CELL-FREE AMMONIA OXIDATION BY *NITROSOMONAS EUROPAEA* EXTRACTS

by

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To my beloved late father,

Kwok Yee

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The author expresses his sincere gratitude to Dr. Isamu Suzuki for his continuing guidance and advice throughout the course of this investigation, and to Dr. H. Lees for his concern and interest.

Gratitude is also expressed to my wife, Amy, for her encouragement, and to Mrs. Barbara Cleugh for the typing of this thesis.

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ABSTRACT

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Cell-free extracts of Nitrosomonas europaea catalyzed the aerobic oxidation of ammonia to nitrite in the presence of serum albumin, Mg^{2+} or polyamines such as spermine.

Some of the general properties of the cell-free ammonia oxidizing system were studied. The activity was optimal around pH 7.7 and 25°C. The system was activated by a phosphate buffer. The K_m value for ammonia was 0.4 mM.

The presence of hydroxylamine or NADH caused inactive extracts to regain their ability to oxidize ammonia.

Spectrophotometric as well as stopped-flow studies of the cytochrome system during the oxidation of ammonia and hydroxylamine led to the formulation of a tentative scheme for the mechanism of ammonia oxidation.

A partial resolution of the system into a membrane fraction and soluble fractions was achieved by centrifugation and Sephadex or Sepharose chromatography.

The results of the present study as well as those reported previously were discussed in relation to the proposed scheme for ammonia oxidation.

INTRODUCTION

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Nitrosomonas europaea, though a tiny microorganism, has a great economic and ecological contribution to soil fertility and nitrogen cycle in nature. In soil, the transformation of ammonia to nitrate provides the form of nitrogen best available to plants.

The oxidation of ammonia to nitrite by Nitrosomonas is the starting point of the steps that bring about the conversion of ammonia to nitrate.

Since it is axiomatic that green plants support all other forms of life, the oxidation of ammonia is therefore of the greatest importance to animal as well as man.

The study of this organism has been largely biochemical as the bacterium requires only a few inorganic chemicals to effect a total synthesis of all the compounds that go to make up a living cell. Unfortunately, the answers to many of these novel biochemical problems presented by the organism still remain largely incomplete in spite of intensive effort by dedicated workers.

Difficulty in the study on the mechanism of ammonia oxidation was due mainly to the lack of cell-free system capable of carrying out this interesting reaction. We have been able to obtain such a system successfully and reproducibly.

It is the purpose of this thesis to present conditions necessary for obtaining a cell-free ammonia-oxidizing system, properties of such a system and some preliminary studies on the mechanism of ammonia oxidation using the cell-free system.

HISTORICAL

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This section will be brief and deal only with the literature pertinent to the present study of Nitrosomonas europaea. The early work on the fundamental principles of the biochemistry and physiology of this organism was written in a book by Lees (1955).

For a complete review of the work on nitrifying bacteria the reader should consult Lees (1960, 1962), Peck (1968), Wallace and Nicholas (1969) and Aleem (1970).

Nature of Organism

Nitrosomonas europaea is a bacterium which has its natural habitat in soil and sewage. The organism is chemoautotrophic since it derives all its energy for growth and cell synthesis by oxidizing inorganic nitrogen ammonia to nitrite and obtains its carbon source from carbon dioxide.

It is also a nitrifying bacterium because its metabolic activity constitutes the first part of the well known process called nitrification; i.e., the conversion of ammonia to nitrite and from nitrite to nitrate.

Although there are certain heterotrophic bacteria according to Fisher, et al., (1956) capable of oxidizing ammonia to nitrite, there is no evidence so far that these organisms can derive energy from such a process, and the rates of oxidation of ammonia are much lower.

A marine form of Nitrosomonas known as Nitrosocystis oceanus had been described by Watson (1965), and appears to be the counterpart of Nitrosomonas in the sea. Research information, however, has been largely confined to the studies done on Nitrosomonas. The use of a generic name will indicate its species.

Discovery of Organism

In the scientific world of eighteenth century the oxidation of ammonia to nitrate which had long been known to occur in soil was looked upon as a purely chemical process.

Pasteur (1862) suggested that the process known as nitrification might be the work of living microorganisms. Unfortunately, he himself did not follow up the subject. Fifteen years later Schloesing and Munty (1877) in a few simple experiments confirmed Pasteur's suggestion of biological origin. Warington (1891) discovered that the process of nitrification occurred in two stages - the oxidation of ammonia to nitrite and of nitrite to nitrate. Subsequently, Winogradsky (1891) using a medium without an organic source isolated the organisms in pure cultures, and named Nitrosomonas coverting ammonia to nitrite and Nitrobacter oxidizing nitrite to nitrate.

The discovery that these organisms can live, grow and reproduce indefinitely in an environment devoid of organic matter led to the concept of chemoautotrophy in microbiology.

Morphology

Winogradsky (1891) described that Nitrosomonas cells were small, Gram-negative, oval in shape and were motile by long, polar flagellum; thus morphologically, Nitrosomonas is a typical eubacterium according to Bisset (1950, 1952).

Ammonia Oxidation

Little was known about intermediary metabolism of N. europaea because of the difficulties encountered in growing and producing sufficient cellular material for study. The mean generation time of this chemoautotroph is between 10 and 12 hours compared to that of 20 minutes for Escherichia coli. The molar growth yield is also disappointing, giving 1.0 - 1.2 g wet weight of cells from 15 liters of medium after 4 days of growth. According to free energy calculation by Baas-Becking, et al., (1927) the growth was only 5 to 10% efficient. During growth of the organism there is a constant need for pH adjustment. It is over the last 18 years or so that biochemical studies have been carried out on this bacterium.

The studies by Lees (1954) and his co-workers; Anderson (1959) and Hofman (1952), during these years have been largely responsible for some understanding of biochemistry and mechanisms in the primary oxidation reaction of this nitrifying organism.

The primary oxidation step by this organism is:

$$NH_4^+ + 1\tfrac{1}{2}\, O_2 \longrightarrow NO_2^- + H_2O + 2H^+$$

The valence change of the nitrogen atom from -3 to +3 in the course of the oxidation involves a net change of 6 electrons. Since biological oxidations proceed by the removal of two electrons at a time, Kluyver and Donker (1926) proposed that the oxidation of ammonia took place in three steps, each consisting of two-electron change. The first experimental evidence showing hydroxylamine as a first step in the oxidation of ammonia was demonstrated by Hofman and Lees (1953). These investigators reported that in the presence of 3×10^{-3} M hydrazine whole cells of Nitrosomonas accumulated hydroxylamine during oxidation of ammonia. This observation coupled with the fact that 10^{-5} M thiourea or allythiourea inhibited the oxidation of ammonia but not of hydroxylamine by Nitrosomonas constituted strong evidence that hydroxylamine was, indeed, an intermediate in the primary oxidation step.

The next intermediate which must exist remains unidentified despite numerous efforts by various research workers. However, there is a number of hypotheses available in literature. All are likely possibilities, but none are substantiated by experimental evidence.

Hyponitrite was postulated by Kluyver and Donker (1926), whereas Lees (1960) suggested nitroxyl-type compound (NOH) as the intermediate. Aleem, et al., (1962) proposed nitrohydroxylamine.

Up to now, the oxidation of ammonia to nitrite can be written in the following way:

$$NH_4^+ \longrightarrow NH_2OH \longrightarrow (X) \longrightarrow NO_2^-$$

where (X) represents the unknown intermediate.

Cell-Free System

The first attempts to obtain cell-free enzyme preparations from Nitrosomonas were made by Imshenetskii and Ruban in a series of papers (1954, 1956, 1957). They reported that autolysates of Nitrosomonas prepared by shaking intact cells with glass powder for twenty-four hours at 40°C, oxidized ammonia and hydroxylamine slowly over a period of 5 days. Unfortunately, no precautions were indicated to eliminate the possibility of whole cells in the autolysates or contamination by other organisms during this long incubation period.

Engel and Alexander (1959) prepared cell-free extracts by sonic oscillation in a Raytheon 10 kc magnetorestrictive oscillator for 15 minutes. They observed a slow disappearance of either ammonia or hydroxylamine for 72 hours in the extracts. Again, the possibility of residual intact cells could not be overlooked.

The first satisfactory report of cell-free extracts of Nitrosomonas was that of Nicholas and Jones (1960). They found that cell-free extracts prepared with an ultrasonic probe oxidized hydroxylamine to nitrite in the presence of added mammalian cytochrome c. The nitrite formed from hydroxylamine in these extracts was only 40-70% of the theoretical value expected from oxygen uptake. Such sonic extracts, however, never oxidized ammonia.

Aleem and Lees (1962) showed the reduction of Nitrosomonas cytochromes by hydroxylamine in sonicated extracts. Anderson (1963) performed experiments with sonicated cell-free extracts and obtained the following results; a) under anaerobic conditions, such extracts decolorized methlene blue in the presence of hydroxylamine with negligible amounts of nitrite formed. Nitric oxide (NO) and nitrous

oxide (N_2O) were produced in amounts equivalent to hydroxylamine disappearance b) under aerobic conditions, methylene blue or mammalian cytochrome c increased nitrite formation from hydroxylamine in the presence of extracts, but nitrite produced did not correspond to oxygen uptake. Hydroxylamine-cytochrome c reductase was purified by Hooper and Nason (1965) from sonicated extracts.

All attempts to find oxidation of ammonia by cell-free extracts of Nitrosomonas were unsuccessful until recently. Suzuki and Kwok (1970) succeeded for the first time in preparing a cell-free system capable of oxidizing ammonia to nitrite by passage of a Nitrosomonas cell suspension through a French Pressure Cell. Simultaneously Watson, et al., (1970) reported a successful preparation of cell-free system from Nitrosocystis oceanus by a similar procedure.

MATERIALS AND METHODS

MATERIALS AND METHODS

Organism and Growth of Organism

A Schmidt Strain of Nitrosomonas europaea was kindly provided by Dr. A.B. Hooper, University of Minnesota. The organism was cultivated in A.T.C.C. medium No. 221 which had the following composition: 3.0 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.004 g $CaCl_2.2H_2O$, 0.1 mg chelated iron, 0.05 g cresol red, and distilled water to make the volume to one liter.

Solutions of $MgSO_4.7H_2O$, $CaCl_2.2H_2O$ and chelated iron were autoclaved separately. These components were then added to the rest of the autoclaved medium after cooling. Chelated iron was prepared by mixing solutions of 0.29 mg $FeCl_3$/10 ml water with 0.6 mg EDTA/10 ml water.

Batch cultures of 15 liters medium were grown in 5 gallon glass carboys fitted with spargers at 28°C. The air was forced through sterilized cotton wool plugs, then through spargers. A batch culture was initiated by inoculating the autoclaved culture medium about 5% of its volume with 4 day old cultures grown in 250 ml flasks (100 ml medium) at 28°C on a rotary shaker at 120 r.p.m.

Cresol red was used as internal pH indicator. During growth of the organism the pH of the medium was maintained constant (pH 8.0) by titrating frequently with 50% (W/V) potassium carbonate.

After four days of growth at which 75% ammonia nitrogen was used up the cells were harvested by Sharples centrifugation at 50,000 r.p.m. The collected pink cells (1 g wet weight per carboy) 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/ml). The cell suspension so obtained was stored at $4^{\circ}C$ and was used within three days.

Preparation of Cell-free Extracts

Cells were disrupted by passing a fresh cell suspension (20 mg wet cells/ml) through a French Pressure Cell at 18,000 p.s.i. The resulting extract was centrifuged at 2,000 x g for 20 minutes to remove whole cells. The cell-free supernatant thus obtained was used as the source of the crude enzyme and for subsequent fractionation by centrifugation and column chromatography. The extract oxidized ammonia only after incubation with various activators such as serum albumin, Mg^{2+} or spermine as described in a later section. In some experiments the cell suspension contained 20 mg bovine serum albumin (Fraction V, Sigma Chemical Co.) per ml before disruption in order to obtain extracts capable of actively oxidizing ammonia, but in most experiments the activation process was carried out after the preparation of extracts.

