BIOCHEMICAL AND STRUCTURAL STUDIES OF THE CELL-FREE AMMONIA-OXIDIZING SYSTEM OF *NITROSOMONAS EUROPAEA*

BY

USHA DULAR

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TO MY PARENTS AND MY HUSBAND

A B S T R A C T

ABSTRACT

A biochemical as well as structural study of the oxidation of ammonia in the cell-free extracts and in the partially resolved and reconstituted system of Nitrosomonas europaea was undertaken with an aim to provide a further insight into the complex mechanism of oxidation.

The cell-free extracts from fresh N. europaea cells oxidized ammonia rapidly in the presence of bovine serum albumin (BSA) or spermine. Extracts prepared from old cells or stored frozen, required $CuCl_2$ and NADH in addition to BSA and spermine.

The ammonia-oxidizing activity of the cell-free extracts was inhibited by metal-binding agents such as KCN, diethyldithiocarbamate, α,α'-dipyridyl and 1, 10-orthophenanthroline and also by mercuric chloride. On the other hand, the oxidation of hydroxylamine was activated by the addition of $CuCl_2$ together with either dipyridyl or o-phenanthroline. The addition of $CuCl_2$ along with either of the

chelators also activated the rate of cytochrome c (mammalian) reduction by either NH_2OH or NADH in the crude cell-free extracts but not in a partially purified preparation of the enzyme NH_2OH-cytochrome c reductase.

Both the NH_2OH-cytochrome c reductase and cytochrome c oxidase activities were inhibited by 0.1 M potassium phosphate.

Partial resolution of the ammonia-oxidizing system could be achieved by chromatography on a Sepharose 6B column under partially anaerobic conditions. The ammonia-oxidizing complex could be resolved into at least three separate fractions, 1, 4 and 6, all of which were required in order to reconstitute the active complex. The fractions that were obtained under completely aerobic conditions, required either the addition of NADH or 2 to 3 hours of preincubation at $4^{\circ}C$.

Although the major component(s) of the membrane fraction (fraction 1) was found to be cytochrome oxidase(s), any attempts to replace it with a partially purified preparation of cytochrome a_1 (19) were not successful.

The active membrane fraction could be obtained only from fresh cells and was unstable during storage. Active fractions 4 and 6 on the other hand, could be obtained from either fresh or old cells and were relatively stable when stored frozen. In addition to the NH_2OH-cytochrome c reductase enzyme, fraction 4 also contained the cytochromes of b-, c- and P-460 types and it could be replaced with a partially purified NH_2OH-cytochrome c reductase preparation (27). The fraction 6 contained a c-type cytochrome(s) and could sometimes be replaced with either NADH or NH_2OH.

An electron microscopic examination of N. europaea cells revealed that in an active ammonia-oxidizing state, the cells appeared contracted and contained a considerable amount of darkly stained particulate matter especially in between the membrane layers. On the other hand, in an inactive state, the cells were more relaxed, swollen and devoid of the darkly stained material.

A striking ultrastructural difference was also observed when an active and inactive cell-free extracts were examined by negative staining. A

highly organized and aggregated membrane structure appeared to have formed when the extracts were activated by spermine or BSA (especially BSA). This organized and aggregated membrane structure could not be detected in the inactive extracts.

The membrane fraction appeared somewhat similar to the inactive extract in that there were many membrane fragments scattered throughout the field. On the other hand, a mixture of fractions 4 and 6 revealed no such fragments but contained globular structures of various sizes arranged in a ring-like manner.

An organized aggregation of large membrane folds with smaller vesicle-like structures was again observed when all the three fractions (1, 4 and 6) were combined to form a highly active (in ammonia oxidation) system.

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INTRODUCTION

Nitrosomonas europaea oxidizes ammonia to nitrite according to the following equation:

$$NH_3 + 1\frac{1}{2}\ O_2 \longrightarrow NO_2^- + H^+ + H_2O$$

Hydroxylamine was shown to be the intermediate in this reaction (41, 26).

The oxidation of hydroxylamine in intact cells and cell-free extracts of *N. europaea* was studied by various workers (2, 5, 22, 55). Hooper and Nason in 1965 (27) characterized the enzyme hydroxylamine-cytochrome *c* reductase. They suggested that the enzyme was a complex consisting of associated proteins, activators and possibly lipids rather than a single simple protein. In addition to the cytochromes of *b*-, *c*- and *a*- types and also flavin (27) *Nitrosomonas* contains cytochrome *o* and cytochrome P-450 (61) or cytochrome P-460 (18) both combining with carbon monoxide with characteristic spectra. It was suggested that cytochrome P-460 might be involved as an oxygenase

in one of the several presumed steps in the aerobic oxidation of ammonia to nitrite by Nitrosomonas (61). This was further supported by the evidence that one of the oxygen atoms in nitrite, formed from the oxidation of ammonia by Nitrosomonas was derived from atmospheric oxygen (62). A homogeneous preparation of hydroxylamine oxidase was purified and characterized by Rees in 1968 (63). He indicated that the terminal oxidase was not physically associated with hydroxylamine oxidase and that an added electron acceptor such as PMS (phenazine methosulphate) was essential for an active hydroxylamine oxidation and the extent of nitrite formation was strongly dependent on the nature of the acceptor employed. During the purification of hydroxylamine oxidase, a fraction containing c-type cytochrome was obtained which was found to inhibit nitrite formation without affecting the oxidation of hydroxylamine. Molecular properties of the enzyme hydroxylamine oxidase were also studied by Rees (63). The enzyme was shown to contain at least one b- and one c-type cytochrome. Electron microscopic examination of the enzyme revealed it to be a spherical particle with a diameter of about 160 Å.

