. Both ammonia and carbon monoxide oxidized hydroxylamine-reduced cytochrome *c*-554, a critical component in the reconstituted system.

The apparent Km values for CO (16 μM), NH_2OH (2-3 μM), NH_2NH_2 (2-3 μM) and cytochrome *c*-554 (1.2 μM) compared favourably with the K_i value for CO (3 μM) in the inhibition of ammonia oxidation (Suzuki *et al*, 1976) and the Km values for NH_2OH (3.6 μM) and NH_2NH_2 (4 μM) in hyroxylamine cytochrome *c* reductase (Hooper & Nason, 1965) and for cytochrome *c*-554 (3.3 μM) in the reconstitution of ammonia-oxidizing activity (Suzuki & Kwok, 1981). Carbon monoxide was a strong inhibitor

of ammonia oxidation. Also the two activities had the same optimal pH.

These results further confirm the concept that the initial reaction of ammonia oxidation is very similar to that of methane oxidation (Wilkinson, 1975; Ferenci et al, 1975; O'Neill and Wilkinson, 1977; Dalton, 1977; Drozd, 1980; Suzuki et al, 1976). Carbon monoxide acts as an alternate substrate both for methane hydroxylase (Ferenci et al, 1975) and for ammonia hydroxylase. The hydroxylation of ammonia to hydroxylamine by Nitrosomonas cells has now been established using $^{15}NH_4Cl$ and $^{18}O_2$ or $H_2{}^{18}O$ (Dua et al, 1979; Hollocher et al, 1981). The availability of a simple essay system is essential in the resolution and purification of ammonia hydroxylase from the membrane system. The interaction of the hydroxylase with carbon monoxide offers advantages over ammonia in studies of the ammonia hydroxylase system. The oxidation of carbon monoxide is a one-step oxidation with carbon dioxide as product, which can be sensitively and continuously assayed using a carbon dioxide electrode (Ferenci et al, 1975). Also, a substrate such as carbon monoxide cannot generate any reductant through further oxidation which might complicate the assay system. The use of carbon monoxide as an alternate substrate and hydroxylamine or hydrazine as electron donor to reduce cytochrome c-554 represents a promising assay system for ammonia hydroxylase.

Experiments so far indicated that the hydroxylase enzyme is tightly bound to the membrane fraction and is sensitive to metal chelators and detergents. Metal chelators like 2,2'-bipyridyl, diethyldithiocarbamate and potassium cyanide inhibited the hydroxylation reaction, an observation which agrees with earlier reports that metal ion such as copper is involved in the reaction (Lees, 1952; Hooper & Terry, 1973).

Detergents such as triton X-100, sodium cholate, and sodium dodecyl sulfate inhibit the reaction to a different extent. The high sensitivity of the activity to Triton X-100, a non-ionic detergent which binds only to hydrophobic proteins and generally does not cause denaturation or loss of biological activity (Schnaitman, C.A., 1981), seems to indicate that the hydroxylase enzyme is hydrophobic in nature. The solubilization and inhibition of the activity by SDS which binds strongly to proteins is probably a result of the unfolding and irreversible denaturation of the proteins.

Although peroxonitrite is unstable in neutral aqueous solution, it is still likely to be involved in ammonia oxidation in N. europaea. Besides the arguments put forward by Suzuki et al (1981), it is found that ammonia accelerated the decomposition of peroxonitrite in alkaline aqueous solution in the presence of Cu^{2+}, suggesting the possible interaction of ammonia with peroxonitrite. It is of interest to study the stability and reactivity of peroxonitrite under hydrophobic conditions. As discussed earlier, ammonia hydroxylase is likely a hydrophobic enzyme. It is possible that peroxonitrite interacts with ammonia more effectively under such hydrophobic conditions than in an aqueous environment.

The effect of $Ni(CN)_4^{=}$ on the reduction of cytochrome c-552 is interesting. The observation that the complex ion which traps nitroxyl can release the inhibition caused by addition of excess hydroxylamine seems to suggest that nitroxyl, a possible intermediate of hydroxylamine oxidation, is formed and can interfere in the reduction of cytochrome c-552 if allowed to accumulate.

The work presented supports the hypothetical scheme of ammonia

oxidation in *N. europaea* proposed by Suzuki *et al* (1981) as discussed in the historical section. The requirement of reduced cytochrome *c*-554 and molecular oxygen for the hydroxylation of ammonia or carbon monoxide is demonstrated. Cytochrome *c*-554 can be reduced effectively by either hydroxylamine or hydrazine or endogenous substrate. NADH stimulates the reduction of the cytochrome in the presence of ammonia. Cytochrome *c*-552, if present, drains electrons to the oxidase, causing a longer lag in ammonia oxidation. The involvement of nitroxyl is supported by the effect of nickel cyanide complex on the reduction of cytochrome *c*-552.

The model of ammonia oxidation proposed (Suzuki *et al*, 1981) is very attractive, but more work is needed to elucidate the mechanism of ammonia oxidation. The purification of the ammonia hydroxylase and the study of the mechanism of ammonia interaction with peroxonitrite are the important aspects of the work that remain to be done.

CONCLUSION

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The work presented showed that *Nitrosomonas* cytochrome *c*-554 could be partially reduced by ammonia, hydroxylamine, or hydrazine in the presence of the *Nitrosomonas* membrane fraction. The reduced cytochrome *c*-554 was oxidized by either ammonia or carbon monoxide, suggesting the role of cytochrome *c*-554 as the electron donor for the hydroxylation of ammonia by the hypothetical ammonia hydroxylase. The use of carbon monoxide as an alternate substrate represented a promising assay system for the ammonia hydroxylase.

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