Partial Resolution of Ammonia-Oxidizing System

1. Centrifugations

Low speed centrifugation (20,000 x g for 30 minutes) was carried out in a Sorvall RC-2 centrifuge at 4°C.

High speed centrifugation (100,000 x g for one hour) was carried out in a Spinco Model L Ultracentrifuge at 4°C.

2. Anaerobic Columns

A column of Sephadex (1 x 25 cm) was prepared with the G-100 Sephadex after swelling in a large volume of water on boiling water bath for 5 hours. When the column had been fully packed the top was sealed with a serum bottle cap which was pierced with a syringe needle connected with a short rubber tubing to a bottle of 0.1 M potassium phosphate buffer (pH 7.5). The solution of buffer had been continuously gassed with nitrogen through a sparger. After the column was equilibrated with this air-free buffer a sample of 2 ml crude extract was applied to the top of the column by injecting with a syringe. The void volume of the column was determined from the elution volume of Blue Dextran 2000. After the passage of buffer equivalent to the void volume three milliliter fractions were collected in small test tubes (0.8 x 7.5 cm). The whole procedure was carried out at room temperature.

The anaerobic procedure for Sepharose 6B (1.5 x11.5 cm) was performed in the same way.

Assay of Ammonia-Oxidizing Activity

The oxidation of ammonia was routinely followed by oxygen uptake in a Gilson Oxygraph (Clark Oxygen Electrode) and was confirmed by the colorimetric determination of nitrite formed. The reaction mixture, in a total volume of 1.5 ml, contained 0.5 ml extract and 1 ml of 0.1 M potassium phosphate buffer (pH 7.5). The addition of ammonia and other reagents was made in microliter quantities with Hamilton microliter syringes. All experiments were carried out at 25°C. When the extract was prepared in the absence of albumin the activation was achieved by the addition of spermine (2 mM) in the Oxygraph vessel unless otherwise indicated. The activity was expressed in μmole O_2 consumed per minute.

Aminco Dual Wavelength Spectrophotometry

Oxygen consumption and cytochrome reduction were recorded simultaneously in an Aminco-Chance Dual Wavelength Spectrophotometer with a vibrating platinum electrode attachment. After the balancing of the instrument at the desired wavelengths (554-545 mμ or 603-580 mμ) the substrate was injected with a microsyringe and the change in transmission and the consumption of oxygen were recorded in strip chart recorders.

Aminco Stopped-Flow Techniques

Kinetic measurements of cytochrome c reduction in extracts were performed in an Aminco-Chance Dual Wavelength Spectrophotometer with an Aminco-Morrow Stopped-flow attachment. The reactions were recorded in an attached Durrum storage oscilloscope with a camera.

The instrument was first balanced optically and electronically at the two wavelengths (λ_1= 554 mμ, λ_2= 545 mμ) with a mixture of buffer and extract present in the light path. The reaction was initiated by mixing a cell-free extract with a solution of substrate. The accuracy of the instrument was confirmed by observing the reaction between cytochrome c and excess ascorbate. The change in transmission was converted to absorbance.

Determination of Nitrite

Nitrite was determined by the method of Bratton et al (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 color development. Aliquots of sample were then brought to a volume of 10 ml with distilled water and the color 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 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. Stored at $4^{o}C$. (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.

Procedure:

To 1 ml sample were 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 color developed was measured in a Klett Summerson colorimeter with a No. 62 filter.

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

Preparation of Nitrohydroxylamine

Nitrohydroxylamine (sodium salt) was prepared according to the method of Angeli (1896). To a solution, rather concentrated, made by adding three equivalents of gram atomic weight of sodium to absolute alcohol was added a solution of hot alcohol saturated with one mole of hydroxylamine hydrochloride. The sodium chloride which formed immediately was separated by means of vacuum filtration. To the clear liquid obtained was added one mole of ethyl nitrate. It was necessary to cool the reacting liquid in cold water. Almost immediately a white precipitate separated and with time the amount increased. After about one hour it was collected on a filter, washed in absolute alcohol and ether, and was placed to dry in a vacuum over sulfuric acid. The mixture obtained in this way was a white powder very soluble in water.

Preparation of Ferrocytochrome c

Reduced cytochrome c was prepared according to Wharton and Tzagoloff (1967). A slight modification of the method was necessary due to the unavailability of potassium ascorbate. One percent solution of cytochrome c (Sigma Chemical Co., Type III, Horse Heart) was dissolved in 0.01 M potassium phosphate buffer containing an excess amount of ascorbic acid. The pH of

solution was adjusted with potassium hydroxide to 7.0. Excess ascorbate was removed by dialysis in size 8 Visking dialysis tubing against 0.01 M phosphate buffer (pH 7.0) overnight with 3 changes of buffer.

RESULTS

RESULTS

Activation of the Ammonia-oxidizing System by Albumin, Mg^{2+} and polyamines

Extracts prepared in the presence of bovine serum albumin (20 mg/ml) oxidized ammonia rapidly with a rate equivalent to 10-20% of that in whole cell system (Fig. 1). The extracts prepared in its absence were inactive.

Dipyridyl at 0.3 mM inhibited the oxidation of ammonia in both the cell-free and whole cell systems. Inhibition by allylthiourea at 10^{-5} M was also observed with an initial lag period of one minute. Dipyridyl and allylthiourea did not inhibit the oxidation when hydroxylamine was used as substrate (Fig. 1).

As shown in Fig. 2 inactive extracts prepared in the absence of albumin became active in the presence of added serum albumin or Mg^{2+}. It was found also that spermine replaced Mg^{2+} or albumin effectively in the activation of inactive extracts. Spermidine was less effective requiring 10 mM for the effect of 0.2 mM spermine. Poly-L-lysine (MW = 140,000) had the same effect as 0.3 mM spermine at a concentration of 100 µg/ml.

Egg albumin, casein and lysozyme did not replace bovine serum albumin. Neither did Ca^{2+} nor Mn^{2+} replace Mg^{2+}. Glycerol or sucrose (20%) and mercaptoethanol or dithiothreitol were not effective in the activation.

Active cell-free extracts were reasonably stable over a period of an hour and half either at 4°C or at room temperature as long as they were stored in small test tubes in order to minimize the exposure to air. However, they lost 50% of activity after four hours. Efforts were made to minimize the exposure to air all through the preparation, fractionation and storage of the extracts.

The extracts could be stored frozen at -20°C without any loss of activity for a day or two, but the storage for longer periods of time resulted in loss of activity.

The ammonia-oxidizing activity of whole cells was, on the other hand, destroyed immediately upon momentary freezing.

Stoichiometry of cell-free ammonia oxidation

The reaction mixtures from the experiments in Fig. 2 were analyzed for nitrite to study the stoichiometry of reaction. As shown in Fig. 2 the amount of oxygen consumed due to ammonia oxidation and the amount of nitrite formed during the oxidation agreed with the following equation:

$$NH_4^+ + 1\tfrac{1}{2}\, O_2 \longrightarrow NO_2^- + H_2O + 2H^+$$

Effect of Activator Concentration

The effect of various concentrations of bovine serum albumin, Mg^{2+} and spermine is shown in Fig. 3 as double reciprocal plots according

to Lineweaver and Burk (1934). The concentration of each activator required for half-maximum activation was estimated as 2.7 mg/ml serum albumin, 5 mM Mg^{2+} and 0.75 mM spermine.

Spermine was the best activator resulting in the highest rate of ammonia oxidation. When two activators of relatively low concentrations were present the activity of extract was greater than that with either one alone. However, such additive effect was not found at saturating concentrations. The activating effect of 2 mM spermine alone was more than that of the simultaneous presence of 2 mM spermine, 10 mM $MgCl_2$ and 20 mg/ml serum albumin.

Effect of Substrate Concentration

As shown in Fig. 4 the rate of ammonia oxidation by the cell-free system increased with increasing substrate concentrations. The apparent K_m value determined from intercepts at the horizontal axis of the double-reciprocal plot was 0.4 mM ammonium sulfate.

Effect of pH on the oxidation of ammonia

An investigation of optimum hydrogen ion concentration for the oxidation of ammonia was carried out in 0.1 M potassium phosphate buffer (Fig. 5). Very little activity was observed below pH 7.0. Enzyme activity increased with increase in pH and the maximum activity occurred around pH 7.7. Further increase in alkalinity caused a sharp drop in the

enzyme activity.

Although the optimum pH was found to be 7.7 in these experiments, all the work reported in this thesis was carried out under the standard conditions described in the Materials and Methods, i.e., pH 7.5.

Effect of Temperature

The optimum temperature for ammonia oxidation was at 25°C as shown in Fig. 6. The solubility of oxygen at various temperatures was taken into account in the calculation of oxygen uptake. The actual amount of oxygen consumed in each experiment was obtained by subtracting the endogenous respiration of which the rate was measured after dipyridyl inhibition. Dipyridyl as mentioned before inhibited ammonia oxidation but it had no effect on endogenous respiration.

Effect of Hydroxylamine and NADH on the Oxidation of Ammonia

The cell-free extracts, after standing for 2 hours or longer, lost the activity to oxidize ammonia even with spermine as activator. The inactive extract regained the ability to oxidize ammonia when a small amount of hydroxylamine or NADH was added (Fig. 7, curves D and F). The amount of oxygen consumed was much more than that required for the oxidation of hydroxylamine or NADH. The actual oxidation of ammonia to nitrite by these hydroxylamine- or NADH-activated extracts was confirmed by the determination

of nitrite produced (Table 1).

In control experiments where ammonia was omitted (curves C and E), hydroxylamine was oxidized faster than NADH, but after completion of each oxidation the rate of oxygen uptake returned to the endogenous level.

In the reaction systems D and F in Table 1 the nitrite recovery due to ammonia oxidation was nearly 90% of that expected from the amount of oxygen consumed.

Hydrazine was also effective in the place of hydroxylamine or NADH whereas succinate, NADPH were less effective. Other electron donors such as GSH, ascorbate, pyruvate, lactate, sodium borohydride, nitrohydroxylamine had no effect on the oxidation.

Effect of phosphate

When the Nitrosomonas cells were ruptured in 0.1 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.5) instead of phosphate buffer, the resulting extracts did not oxidize ammonia even with the addition of hydroxylamine or NADH when tested in the HEPES buffer. Hydroxylamine was not even oxidized. Spermine, however, could activate these inactive extracts toward ammonia oxidation and there was a further activation when hydroxylamine or NADH was added resulting in an increased rate of oxidation.

When these extracts were tested in 0.1 M potassium phosphate buffer, (pH 7.5), however, the addition of NADH or hydroxylamine alone initiated the oxidation of ammonia. Phosphate could be replaced by the same concentration of sulphate.

When the cell-free extracts were prepared in 0.1 M Tris-HCl buffer (pH 7.5) and tested in the same buffer no ammonia oxidation was observed even with the addition of hydroxylamine or NADH. Neither hydroxylamine nor NADH was oxidized under those conditions. When the extracts were tested in 0.1 M phosphate buffer or in the presence of spermine, hydroxylamine or NADH was oxidized, but there was still no oxidation of ammonia.

Spectrophotometric Study of the Ammonia-oxidizing System

A differencespectrum of the active cell-free extract of Nitrosomonas europaea with and without ammonia was measured in a Shimadzu Multipurpose Recording Spectrophotometer. As shown in Fig. 8 there were three absorption peaks (525 mμ, 554 mμ, and 603 mμ) observed. An identical difference spectrum was obtained with the intact cells also. With hydroxylamine or NADH instead of ammonia the difference spectrum was not qualitatively different from that with ammonia.

From the spectrum in Fig. 8 two wavelength pairs were selected for the study of cytochrome reduction in an Aminco Dual Wavelength Spectrophotometer, 554-545 mμ and 603-580 mμ.