Hydroxylamine-nitrite reductase, which catalysed the reaction of hydroxylamine, nitrite and oxygen to produce nitrous oxide (N_2O) and nitric oxide (NO) was characterized by Hooper (29). He pointed out that hydroxylamine-nitrite reaction apparently did not occur during the normal nitrification process by Nitrosomonas.

Ammonia oxidation by Nitrosomonas was reviewed by several workers (4, 69, 80). Hooper and Terry (32) studied the effect of various specific inhibitors on ammonia oxidation in intact cells of N. europaea. Isolation of a membrane envelope fraction by freezing and thawing of Nitrosomonas cells was accomplished by Hooper, Erickson, and Terry (31). They studied the electron transport components obtained in the supernatant fraction and membrane envelope obtained in the particulate fraction. They also presented electron microscopic evidence that the release of proteins by freezing and thawing resulted in the disappearance of electron dense material from between the closely associated internal membranes of Nitrosomonas. It was further pointed out that the cytochromes and electron transport enzymes might be concentrated between the closely associated cytoplasmic membranes of the cell in an arrangement which facilitated interaction with membrane-bound enzymes such as a-type cytochrome oxidase (31).

Restoration by hydroxylamine, of the ammonia-oxidizing activity of inactive Nitrosomonas spheroplasts was achieved by Suzuki and Kwok in 1969 (70). At the same time, the effect of hydroxylamine in diminishing the lag observed during ammonia oxidation by intact cells of Nitrosomonas was reported by Hooper (30).

The cell-free extracts oxidizing ammonia were independently and simultaneously obtained by Watson, Asbell, and Valois (83) from a marine organism Nitrosocystis oceanus and by Suzuki and Kwok (71) from Nitrosomonas europaea. Both of these workers used French Pressure Cell for obtaining cell-free extracts. The cell-free system from Nitrosocystis required sea water, magnesium and ATP (adenosine triphosphate) for active ammonia oxidation and most of the activity was found in the membrane fraction consisting of vesicles which were covered with 80 to 100 Å particles (83). On the other hand, the Nitrosomonas system required activation by spermine, magnesium or BSA (bovine serum albumin) when the extracts were prepared in 0.1 M potassium phosphate buffer (pH 7.5). In both the cases, the ammonia-oxidizing activity of the extracts was 10 to 20% of that of intact cells.

The properties of Nitrosomonas cell-free system was studied by Kwok (39). The optimum pH and temperature for ammonia oxidation were 7.7 and 25°C respectively. The K_m for ammonia was estimated to be approximately 0.4 mM agreeing with the value for whole cells (0.3 mM). It was further observed that the cytochromes were first reduced before oxygen consumption started and when all the oxygen was depleted from the system, there was reoxidation of some of these reduced cytochromes. From these observations, it was proposed that the priming effect of reduced cytochromes initiated the oxidation of ammonia. The two stage reduction of the Nitrosomonas cytochromes by hydroxylamine was observed in stopped flow experiment by Kwok (39). This was believed to indicate that two consecutive reactions might be involved in the oxidation of hydroxylamine to nitrite with an intermediate of the oxidation level (NOH).

That NH_3 rather than NH_4^+ was the substrate for oxidation in Nitrosomonas was suggested by the observation that ammonia showed higher affinity at a higher pH values (73). A mechanism of ammonia oxidation was proposed by Suzuki (72) in which the involvement of hypothetical ammonia hydroxylase catalysing the reaction: $NH_3 + O_2 + AH_2 \longrightarrow NH_2OH + H_2O + A$ was indicated. An

unidentified electron or hydrogen carrier A might possibly be cytochrome P-460. This cytochrome P-460, the properties of which were found to be similar to those of cytochrome P-450 from Pseudomonas or from mammalian microsomes was purified by Erickson and Hooper in 1972 (18).

The present study was undertaken in order to further our present knowledge of the mechanism of the oxidation of ammonia in cell-free extracts of N. europaea. An attempt was made to study the characteristics of partially resolved and reconstituted system active in ammonia oxidation. A comparative structural study by electron microscopic examination of functionally active and inactive cells, cell-free extracts, as well as partially resolved and reconstituted system is also presented.