The extracts also reduced horse heart cytochrome c with hydroxylamine and oxidized reduced horse heart cytochrome c with oxygen indicating the presence of hydroxylamine-cytochrome c reductase and cytochrome oxidase in these extracts.

As seen in Figs.9 and 10 obtained by dual-wavelength spectroscopy, both cytochromes (554-545 mμ and 603-580 mμ) were reduced rapidly upon addition of ammonia. The oxygen consumptions also shown in these figures, however, started only after near completion of the cytochrome reduction. When all the oxygen in the system was used up there was a partial reoxidation of both cytochromes.

With hydroxylamine as substrate (not shown in figures) cytochromes were reduced more rapidly and completely and the oxygen consumption started without any lag period. The oxygen uptake corresponds to the amount of hydroxylamine added, after which cytochromes were oxidized to the original level by cytochrome oxidase. The same process was repeated after a successive addition of hydroxylamine.

Stopped-flow Spectrophotometric Study

The rate of cytochrome c (554 mμ peak) reduction was studied in an Aminco Stopped-Flow apparatus. With hydroxylamine as substrate the reduction measured (554-545 mμ) occurred in two stages with reaction half times of 70 milliseconds and 2 seconds as shown in Figs. 11a and 11b. After completion of hydroxylamine oxidation there was a slow reoxidation of cytochrome (half time of 13 seconds) because of the action of cytochrome oxidase (Fig. 11c).

When ammonia was used as substrate instead of hydroxylamine there was an initial lag period of 5-10 seconds before the reduction of cytochrome (Fig. 12). The reduction, once started, proceeded in one stage with the reaction half time of 7 seconds.

Partial Resolution of the Ammonia-oxidizing System

When the active cell-free extracts were centrifuged at either a low or a high speed as described in Materials and Methods, neither the supernatant nor the pellet alone oxidized ammonia. When both fractions were combined, however, ammonia was oxidized at 10% the rate of original extracts.

A passage of active extracts through Sephadex columns inactivated the extracts and no ammonia-oxidizing activity was observed in any eluate

fraction or combination of fractions. In an anaerobic column as described in Materials and Methods the activity was recovered indicating the sensitivity to exposure to oxygen.

In Sephadex G-100 the pink color of cytochromes moved with the front and was collected in the first fraction. There was no ammonia oxidation when any one of the fractions collected was tested alone. The ammonia-oxidizing activity was found when the fraction 1 was combined with fraction 4. The absorption spectrum of fraction 4 had a peak around 260 mμ. It was not replaced, however, by ATP, AMP, and cyclic AMP and not removed by treatment with charcoal. The active component in the fraction 4 was removed by the treatment with Dowex 50 W-X8 (H^+-form), a cationic ion-exchange resin. The following metal ions were individually tested, but could not replace the fraction 4: Fe^{3+}, Ca^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+} at 3×10^{-4} M.

The fraction 1 was obviously a large molecular weight fraction, possibly a membrane fraction excluded from the Sephadex. The hydroxylamine-cytochrome c reductase activity was also present in this fraction.

In Sepharose 6 B the ammonia-oxidizing activity was recovered when the fractions, 1, 3, and 5 were combined. The fraction 3 had the hydroxylamine-cytochrome c reductase activity and could be replaced by an approximately equivalent amount of the enzyme purified according to Hooper and Nason (1965). The fraction 1 was probably a membrane fraction excluded from

Sepharose 6B and fraction 5 was probably equivalent to a small molecular weight fraction equivalent to the fraction 4 of the Sephadex G-100 experiment.

FIGURES

Fig. 1. Oxidation of ammonia by Nitrosomonas cells and French Pressure Cell extracts. Oxygen consumption was followed in a Gilson Oxygraph (reaction volume, 1.5 ml extract or cell suspension) as described in Materials and Methods. At the times indicated by arrows additions were made as follows: 1.7 mM $(NH_4)_2SO_4$, 0.3 mM α,α'-dipyridyl and 33 μM $NH_2OH.HCl$. Extracts were prepared in the absence (I, 1.1 mg extract protein/ml) and in the presence (III) of bovine serum albumin from a cell suspension of 20 mg/ml. In experiment II the original cell suspension was diluted 10-fold (2 mg cells/ml).

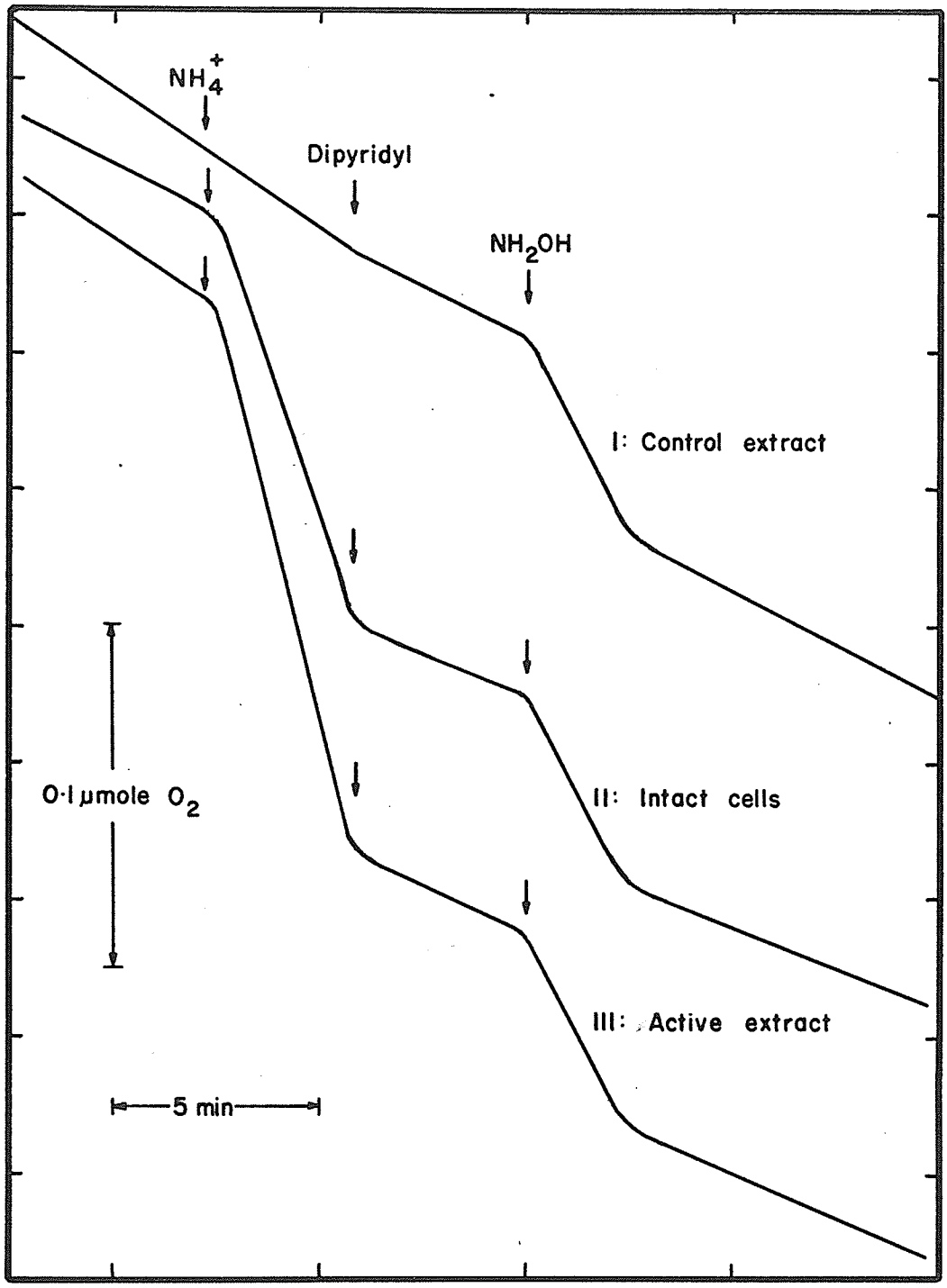

NH_4^+
Dipyridyl
NH_2OH
I: Control extract
II: Intact cells
III: Active extract
0·1 µmole O_2
5 min

Fig. 2. Activation of Nitrosomonas extracts by Mg^{2+}, spermine and bovine serum albumin and stoichiometry of ammonia oxidation. The Oxygraph reaction vessel contained 1.5 ml of a cell-free extract (1.1 mg extract protein/ml) prepared in the absence of bovine serum albumin. Additions: 10 mM $MgCl_2$, 2 mM spermine, 20 mg/ml bovine serum albumin, 1.7 mM $(NH_4)_2SO_4$ and 0.3 mM α,α'-dipyridyl.

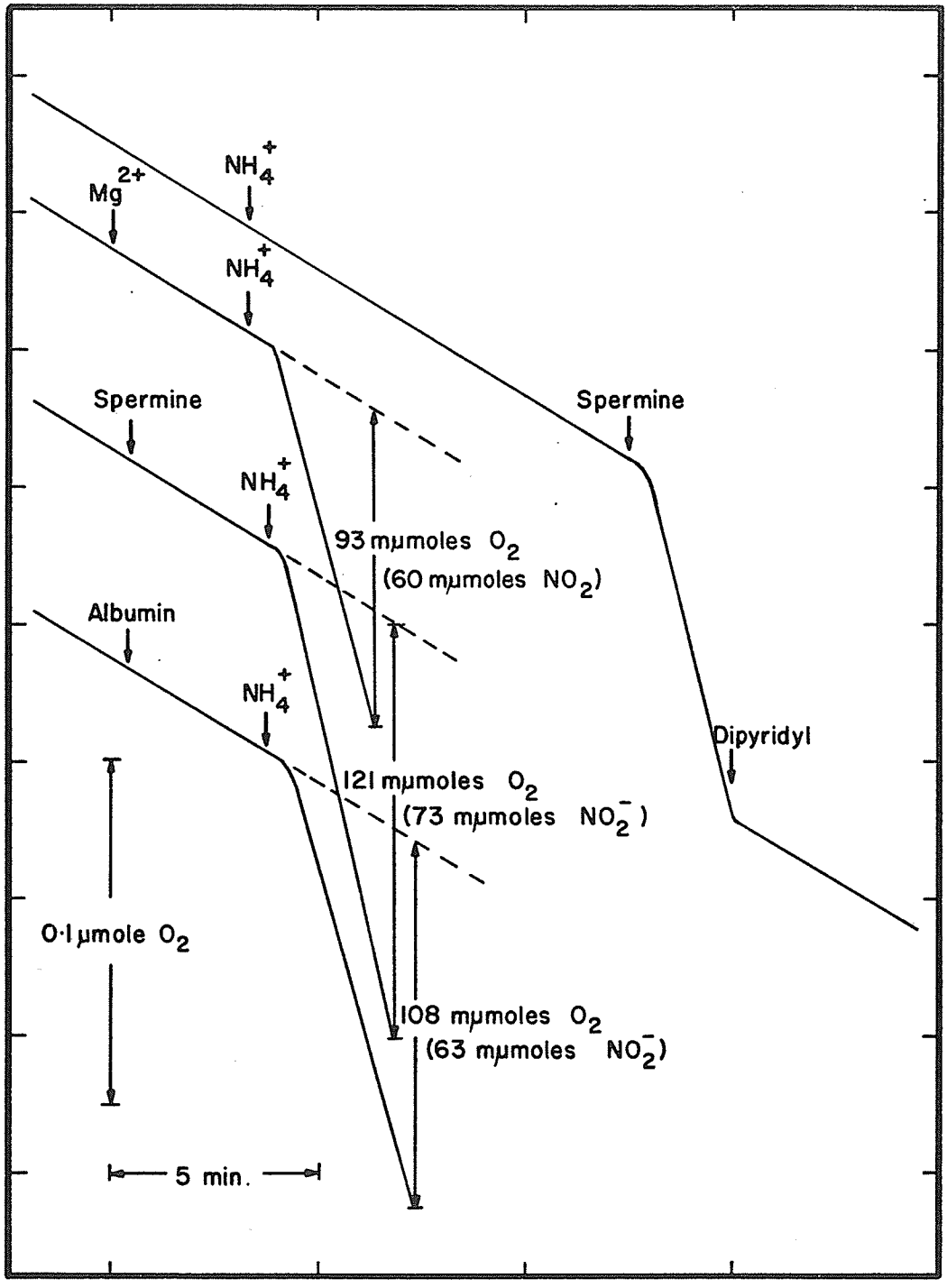

Mg^{2+}
NH_4^+
NH_4^+
NH_4^+
NH_4^+
Spermine
Spermine
Albumin
Dipyridyl
93 mμmoles O_2
(60 mμmoles NO_2)
121 mμmoles O_2
(73 mμmoles NO_2^-)
108 mμmoles O_2
(63 mμmoles NO_2^-)
0·1 μmole O_2
5 min.