H I S T O R I C A L

HISTORICAL

One among the many unceasing and important contributions of microorganisms toward the constant circulation of the essential element, nitrogen, is a process known as nitrification. As early as in 1862, Pasteur suggested that the oxidation of ammonia to nitrate (nitrification) might be the work of microorganisms present in the soil (cited in ref. 3). However, the conclusive evidence came after fifteen years in 1877 when Schloesing and Muntz (66) demonstrated the biological origin of the process. That this process occurs in two steps (first, the oxidation of ammonia to nitrite and second, the oxidation of nitrite to nitrate) was first established by Warrington in 1891 (81). The organisms responsible for bringing about nitrification process were subsequently isolated from soil and were termed Nitrosomonas and Nitrobacter by Winogradsky (86).

The organisms oxidizing ammonia to nitrite belong to the genus Nitrosomonas and were described by Winogradsky (86) as Gram-negative, oval cells of

about 1.0 to 1.5 micron in size and motile with a single polar flagellum. Winogradsky also observed that nitrification occurred under aerobic conditions only. Any device for increasing the oxygen supply such as an introduction of a stream of air bubbles into liquid cultures resulted in an increase in nitrite formation from ammonia by Nitrosomonas (23, 40). Pure oxygen however, was found to cause an irreversible drop in the rate of respiration (51). The nutritional requirements of Nitrosomonas were found to include calcium, magnesium, phosphorus, iron and probably copper (49, 50).

The member of the genus Nitrosomonas fulfil their major energy and carbon needs by the oxidation of ammonia and the fixation of carbon dioxide. The overall reaction in the oxidation of ammonia to nitrite is:

$$NH_3 + 1\tfrac{1}{2}\ O_2 \longrightarrow NO_2^- + H_2O + H^+$$

Kluyver and Donker in 1926 (38) suggested that hydroxylamine was an intermediate in this reaction. Using hydrazine as an inhibitor, Lees (41) and Hofman and Lees (26) demonstrated the accumulation of hydroxylamine during the oxidation of ammonia by intact

Nitrosomonas cells. Since thiourea or allylthiourea powerfully inhibited the oxidation of ammonia but not hydroxylamine, it was inferred that the enzyme system oxidizing ammonia to hydroxylamine involved a copper protein (42). Thus the first reaction in the oxidation of ammonia was formulated as:

$$NH_4^+ + \tfrac{1}{2}\ O_2 \longrightarrow NH_2OH + H^+$$

Hyponitrite was suggested to be the possible intermediate in the oxidation of hydroxylamine to nitrite (38). Lees (43) suggested an unstable nitroxyl-type (NOH) intermediate, Aleem et al (1) proposed nitro-hydroxylamine whereas Hughes and Nicklin (33) suggested peroxonitrite (ONO_2^-) as a possible intermediate. However, none of these have been substantiated by experimental evidence and up until today the oxidation of ammonia to nitrite can be written as:

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

where (X) represents the unknown intermediate.

OXIDATION OF HYDROXYLAMINE

Although Imshenetskii and Ruben (34, 35) and Engel and Alexander (15) first reported slow oxidation of ammonia and hydroxylamine in the autolysates of Nitrosomonas, the possibility of the intact cells in their preparations could not be overlooked. The first conclusive report of hydroxylamine oxidation in cell-free extracts of Nitrosomonas was presented by Nicholas and Jones in 1960 (55). They demonstrated that cell-free extracts prepared by ultrasonic probe oxidized hydroxylamine to nitrite in the presence of a suitable electron acceptor such as cytochrome c or phenazine methosulphate. However, ammonia was not oxidized by their preparation although it stimulated the oxidation of hydroxylamine. They achieved fourty-four-fold purification of hydroxylamine-oxidizing enzyme system by precipitation with ammonium sulfate and fractionation on DEAE-cellulose columns. Subsequently in 1963, Aleem and Lees (2) showed that hydroxylamine oxidation by intact cells or cell-free preparations of Nitrosomonas was mediated by cytochromes of b-, c- and a-type components. These workers partially purified (85 fold) hydroxylamine-cytochrome c reductase enzyme complex which also contained cytochrome oxidase activity.

Since 60% inhibition of hydroxylamine-cytochrome c reductase caused by 0.5 mM atebrin (flavin analog) was completely reversed by the addition of 1 mM FMN, the involvement of flavin was indirectly indicated (2). Similar results were independently reported by Falcone, Shug and Nicholas (21, 22). Nicholas et al (56) also observed that the paramagnetic resonance spectra of Nitrosomonas particles contained a copper protein exhibiting a signal around 2.1 g. Upon the addition of hydroxylamine, the copper signal underwent complex changes indicating the involvement of the metal in the oxidase system. Hooper and Nason (27) compared the properties of the electron transport system involved in hydroxylamine oxidation by Nitrosomonas europaea and Nitrosocystis oceanus. The presence in Nitrosomonas cells of a P-450-like cytochrome and a soluble terminal oxidase identified as cytochrome of o-type was reported by Rees and Nason (61). It was believed that the P-450-like cytochrome, which in other biological systems (20) plays a role in certain oxygenase reactions (e.g. in activation of atmospheric oxygen for substrate hydroxylation), might be involved as an oxygenase in the oxidation of ammonia to nitrite. In keeping with this, a report was made that at least one of the oxygen atoms in the nitrite arising from the oxidation of ammonia was in

fact derived from atmospheric oxygen (62). Rees (63) purified the enzyme hydroxylamine oxidase to a homogeneous preparation with a molecular weight of about 200,000. The purified preparation contained cytochromes of b- and c-type but no flavin. Maximum nitrite formation occurred only in the presence of phenazine methosulphate. Rees presented evidence that hydroxylamine oxidase was essentially a spherical particle with a diameter of about 160 Å (63). Peroxidase was purified from Nitrosomonas (6) but no evidence was obtained in support of its possible involvement in ammonia oxidation. Also, a nitrite-reducing enzyme was characterized from N. europaea by Hooper in 1968 (29). He suggested that nitrite reductase and terminal oxidase might possibly be separate enzymes competing with each other for the electrons originating from hydroxylamine and that terminal oxidase activity was rate limiting for hydroxylamine oxidation and the rate of nitrite reduction was greater than the rate of reduction of oxygen. In Hooper's study the final products of nitrite reduction were nitrous and nitric oxides (N_2O and NO).

Solubilization and purification of cytochrome a_1 and also a CO-binding heme protein referred to as

cytochrome P-460 was reported in 1972 by Erickson, Hooper, and Terry (19) and by Erickson and Hooper (18) respectively. Tronson, Ritchie, and Nicholas (78) purified c-types cytochromes from cell-free extracts of N. europaea. Yamanaka and Shinra (88) also reported the purification, properties and function in hydroxylamine oxidation of cytochromes c-552 and c-554. They further observed that cytochrome c-552 which was contended as the cytochrome c of the organism in a functional sense did not react directly with the enzyme hydroxylamine-cytochrome c reductase while cytochrome c-554 reacted slowly with the enzyme. However, in the presence of a small amount of cytochrome c-554, which was shown to be autooxidizable, cytochrome c-552 was found to be reduced fairly rapidly by hydroxylamine-cytochrome c reductase with hydroxylamine as a substrate. From their results, Yamanaka and Shinra (88) concluded that electron transfer from hydroxylamine to oxygen in N. europaea occurred as follows:

NH_2OH ⟶ NH_2OH-cytochrome c reductase ⟶ cytochrome c-554 ⟶ cytochrome c-552 ⟶ cytochrome oxidase ⟶ Oxygen.

OXIDATION OF AMMONIA

Although a considerable amount of knowledge had accumulated on the mechanism of the oxidation of hydroxylamine to nitrite in the cell-free extracts of Nitrosomonas, the mechanism of ammonia oxidation could not be studied intensively until recently because of the difficulties encountered in obtaining active cell-free extracts. Engel and Alexander (16) prepared Nitrosomonas extracts by sonic oscillations. A very slow formation of nitrite was detected when these extracts were incubated either with ammonia or hydroxylamine. However, the possibility of residual cells could not be eliminated. Rees and Nason (62) found a small incorporation of ^{18}O from $^{18}O_2$ into nitrite formed during the oxidation of ammonia by Nitrosomonas cells. Hooper in 1969 (30) observed a lag in ammonia oxidation by a freshly diluted suspension of resting cells of Nitrosomonas. The lag was diminished when the cells were preincubated in a dilute suspension or when a small quantity of hydroxylamine was included in the reaction mixture. It was suggested that the oxidation of hydroxylamine was probably coupled to ATP (adenosine triphosphate) synthesis and the generation of reduced pyridine nucleotide and that one of those compounds was possibly required directly or indirectly for activation of the ammonia oxidation process.

The ammonia-oxidizing activity of inactive spheroplasts could be restored by preincubation with magnesium or by the addition of hydroxylamine thus suggesting a possible structural requirement of cell membranes for ammonia oxidation (70). Watson, Asbell and Valois (83) and Suzuki and Kwok (71) simultaneously and independently obtained cell-free extracts capable of oxidizing ammonia. Watson, Asbell and Valois's system was obtained by rupturing Nitrosocystis oceanus, a marine organism, in sea water by means of a French Pressure Cell. These extracts required sea water, magnesium and ATP for ammonia oxidation. It was suggested that magnesium might have been essential to maintain the membrane in a biochemically and structurally active state (83). Cell-free extracts obtained by Suzuki and Kwok (70) from N. europaea cells by means of a French Pressure Cell, oxidized ammonia only when activated by BSA, magnesium or spermine. Egg albumin, casein or lysozyme did not replace BSA. Spermidine was not as effective but poly-L-lysine had the same effect as spermine. Glycerol, sucrose, mercaptoethanol or dithiothreitol were ineffective. Dipyridyl was found to inhibit ammonia oxidation in the cell-free system as well as intact cells (70).