Fig. 3. Effect of Mg^{2+}, spermine and bovine serum albumin concentrations on the rate of ammonia oxidation. v, mμmoles O_2 consumed/min. The reaction vessel contained 1.5 ml of Nitrosomonas extract (1.0 mg protein/ml) prepared in the absence of albumin. Mg^{2+}, spermine or albumin was added before the initiation of reaction with 1.7 mM $(NH_4)_2SO_4$.

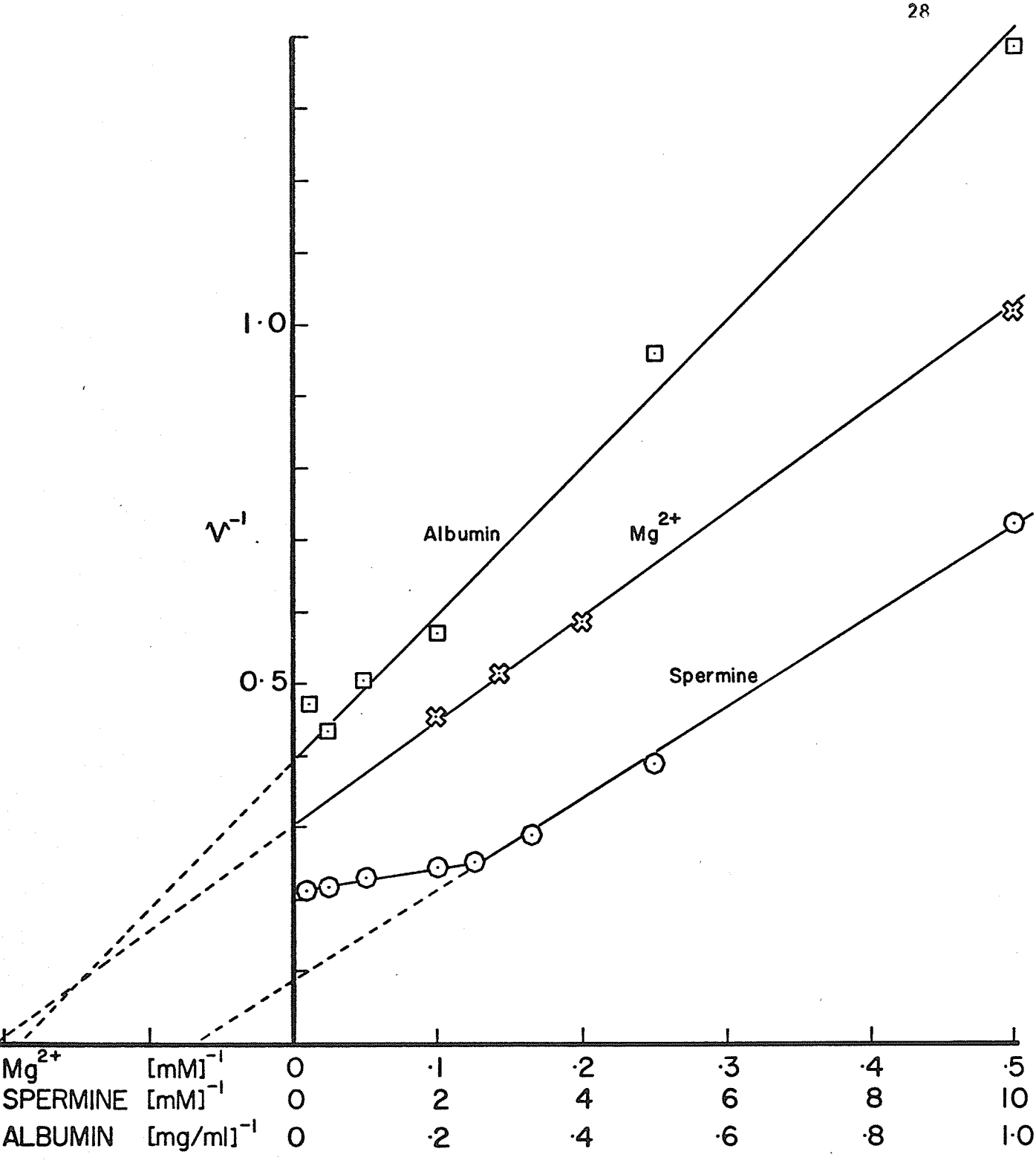
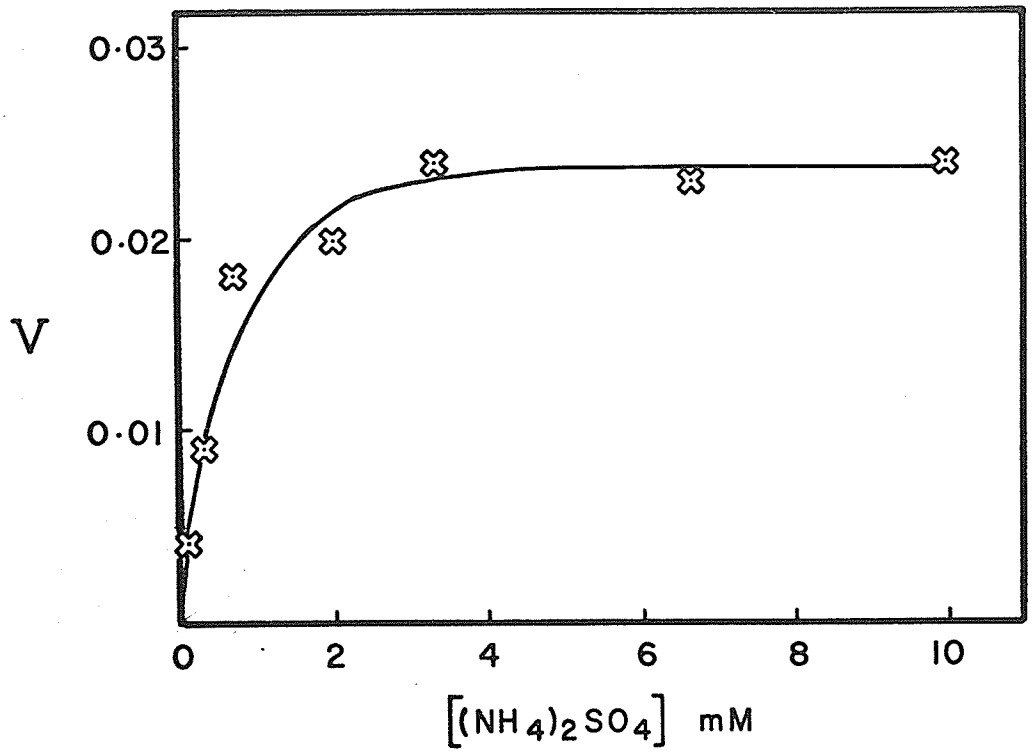

1·0
0·5
v^{-1}
Albumin
Mg^{2+}
Spermine
Mg^{2+} $[mM]^{-1}$ 0 ·1 ·2 ·3 ·4 ·5
SPERMINE $[mM]^{-1}$ 0 2 4 6 8 10
ALBUMIN $[mg/ml]^{-1}$ 0 ·2 ·4 ·6 ·8 1·0

Fig. 4. Effect of substrate concentration on the rate of ammonia oxidation by cell-free extracts. The reaction was carried out in an Oxygraph at 25°C. The reaction mixture, in a total volume of 1.5 ml, contained 0.5 ml cell-free extract and 0.1 M potassium phosphate (pH 7.5). The system was activated with 2 mM spermine before the addition of ammonium sulfate.


0·03
0·02
0·01
V
0
2
4
6
8
10
[(NH4)2SO4] mM

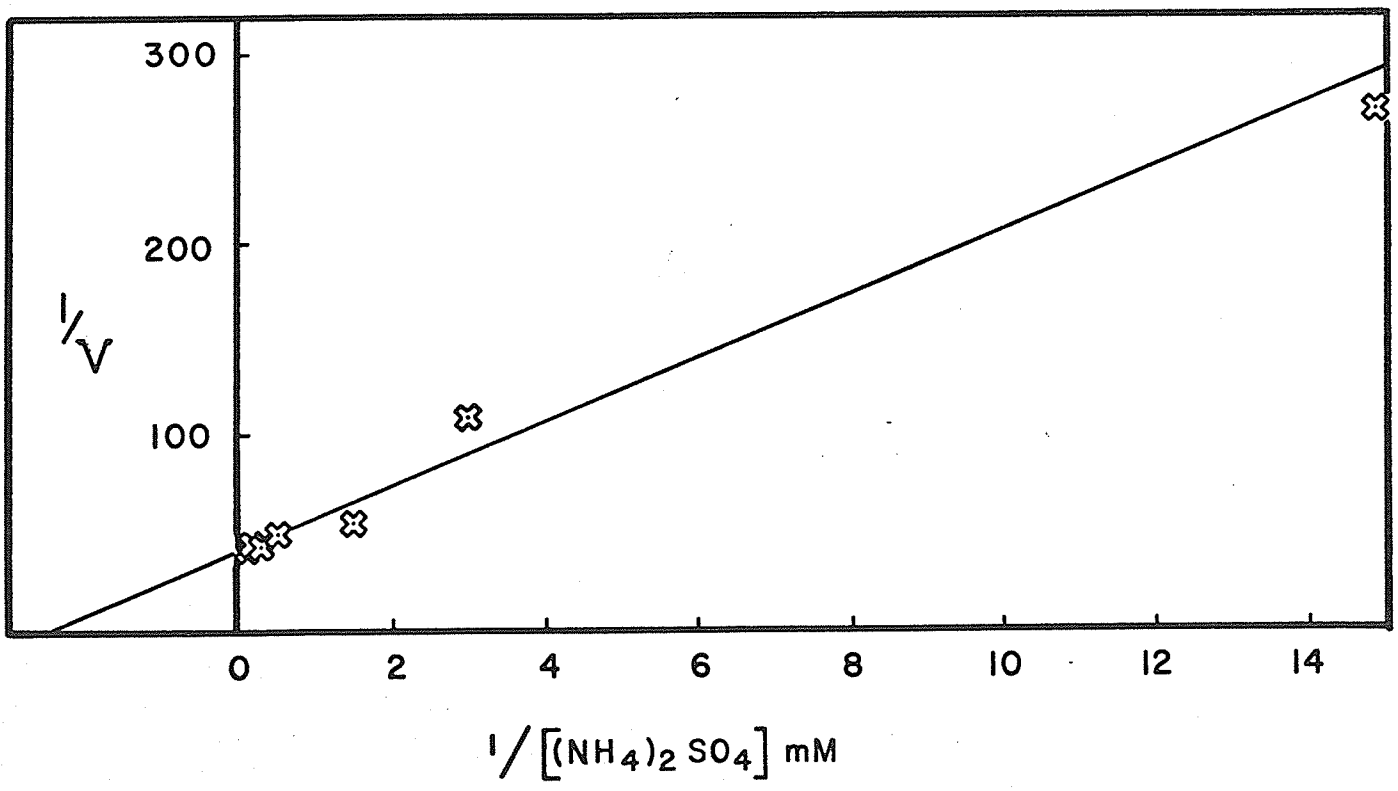

300
200
100
1/V
0
2
4
6
8
10
12
14
1/[(NH4)2SO4] mM

Fig. 5. Effect of pH on the cell-free ammonia-oxidation. The reaction was carried out in an Oxygraph at 25°C. The reaction mixture, in a total volume of 1.5 ml, contained 0.5 ml cell-free extract and 0.1 M potassium phosphate buffer of various pH's. The system was activated by 2 mM spermine before the addition of 1.7 mM ammonium sulfate. The pH of the reaction mixture was determined after the measurement of the rate of ammonia oxidation.