STRUCTURAL STUDIES

A comparison of the ultrastructure of Nitrosocystis, Nitrosomonas and Nitrobacter was carried out by Murray and Watson (54). They found that despite the physiological correspondence of activities, Nitrosocystis and Nitrosomonas showed different organizations of the internal structure. The cell envelope of Nitrosocystis was shown to be composed of seven distinct layers and also an elaborate membrane structure was found across the entire cell (82). In contrast, N. europaea cells were observed to have several layers of membranes traversing around the entire cell but no membranes were observed across the cell (54).

The studies presented by Rees (63) indicated that the reduction in the hydroxylamine-oxidizing activity that invariably accompanied lysis of N. europaea cells was due to the concomitant physical separation of the terminal oxidase from hydroxylamine oxidase. Ritchie and Nicholas (65) reported that the addition of BSA to the cell suspension prior to cell disruption protected hydroxylamine oxidase. They suggested that a physical association between hydroxylamine oxidase and cytochrome oxidase was protected by BSA.

Hooper, Erickson, and Terry (31) described an intact membrane-envelope complex obtained by freezing and thawing of N. europaea cells. This complex contained approximately 50% of the cell protein and more than 90% of the ubiquinone and a-type mammalian cytochrome oxidase activity. The membrane fraction was described as a torn outer layer covering several large flattened vesicles approximately one-fifth the size of the entire complex. However, the ammonia-oxidizing activity of this complex was not reported. Watson, Asbell, and Valois (83) found that membrane pellet of Nitrosocystis which was active in ammonia oxidation, consisted of vesicles covered with 80 - 100 Å doughnut shaped particles. It was further pointed out that most of the enzymes involved in the oxidation of ammonia were particulate and membrane bound. Moreover, when the cells were crushed in the presence of magnesium, these particles were arranged in a rectilinear array suggesting that magnesium might be playing an important role of maintaining an ordered spatial arrangement of these particles on the membrane (83).

MATERIALS AND METHODS

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MATERIALS

Spermine (tetrahydrochloride), bovine serum albumin (fraction V), cytochrome c (type III, from horse heart), lysozyme (egg white), L-ascorbate (Na salt), valinomycin (crystalline), o-phenanthroline (1, 10-phenanthroline monohydrate), α,α'-dipyridyl (2,2'-bipyridyl), bathocuproin disulfonate (Na salt), 2, 4-dinitrophenol, dicumarol (bis-hydroxycoumarine), phospholipase A and TTA (2-theonyl tri fluoroacetone) were obtained from Sigma Chemical Company, St. Louis, U.S.A. NADH (nicotinamide adenine dinucleotide, reduced, disodium salt) was obtained from P. L. Biochemicals Inc., Wisconsin, U.S.A. Diethyldithiocarbamate (Na salt), lead nitrate and sodium citrate were from Fisher Scientific Corporation, N.J., U.S.A. Potassium cyanide, mercuric chloride and phosphotungstic acid were purchased from J.T. Baker & Co. N.J., U.S.A. Sodium azide, EDTA (ethylenediaminetetraacetic acid, Na salt) and uranyl acetate form B.D.H. Chemicals, England. CCCP (carbonyl cyanide m-chlorophenyl

hydrazone) and deoxyribonuclease (pancreatic) were from Calbiochem, California, U.S.A. Sepharose 6B and Blue Dextran 2000 were from Pharmacia, Sweden. Nitrogen and carbon monoxide gas cylinders were obtained from Union Carbide Co., Canada.

ORGANISM

Nitrosomonas europaea (Schmidt strain) kindly provided by Dr. A. B. Hooper was grown in batch cultures, harvested and washed as described by Kwok (39). The cell suspension (20 mg wet cells per ml) in 0.1 M potasium phosphate buffer was stored at 4°C. The cells thus obtained were considered as fresh cells when they were used within 3 to 4 days and as old cells if they were stored for longer periods, the maximum being 6 to 8 weeks.

METHODS

Standard Method for the Preparation of Cell-Free Extracts

All the procedures were carried out at 4°C unless otherwise mentioned. Fresh cells were washed once and were resuspended (30 mg wet cells per ml) in

0.1 M potassium phosphate buffer (pH 7.5) containing BSA (20 mg per ml) unless otherwise indicated. This cell suspension was then passed through a French Pressure Cell (Aminco) at 18,000 p.s.i. The resultant cell homogenate was centrifuged at 2,000 x g for 20 minutes. The supernatant, "cell-free extract", which contained 3.8 mg of extract protein per ml, was carefully aspirated with a syringe and was kept at $4^{o}C$ until further used. The preparation of the cell-free extract under nitrogen atmosphere was carried out in the same manner as described above except that the cells were suspended in buffer without BSA and nitrogen gas was gently bubbled on the surface of the cell suspension before its passage through the French Pressure Cell.

Determination of Protein

Protein was determined by the colorimetric method of Lowry *et al* (46). Crystalline BSA was used as the reference protein.

Assay of Ammonia-oxidizing Activity

Ammonia oxidation was routinely followed by

oxygen uptake in a Gilson Oxygraph (G.M.E., Wisconsin, U.S.A.) using a Clark Oxygen Electrode at 25°C. The reaction mixture contained 0.5 ml of cell-free extract (3.8 mg extract protein per ml) or 0.5 ml of Sepharose 6B fraction(s) and 0.1 M potassium phosphate buffer (pH 7.5) in a total volume of 1.5 ml. Spermine and ammonium sulfate (and/or other reagents if and when used) were added in microlitre volumes by using Hamilton microsyringes equipped with long needles. The reaction mixture was constantly stirred with a small magnetic stirring bar. The activity was expressed in terms of nmoles of oxygen consumed per minute.

Centrifugations

Low speed centrifugations were carried out in a Sorvall RC-2B centrifuge at 4°C. High speed centrifugations were done in a Beckman model L2-65 B ultracentrifuge using a 60 Ti rotor at 4°C.

Determination of Nitrite

Nitrite was determined by the method of Bratton and Marshall (8). The reagents consisted of 0.12% of N-(1-naphthyl)-ethylenediamine dihydrochloride in

distilled water and 1% sulfanilic acid in 20% HCl.

In the experiments of Table 7, 0.5 ml of a reaction mixture was diluted with distilled water to 1 ml and 1 ml each of sulfanilic acid and N-(1-napthyl)-ethylenediamine dihydrochloride solutions were added to it. The tubes were incubated at room temperature (25°C) for 10 minutes to insure maximum color development. The volume of each reaction sample was then made up to 10 ml with distilled water. If any turbidity was observed, the solutions were centrifuged to remove it. The intensity of color was measured in a Klett Summerson colorimeter using No. 54 filter.

A standard curve was prepared with 0.5 ml of sample (cell-free extract diluted 1:3 with 0.1 M potassium phosphate buffer, pH 7.5) and was treated in the same manner using known concentrations of sodium nitrite. Zero adjustment was done with reagent blank consisting of no added nitrite.

Spectrophotometric Analyses

All the spectrophotometric analyses were carried out at room temperature using a Shimadzu Multipurpose Recording Spectrophotometer Model MPS-50L with 1 cm light path. NADH oxidation was followed by measuring the change in absorption at 340 nm. Cytochrome *c* reduction or oxidation was measured by following the change in optical density at a fixed wavelength of 550 nm.

Preparation of Reduced Cytochrome c (Mammalian)

Reduced cytochrome c was prepared according to the method of Wharton and Tzagoloff (85) with slight modification.

One percent solution of cytochrome c (type III, from horse heart) was prepared in 0.01 M potassium phosphate buffer containing an excess amount of ascorbic acid. The pH of the solution was adjusted to 7.0 with potassium hydroxide. Excess of ascorbate was removed by dialysis in size 8 Visking dialysis tubing against 0.01 M potassium phosphate buffer (pH 7.0) overnight with 3 changes of buffer.

Preparation of Partially Purified NH_2OH-Cytochrome c Reductase

Unless otherwise mentioned, all the steps were carried out at 4°C. NH_2OH-Cytochrome c reductase was partially purified (up to fraction 4, ref. no. 27) by the method of Hooper and Nason (27) with slight modification.

N. europaea cell suspension (5 g wet cells per 100 ml) in tris-HCl buffer (0.1 M, pH 8.0) was sonicated for 15 minutes in a 10 Kc Ratheon sonicator with the use of maximum power. The sonicate was centrifuged for 15

minutes at 10,000 x g and the clear red supernatant (6.5 mg protein per ml) was centrifuged at 100,000 x g for 2 hours. The resultant supernatant was dialyzed against 1 mM potassium phosphate buffer (pH 7.5) overnight with three changes of buffer. The dialyzed supernatant (fraction 2) was then added at the rate of three drops per minute on the top of a column (1.5 x 30 cm) of washed DEAE-cellulose (Sigma) which had been packed under pressure and equilibrated with 1 mM potassium phosphate buffer (pH 7.5). The column was perfused with 250 ml of the same buffer. A linear gradient elution procedure (0 - 0.5 M KCl) was employed as described by Hooper and Nason (27). A pooled enzyme fraction (eluted at approximately 0.2 M KCl, fraction 3, Hooper and Nason) was concentrated by ammonium sulfate fractionation (0.5 g per ml) and the concentrated precipitate was dissolved in a minimum volume of 0.1 M potassium phosphate buffer (pH 7.5). The concentrated enzyme preparation was dialyzed overnight with three changes of buffer. The dialyzed, partially purified NH_2OH-cytochrome c reductase preparation (fraction 4, 1.5 mg protein per ml) thus obtained was assayed in 0.01 and 0.1 M potassium phosphate buffer (pH 7.5) according to Hooper and Nason (27) and was stored at -20°C until required. This preparation did not show any cytochrome oxidase activity when it was assayed in

0.1 M potassium phosphate buffer (pH 7.5) with reduced cytochrome c (type III, from horse heart) as a substrate.

Preparation of Membrane Envelope Complex

Membrane envelope complex from N. europaea cells was prepared as described by Erickson, Hooper and Terry (19).