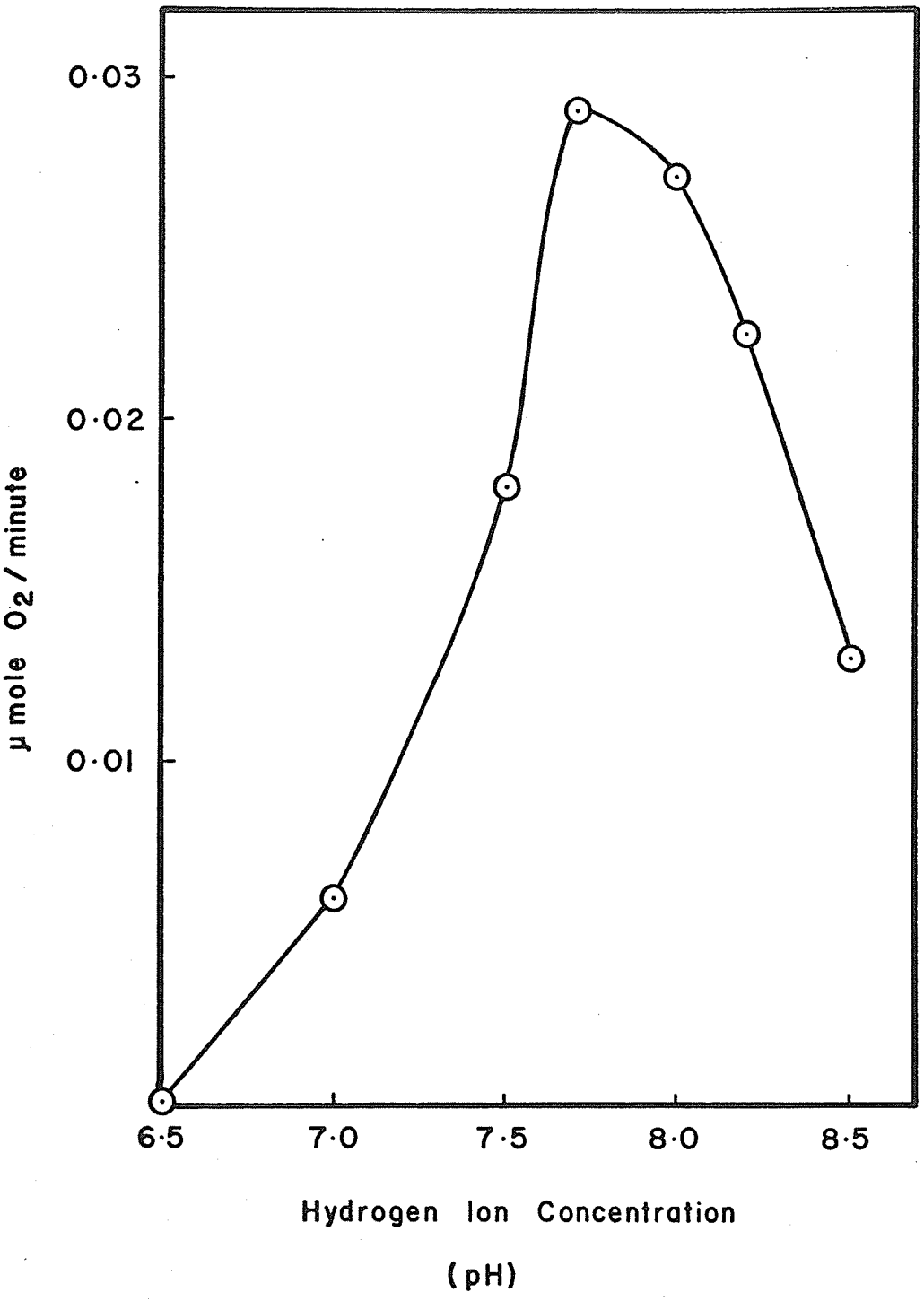

0·03
0·02
0·01
µ mole O_2 / minute
6·5
7·0
7·5
8·0
8·5
Hydrogen Ion Concentration
(pH)

Fig. 6. Effect of temperature on the oxidation of ammonia by cell-free extracts. The reaction was carried out in an Oxygraph at desired temperatures. The reaction mixture, in a total volume of 1.5 ml, contained 0.5 ml cell-free extract and 0.1 M potassium phosphate (pH 7.5). Activator: 2 mM spermine. Substrate: 1.7 mM ammonium sulfate.

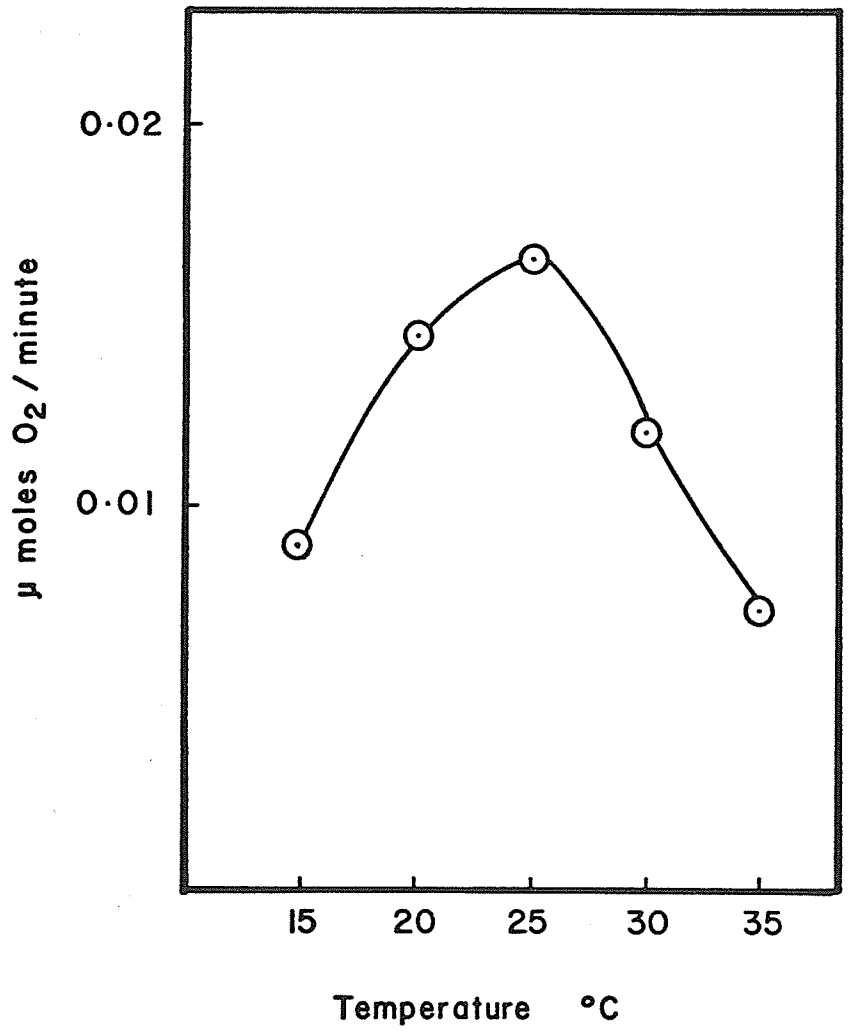

0·02
0·01
μ moles O2 / minute
15
20
25
30
35
Temperature °C

Table 1

Effect of hydroxylamine and NADH on the oxidation of ammonia by Nitrosomonas cell-free extracts.

Reaction system*	O_2 consumed μmole	Nitrite found μmole	O_2 consumed due to ammonia oxidation μmole	Nitrite formed from ammonia μmole
A. Extract	0.03	0.01		
B. Extract + $(NH_4)_2SO_4$	0.02	0.01		
C. Extract + hydroxylamine	0.06	0.05		
D. Extract + $(NH_4)_2SO_4$ + hydroxylamine	0.28	0.18	0.22	0.13
E. Extract + NADH	0.02	0.02		
F. Extract + $(NH_4)_2SO_4$ + NADH	0.26	0.16	0.24	0.14

*The reaction was carried out in an Oxygraph at 25°C. The reaction mixture, in a total volume of 1.5 ml, contained 0.5 ml cell-free extract and 0.1 M potassium phosphate (pH 7.5). Additions: ammonium sulfate 2.5 μmoles, hydroxylamine 0.05 μmole, NADH 0.05 μmole.

Fig. 7. Time course of oxygen utilization by Nitrosomonas cell-free extracts activated by hydroxylamine or NADH. At the times indicated by arrows, either 2.5 μmoles of ammonium sulfate, 0.05 μmole of hydroxylamine or 0.05 μmole NADH were added. The reaction systems (A-F) were identical to those of Table 1. The stars indicate the times when the reaction was stopped and the reaction mixtures were analysed for nitrite.

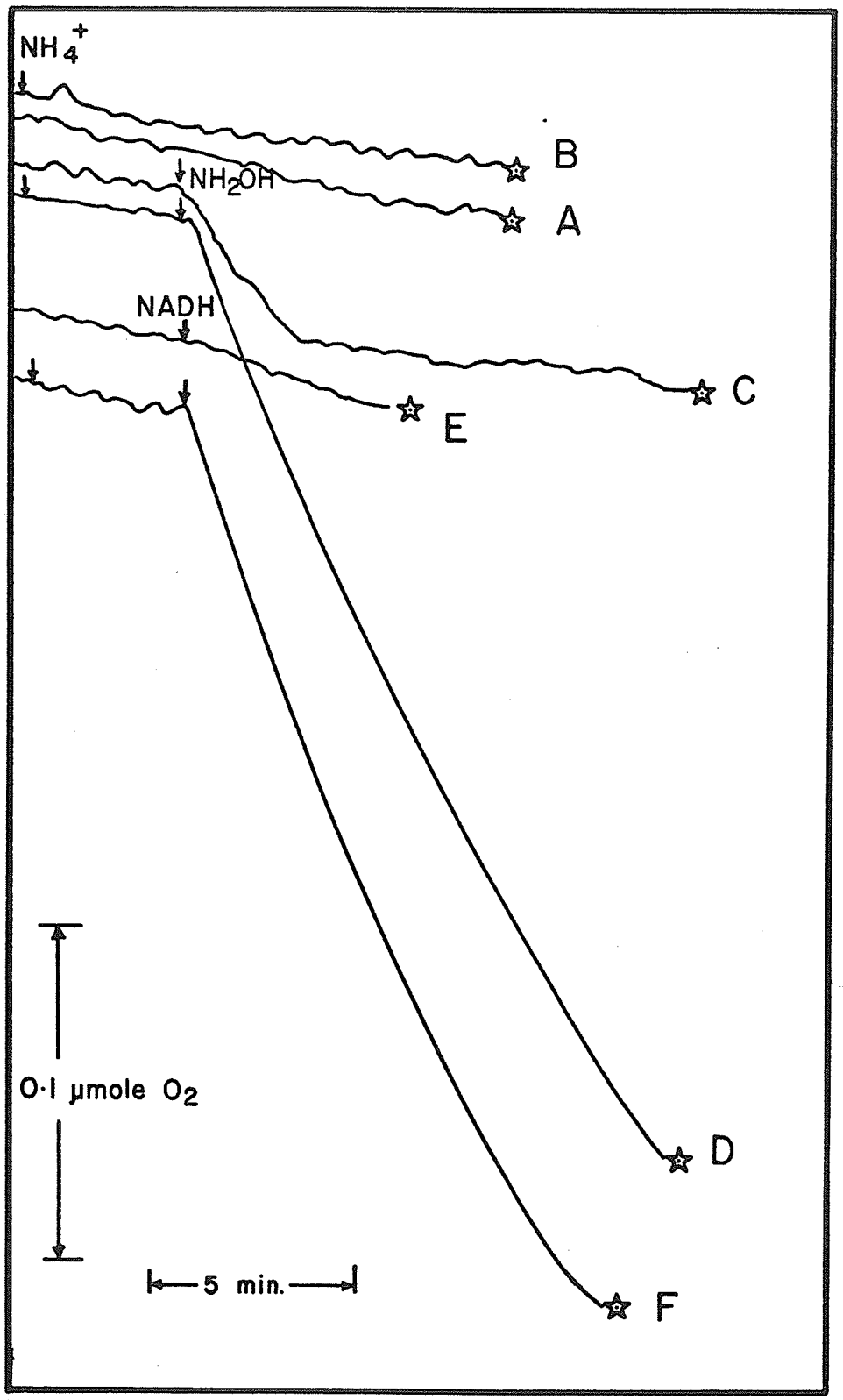

NH4+
B
NH2OH
A
NADH
C
E
D
F
0·1 µmole O2
5 min.