Cell-suspension of N. europaea (0.2 g wet cells per ml) in 0.05 M potassium phosphate buffer (pH 7.5) containing a small amount of pancreatic deoxyribonuclease was frozen at -10°C and thawed three times. The resultant homogenate was centrifuged at 20,000 x g for 20 minutes (at 4°C). The supernatant was carefully aspirated by means of a syringe and was designated as "freeze-thaw supernatant".

The particulate fraction was washed 6 times with 0.1 M potassium phosphate buffer (pH 7.5) and finally suspended in the same buffer (400 mg wet weight per ml) to yield a membrane envelope complex (40 mg protein per ml) which was stored at -20°C until further used for the preparation of S_1, S_2 and cytochrome a_1.

Preparation of S_1, S_2 and Cytochrome a_1

All the steps were carried out at 4°C unless otherwise mentioned. The membrane envelope complex

obtained as described above, was passed through a French Pressure Cell for three times at 16,000 p.s.i. The resultant cell-homogenate was centrifuged at 20,000 x g for 20 minutes. The cloudy brown supernatant was further centrifuged at 78,000 x g for 2 hours to yield a red gelatinous pellet and a clear brownish yellow supernatant. The supernatant, designated as S_1 was saved and stored at -20°C while the pellet was suspended in 0.1 M potassium phosphate buffer (pH 7.5) which contained 5% KCl (w/v), stirred for 12 hours and centrifuged at 78,000 x g for 2 hours. The supernatant thus obtained was designated as S_2 (2.2 mg protein per ml) and was stored at -20°C. The pellet was washed twice and suspended in 0.1 M potassium phosphate buffer (pH 7.5) containing 0.5% triton X-100 (v/v) and was used for partial purification of cytochrome a_1 according to Erickson, Hooper and Terry (19) up to fraction 7 (30 mg protein per ml).

Partial Resolution of the Ammonia-oxidizing System from the Cell-free Extracts

(1) Differential Centrifugation Method

The cell-free extract (15 ml) obtained by the standard method of preparation as described earlier was divided into 2 parts; one was kept at 4°C under nitrogen atmosphere for 1 hour while the other was centrifuged at 100,000 x g for 1 hour at 4°C under nitrogen atmosphere. The supernatant was aspirated carefully into another tube. The pellet was washed twice and was suspended in 1/10th of the original volume of 0.1 M potassium phosphate buffer

(pH 7.5) and stored at 4°C until assayed.

Assay of the ammonia-oxidizing activity of the supernatant (1.6 mg protein per ml) and/or pellet (0.5 ml and 0.05 ml respectively) was carried out as described earlier.

(2) Column Chromatography

A glass column (1.8 x 45 cms) was packed with Sepharose 6B which had been washed first with distilled water and then with 0.1 M potassium phosphate buffer (pH 7.5). The column was then equilibrated with the same buffer. In case of the anaerobic columns, the buffer was continuously bubbled with nitrogen gas through a sparger and the column was equilibrated with this nitrogen-bubbled buffer for at least one hour (60 ml of buffer) prior to the application of the sample. For partially anaerobic columns, nitrogen bubbling was started as soon as the elution of the 1st fraction (membrane fraction) began and was continued until both the remaining fractions (fractions 4 and 6, see below) were eluted. Void volume of the column was determined from the elution volume of Blue Dextran 2000 and the flow rate was adjusted approximately to 0.8 to 1.0 ml per minute.

A sample of 4 ml of the cell-free extract (or 1.5 ml of 100,000 x g pellet) obtained as described earlier was applied to the top of the column by care-

fully injecting it onto the sides of the column with a syringe. As soon as all the sample went into the Sepharose, the column was eluted with 0.1 M potassium phosphate buffer (pH 7.5). Whenever anaerobic conditions were required, the column was tightly closed with a serum bottle cap or a rubber stopper and the sample was injected through it. A needle was pierced through the stopper and it was in turn connected through a rubber tubing to a bottle of nitrogen-bubbled buffer.

Fraction 1 which was a large molecular weight membrane fraction, immediately separated out from a brown band which contained fraction 4 and fraction 6 and was excluded as a cloudy buff white fraction. The brown band moved slowly and separated into two fractions when it had travelled approximately 2/3 the length of the column. The whole fractionation procedure was carried out at room temperature and it did not take longer than 2 to 2.5 hours. Each fraction (1.5 x volume of the original sample) was collected in a long narrow tube kept in a bucket of ice and was stoppered with a cork stopper immediately. The protein contents of fractions 1, 4 and 6 were 1.4, 1.6 and 0.78 mg per ml respectively.

The fractions were assayed for their ammonia-oxidizing activity as described earlier using 0.5 ml of each fraction, 2 mM spermine and 1.7 mM ammonium sulfate. Potassium phosphate buffer (0.1 M, pH 7.5) was used to make up the volume to 1.5 ml whenever required.