Fig. 8. Difference spectrum of an active cell-free extract of Nitrosomonas europaea. The extract was prepared from a cell suspension containing bovine serum albumin as described in Materials and Methods. The experiment was performed in a Shimadzu Multi-purpose Recording Spectophotometer with 1 cm light path. Both sample and reference cuvettes contained 1 ml cell-free extract. To the sample cuvette 15 µl of 0.1 M $(NH_4)_2SO_4$ were added to reduce the cytochromes. To the reference cuvette 15 µl of water were added.

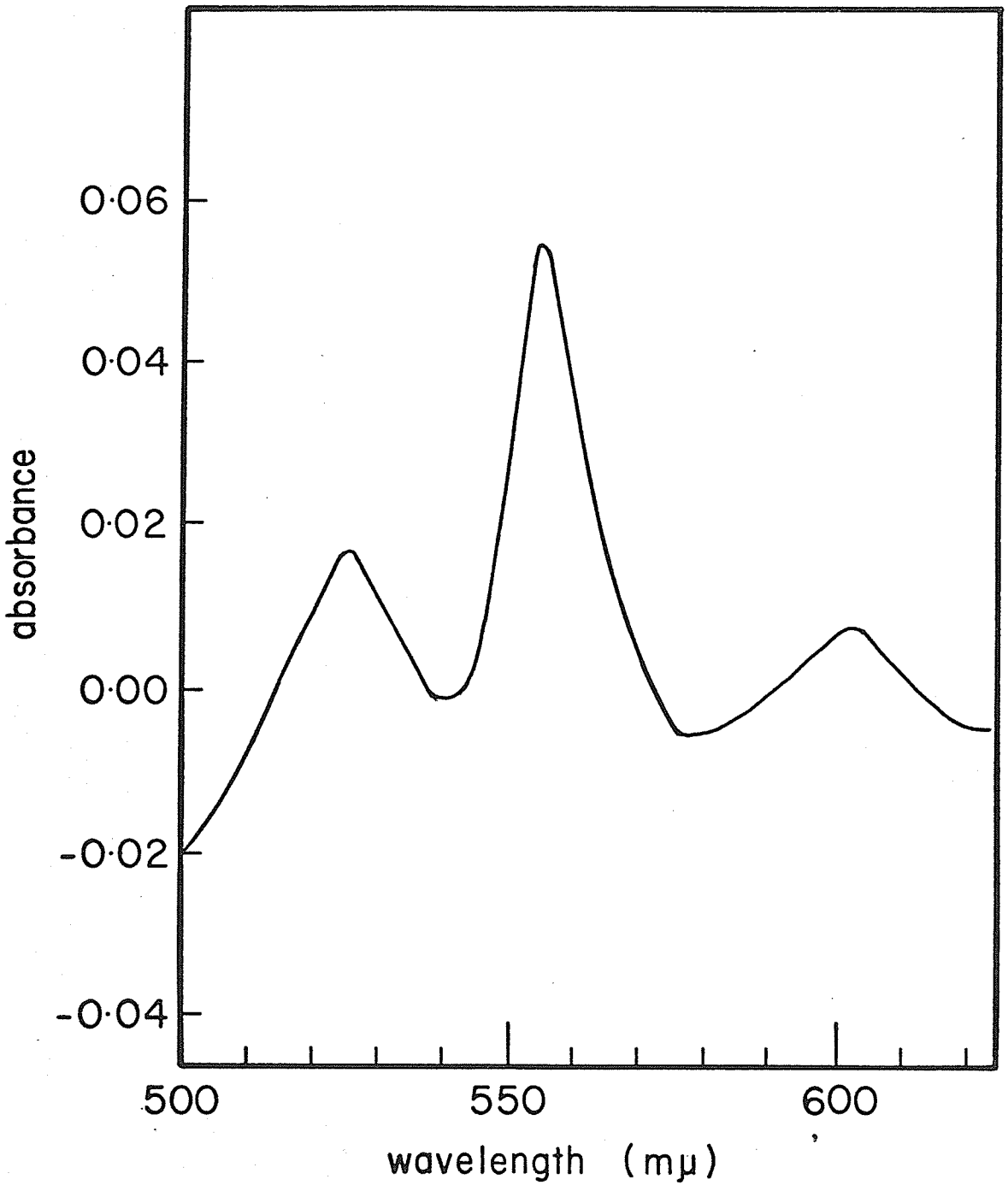

0·06
0·04
0·02
0·00
-0·02
-0·04
absorbance
500
550
600
wavelength (mμ)

Fig. 9. Time course of the reduction of a Nitrosomonas cytochrome (554 mμ peak) and oxygen consumption during ammonia oxidation. The reaction mixture, in a total volume of 3 ml, contained 2 ml of cell-free extract and 0.1 M potassium phosphate (pH 7.5). The system was activated with 2 mM spermine before the addition of 1.7 mM ammonium sulfate to initiate the reaction. The reduction of cytochrome was measured in an Aminco-Chance Dual Wavelength Spectrophotometer as described in Materials and Methods as $T_{554-545\ m\mu}$ (A) and the oxygen consumption was measured with a vibrating platinum electrode as mμmoles (B).

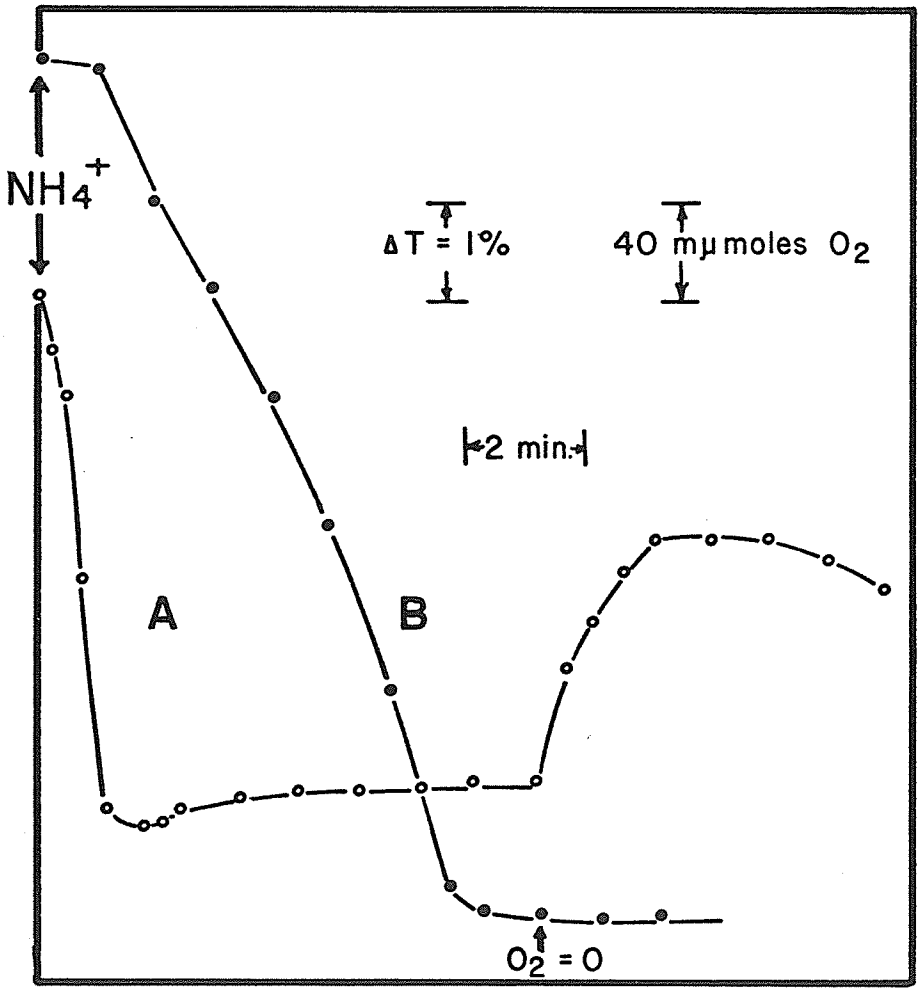

NH_4^+
ΔT = 1%
40 mμ moles O_2
2 min.
A
B
O_2 = 0

Fig. 10. Time course of the reduction of a Nitrosomonas cytochrome (603 mμ peak) and oxygen consumption during ammonium oxidation. Experimental conditions were the same as those of Fig. 9. A: $T_{603-580\ m\mu}$ and B: oxygen consumption as μmoles.

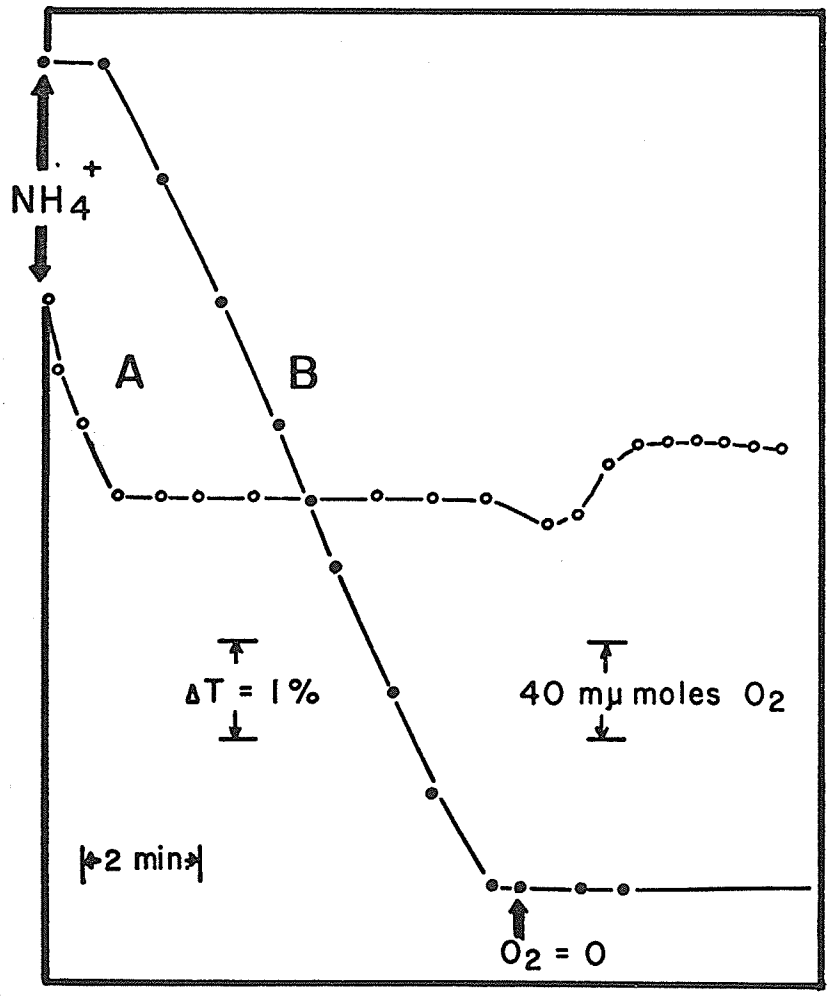
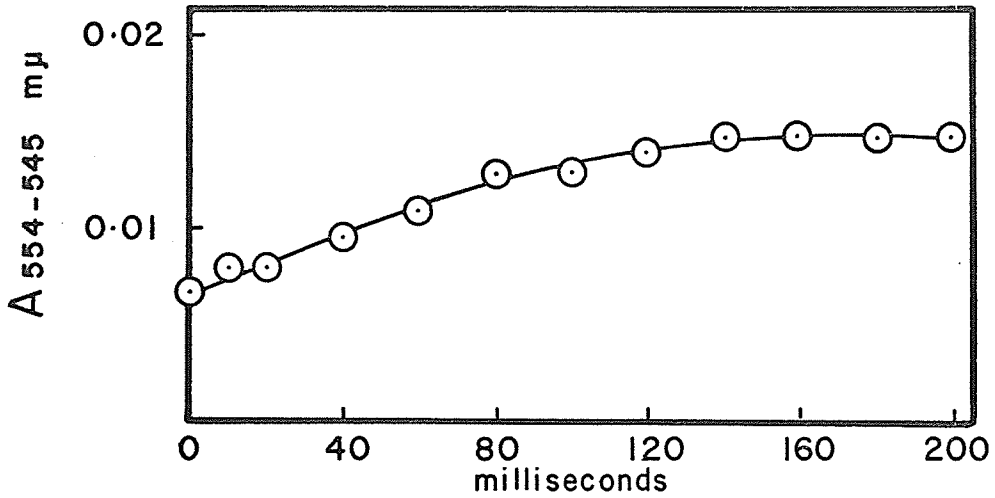
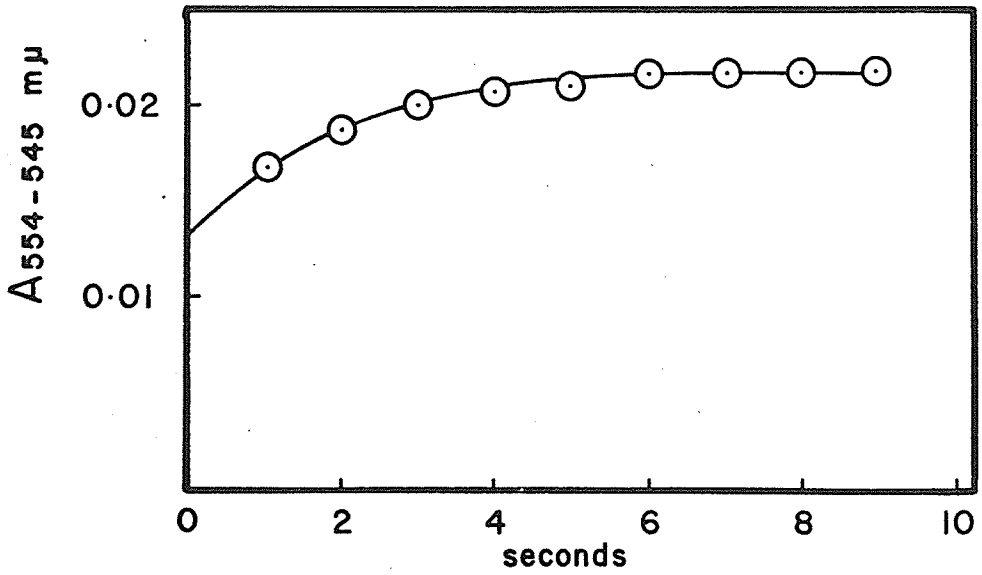

NH4+
A
B
ΔT = 1%
40 mμ moles O2
2 min
O2 = 0

Fig. 11. Kinetics of the reduction of a Nitrosomonas cytochrome (554 mμ peak) during hydroxylamine oxidation. Experiments were performed in an Aminco-Chance Dual Wavelength Spectrophotometer with an Aminco-Morrow Stopped-Flow attachment as described in Materials and Methods. The reaction was initiated by mixing a cell-free extract in 2 mM spermine with a solution of 6×10^{-5} M hydroxylamine. $A_{554-545\ m\mu}$ changes were recorded in a storage oscilloscope with three different time/division scales (a, b, c).


0·02
0·01
A554-545 mμ
0
40
80
120
160
200
milliseconds


0·02
0·01
A554-545 mμ
0
2
4
6
8
10
seconds

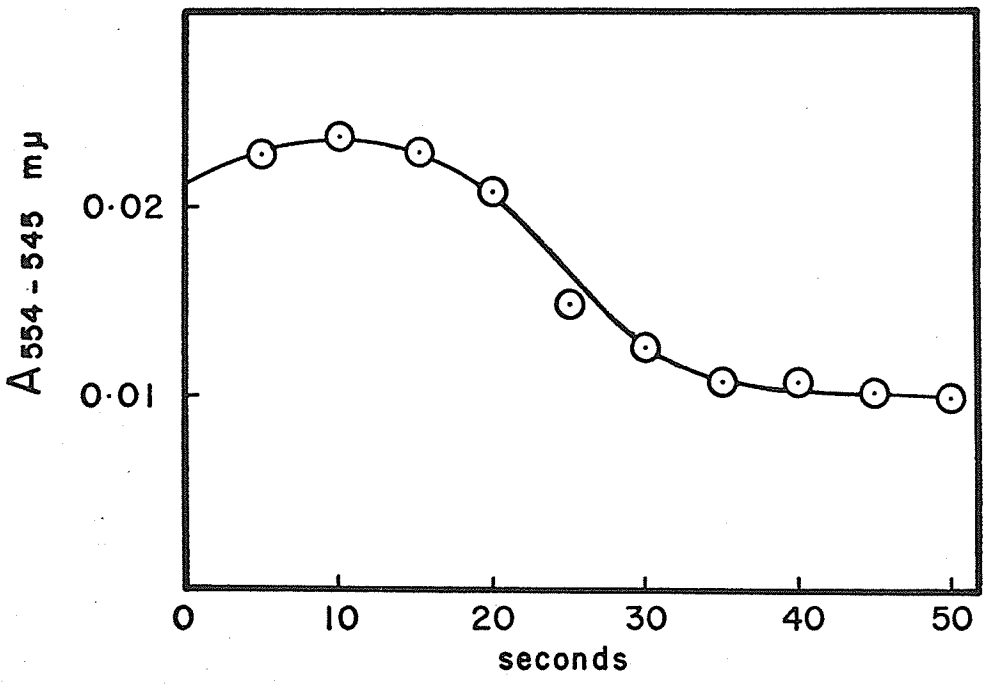
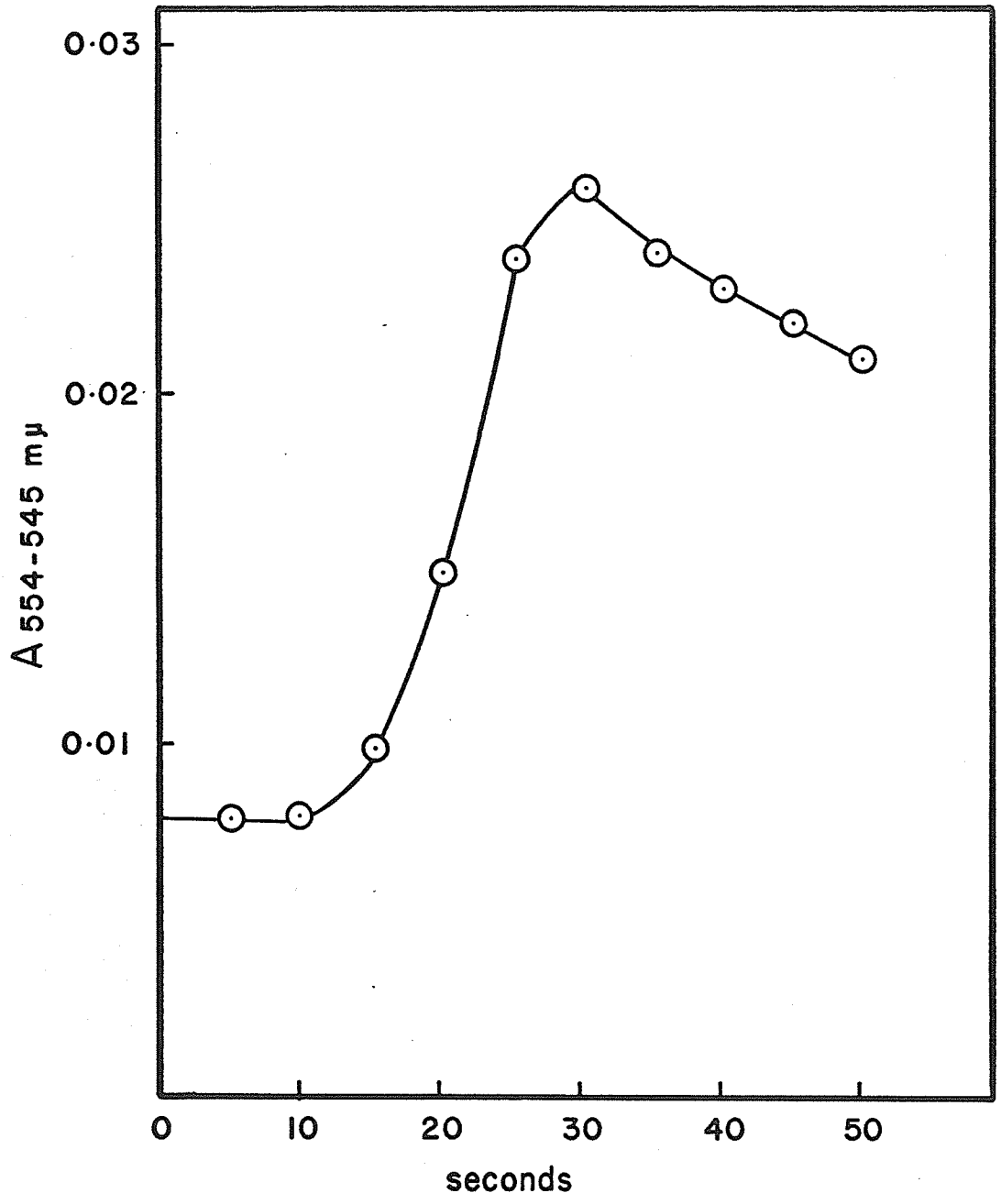

0·02
0·01
A554-545 mμ
0
10
20
30
40
50
seconds

Fig. 12. Kinetics of the reduction of a Nitrosomonas cytochrome (554 mμ peak) during ammonia oxidation. Experimental conditions were the same as those of Fig. 11, except that a solution of 3.4×10^{-3} M ammonium sulfate replaced the hydroxylamine solution as substrate.


0·03
0·02
0·01
A 554-545 mμ
0
10
20
30
40
50
seconds

DISCUSSION

DISCUSSION

The results in the previous sections conclusively established that the oxidation of ammonia could be achieved by the cell-free extracts of Nitrosomonas europaea. The possibility of surviving intact cells accounting for the activity of cell-free extracts was entirely eliminated. Microscopic examination of extracts showed that the number of intact cells, if present, was less than 0.2% of that in the cell suspension, while the activity of extracts was 10-20% of the activity of whole cells. In control experiments where whole cells were centrifuged at 2,000 x g, the clear supernatant did not oxidize either ammonia or hydroxylamine. Finally, the ammonia-oxidizing activity of the extracts was lost completely after storage at $4^{\circ}C$ for 6 or 7 hours, while the activity of whole cells was stable for at least several days.

Out of various methods of disrupting cells tested, i.e., sonic oscillation, osmotic rupture of spheroplasts and French Press passage, only the last method produced active extracts.

In the preparation of active extracts, however, it was essential to start with very active whole cells of N. europaea. The very active cells were characterized with deep red color compared to the pink color of less active cells. The cells with deep red color oxidized ammonia faster than the cells with pink color. The deep red cells always produced extracts capable of oxidizing ammonia after activation with spermine, serum albumin or Mg^{2+}, but the extracts prepared from pink cells often required further activation by hydroxylamine or NADH as well.

Iron was important in obtaining cells with deep red color. When $FeCl_3$ solution was stored before the preparation of growth medium iron was lost from the solution as ferric hydroxide on the flask wall and the resulting medium produced pink cells. It was therefore stored after mixing with EDTA.

Even in the presence of an adequate quantity of iron in the growth medium cells with deep red color were not obtained unless the medium was constantly kept around pH 8.0 with the addition of K_2CO_3 solution. The neutralization process was particularly critical toward the end of the growth period because of accelerated rate of nitrite formation from ammonia. The cells were harvested as soon as the pH started to change after the last neutralization ($\sim$ 2 hours).

The deep red color of active cells was due to high concentrations of cytochromes in these cells. The requirement for iron was probably for the synthesis of cytochromes. These cytochromes were synthesized only when the supply of iron was sufficient and when the cells were oxidizing ammonia rapidly, i.e., around pH 8.0.

These active cells, when freshly harvested, oxidized ammonia rapidly, but oxidized hydroxylamine very slowly, if at all. After storage at $4^{\circ}C$ for 2-3 weeks the ammonia-oxidizing activity decreased and now the hydroxylamine-oxidizing activity increased to the level where hydroxylamine was oxidized faster than ammonia confirming the results of Engel and Alexander (1958) and Anderson (1959). These "aged" cells did not have the deep red color of fresh cells and produced

inactive cell-free extracts. In fact when a suspension of aged cells was centrifuged the supernatant contained cytochromes leaked out from the cells.

The activation effects of bovine serum albumin , Mg^{2+} or polyamines on ammonia oxidation by N. europaea cell-free extracts seem to be related to their effects on biological membranesor particle systems: protein synthesis by ribosomes where polyamines can replace Mg^{2+} as reported by Cohen and Lichtenstein (1960), Takeda (1969), Takeda and Igarashi (1969) and Bachrach (1970) and mitochondria where it is well known that serum albumin activates oxidative phosphorylation. The precise mechanism of these activation processes is not known at this time whether it is due to charge neutralization or hydrophobic effect, but our centrifugation studies showed that in the presence of these activators the amount of precipitate increased considerably suggesting the association of smaller membrane pieces to larger structures. A preliminary electron microscopic study also confirmed these results. Obviously a further critical work is necessary in this area.

The optimum activity of cell-free ammonia-oxidizing system occurred around pH 7.7 agreeing with the optimum pH for growth of this organism. The optimum temperature obtained around 25°C also agreed with that with whole cells.

The concentration of substrate, ammonium sulfate, required for half-maximum activity was estimated as 0.4 mM for the cell-free system coinciding with the value obtained with whole cells, 0.3 mM.

In the studies carried out by centrifugation, the supernatant together with the pellet were required for ammonia oxidation indicating

the presence of at least two components in the ammonia-oxidizing system. In column chromatography the ammonia-oxidizing activity was found in a combination of fractions 1 and 4 in Sephadex G-100 or fractions 1, 3 and 5 in Sepharose 6 B. The fraction 1 of Sephadex G-100 experiment probably consisted of a membrane fraction and some large molecular weight proteins, e.g., hydroxylamine-cytochrome c reductase.

The fraction 3 of Sepharose 6 B experiment was shown to be hydroxylamine-cytochrome c reductase. Hooper and Nason (1965) determined that the molecular weight of hydroxylamine-cytochrome c reductase was between 100,000 to 200,000. This molecular weight range agrees with the exclusion in Sephadex G-100 and the fractionation in Sepharose 6 B. The membrane fraction, the fraction 1 in Sepharose 6 B, was excluded from the agarose gel with an exclusion limit of 4,000,000.

The small molecular weight fraction (fraction 4 in Sephadex G-100 and fraction 5 in Sepharose 6 B) remains unidentified. The active component sometimes moved with the membrane fraction without separation.

With extracts prepared in HEPES buffer there was no ammonia oxidation in the presence of hydroxylamine or NADH unless phosphate was present. This effect of phosphate agrees with the finding by Droogenbroeck and Laudelout (1967) who reported that phosphate was required for the growth of Nitrosomonas. Crude sonicated extracts of Nitrosomonas prepared by Nicholas and Jones (1960) oxidized hydroxylamine, but not ammonia and required phosphate for the hydroxylamine oxidation with oxygen in the presence of horse heart cytochrome c. The fact that phosphate can be replaced by sulfate and that the extracts can be

activated with spermine in the absence of phosphate suggest that the effect of phosphate is ionic influencing the physico-chemical structure of the ammonia-oxidizing system.

In Tris-HCl buffer there was no oxidation of ammonia even in the presence of phosphate or spermine. Thus the buffer obviously inhibits the cell-free ammonia oxidation. Rees (1968) reported with whole cells that 0.1 M sodium or potassium chloride inhibited the production of nitrite without affecting the rate of hydroxylamine disappearance. The inhibitory component in the Tris buffer, therefore, could be chloride ions, but was not investigated further.

The response of cytochromes (554 mμ and 603 mμ) in N. europaea extracts to the addition of ammonia was very interesting. Cytochromes were first reduced before oxygen consumption started. This observation together with the effect of NADH and hydroxylamine led to the idea that the priming effect of reduced cytochromes initiated the oxidation of ammonia. When all the oxygen was depleted from the system there was some reoxidation of reduced cytochromes. The reoxidation of reduced cytochromes could be due to the reversed election flow reported by Aleem (1966) or nitrite reductase reported by Hooper (1968) and by Wallace and Nicholas (1968), but requires further investigation before the actual mechanism is elucidated.

In the cell-free ammonia oxidation there was always a lag period of 0.5 - 1 min even in the very active state of extracts before the initiation of oxygen uptake. Small amounts of NADH or hydroxylamine present not only abolished this lag-phase but also activated ammonia oxidation. In fact the rate of NADH oxidation measured at 340 mμ was accelerated when ammonia was present.

If ammonia was added after complete oxidation of either NADH or hydroxylamine no ammonia oxidation could be observed. This showed that catalytic amounts of NADH or hydroxylamine initially reduced cytochromes which primed the oxidation of the substrate, ammonia.

The priming effect of hydroxylamine on ammonia oxidation was also observed with N. europaea spheroplasts by Suzuki and Kwok (1969) and with whole cells by Hooper (1969).

A tentative scheme for ammonia oxidation by N. europaea based on the results described here and those reported previously is represented by the following equations:

(1) $NH_4^+ + O_2 + AH_2 \xrightarrow{\text{oxygenase}} NH_2OH + A + H_2O + H^+$

(2) $NH_2OH + A \xrightarrow{NH_2OH\text{-cyt-reductase}} (NOH) + AH_2$

(3) $H_2O + (NOH) + A' \xrightarrow{(NOH)\text{-cyt-reductase}} NO_2^- + A'H_2 + H^+$

(4) $A'H_2 + \frac{1}{2}O_2 \xrightarrow{\text{cyt-oxidase}} H_2O + A'$

Sum: $NH_4^+ + 1\frac{1}{2}O_2 \longrightarrow NO_2^- + 2H^+ + H_2O$

Where A,A' = 2 cytochromes and AH_2,$A'H_2$ = 2 reduced cytochromes + $2H^+$ which can be oxidized either by equation (1) or (4).

Rees and Nason (1965, 1966) suggested that ammonia oxidation by Nitrosomonas involved an oxygenase. Suzuki and Kwok (1969) proposed a mixed-fuction type of reaction between ammonia and hydroxylamine based on the effect of hydroxylamine on the spheroplasts of N. europaea. In

the equation (1) A represents a cytochrome or cytochromes reduced by hydroxylamine or NADH to AH_2 which will initiate the oxidation of ammonia.

The equation (2) is catalyzed by hydroxylamine-cytochrome c reductase. (NOH) is the hypothetical nitroxyl intermediate suggested by Lees (1954) and is further oxidized to NO_2^- according to equation (3). A' may or may not be the same cytochrome as A.

Finally reduced cytochromes (AH_2 or $A'H_2$) will be oxidized by cytochrome oxidase.

When the oxidation of ammonia is going under ideal conditions as in active cells or cell-free extracts, all the reactions (1) to (4) must proceed at the same steady-state rate. At the initiation of ammonia oxidation, however, AH_2 has to be supplied. The addition of ammonia to active extracts leads to the reduction of cytochromes after a lag period of 5 - 10 seconds as observed in stopped-flow experiments (Fig. 12). During the reduction there was no oxygen uptake (Fig. 9). This was obviously the period when the steady-state level of AH_2 was built up to initiate the rapid oxidation of ammonia with concomitant oxygen uptake. The mechanism for AH_2 build-up is unknown, but possibly reactions (1) to (3) proceed faster than reaction (4) until reduced cytochromes are built up to a sufficient concentration. There was in fact a slight over-reduction of cytochrome (554 mμ peak) before the initiation of oxygen uptake after which the steady-state level of cytochrome reduction was maintained (Figs. 9 and 12).

The priming effect of hydroxylamine or NADH is easily understandable in this scheme. The increased rate of NADH oxidation with ammonia may

be explained as follows. NADH reduces cytochromes which can be oxidized by equation (1) as well as by equation (4) in the presence of ammonia.

In the stopped-flow experiments hydroxylamine reduced the N. europaea cytochrome in two stages, possibly representing reactions (2) and (3), with reaction (2) faster than reaction (3). With ammonia as substrate the reduction process took place in one stage. This latter experiment suggests a tight coupling of reactions (1) and (2) and the reduction of cytochrome observed was possibly due to reaction (3). The reaction half times observed in Figs. 11 and 12 agree with this interpretation.

The reduction of nitrite in the presence of hydroxylamine observed by Hooper (1968) in a soluble enzyme system from N. europaea may be explained by a combination of equations (2) and (3), the equation (3) going from right to left.

Further support for the role of reduced cytochrome, AH_2, in ammonia oxidation comes from studies on growing cultures of these ammonia-oxidizing organisms. If the cultures of N. europaea were aerated too vigorously the growth of organism was inhibited and cells gradually lysed according to our own observation. Gould (1959) also reported that cultures failed to grow in vigorously aerated medium. Gundersen (1966) studied the growth of Nitrosocystis oceanus and found that 90% oxygen was toxic and that the organism could grow considerably better on plates incubated at 2% oxygen than in air. He concluded that low partial pressures were tolerated much better by this organism than high partial pressures of oxygen. These inhibitory effects of oxygen may be due to the competition for AH_2 with ammonia and a high oxygen concentration may inhibit the ammonia oxidation by making the steady-state

concentration of AH_2 too low for good growth (possibly through cytochrome oxidase).

In our studies with artificial electron acceptors (unpublished data) dichlorophenol indophenol, phenazine methosulfate, or methylene blue at 1.5×10^{-4} M inhibited the cell-free ammonia oxidation completely if added before ammonia. However, the inhibition was only about 50% if these dyes were added in the middle of the ammonia oxidation. These dyes probably oxidized AH_2 preventing the initiation of ammonia oxidation, but once the oxidation reached a steady-state level their effects were reduced possibly because of a tight coupling of all the 4 reactions.

The mechanism of ammonia oxidation by nitrifying bacteria is still far from solution. The availability of a cell-free ammonia-oxidizing system, however, should lead to a better understanding of the mechanism. The scheme proposed here is consistent with most observations and data in this work as well as in the literature.

A further work is obviously needed before a more detailed mechanism can be proposed that incorporates the energy aspect, specific enzymes and individual cytochromes.

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