DEAE-cellulose Fractionation of S_1

DEAE-cellulose powder (Schleicher & Schuell Inc., New Hampshire) was washed with 0.1 N HCl, followed by the washing with several changes of distilled water, and finally with 0.1 M potassium phosphate buffer (pH 7.5). A glass column (2 x 28.5 cm) was packed with this DEAE-cellulose and was equilibrated with the same buffer anaerobically as described earlier.

A sample (3 ml) of S_1 was carefully applied on the top of the column and was allowed to adsorb on DEAE cellulose. The column was then washed with nitrogen-bubbled equilibrating buffer (0.1 M potassium phosphate, pH 7.5). A brown fraction (9 ml) which eluted during the washing of the column with the equilibrating buffer was designated as AS_1 (0.16 mg protein per ml). A dark brown portion remained on the top of the column most of which could be eluted with either 0.25 M potassium phosphate buffer or with 0.1 M potassium phosphate buffer containing 0.25 M

KCl. This fraction was labeled as AS_2 (0.21 mg protein per ml).

One ml out of 9 ml of each of AS_1 and AS_2 fractions was saved as control and the rest was concentrated by powdered sucrose separately at room temperature as described below. Ten fold concentration could be achieved in about 2 hours. Both the dilute (control) and the concentrated AS_1 and AS_2 were stored in small cork-stoppered tubes at $-20^{\circ}C$ until further used.

Concentration of Fractions by Powdered Sucrose

Concentrations of (4 + 6) (Sapharose 6B fractions 4 & 6 combined) and AS_1 and AS_2 (separately) fractions were carried out at room temperature in a dialysis tubing which had been boiled in and washed with 0.1 M potassium phosphate buffer (pH 7.5) containing 5 mM EDTA. The dialysis tubing was filled with the fraction and tied tightly with a piece of string. It was then put into a glass dish containing powdered sucrose. Sucrose powder was changed several times. Five fold concentration of (4 + 6) fraction and ten fold concentration of AS_1 and AS_2 was achieved in about 2 to 2.5 hours.

Electron Microscopic Studies

All the electron microscopic examinations were carried out on an AEI (Associated Electrical Industries Ltd., England) electron microscope Model EM 6B operated at 60 Kv.

(1) Reagents Used

(i) Fixative (Hess (25) method) Ten ml of fixative were prepared fresh for every experiment by mixing the following reagents:

Gluteraldehyde (25%, K & K Lab., U.S.A.) ..... 1.2 ml
Acrolein (Eastman Kodak & Co., N.Y., U.S.A.)..... 0.3 ml
Distilled Water ..... 3.5 ml
0.2 M Cacodylate buffer (City Chem., N.Y., U.S.A.) ..... 5.0 ml

(ii) Uranyl acetate (B.D.H. Chem., England). 0.5% aqu. sol.

(iii) Osmium tetroxide (Steven's Met. Corp., U.S.A.). 1% aqu. sol.

(iv) Ethyl alcohol. 50%, 70%, 90% & absolute

(v) Embedding mixture. Methyl methacrylate

(Matheson Coleman & Bell, U.S.A.) 15 ml, butyl methacrylate (same as above) 85 ml, divinyl benzene (5%, Dow Chemicals) 5 ml and benzoyl peroxide (Matheson, Coleman & Bell) 1 g. These chemicals were mixed and allowed to age at 4°C for at least 24 hours before use.

(vi) Stains. Lead citrate stain was prepared according to Reynolds method (64) as follows:

A mixture of 1.33 g of lead nitrate and 1.76 g of sodium citrate was suspended in 30 ml of distilled water (CO_2-free) in a 50 ml volumetric flask. The suspension was allowed to stand at room temperature for 30 minutes with intermittent shaking. Eight ml of 1 N NaOH were then added to the flask and the contents were mixed well. The volume was made up to 50 ml with distilled water (CO_2-free). Upon mixing by inversion, lead citrate dissolved completely and if there was any turbidity remained, the solution was centrifuged to remove it.

Potassium phosphotungstate for negative staining was prepared as follows:

One per cent solution of phosphotungstic acid (J.T. Baker & Co., U.S.A.) was prepared in distilled water and the pH of the solution was adjusted to 7.4 with concentrated KOH solution.

(2) Methods

Intact cells. Fresh active N. europaea cells, which oxidized ammonia rapidly and old cells (preserved for 6 to 8 weeks at 4°C) which oxidized ammonia either very slowly or not at all, were incubated with 1.7 mM ammonium sulfate for 1 minute. Immediately after the incubation, both the cell-suspensions were prefixed with the fixative mixture for 2 hours at room temperature. The cells were then pelleted by centrifugation at 20,000 x g for 20 minutes (at 4°C) and embedded in 2% agar. The gelled agar was cut into tiny square cubes. The cubes were washed with four changes (at 15 minutes intervals) of 0.1 M cacodylate buffer (pH 7.5), post-fixed with 1% OsO_4 solution for 2 hours at 4°C, washed again with four changes of the same buffer and were then allowed to stand in 0.5% uranyl acetate solution overnight at room temperature. On the following day, the agar cubes were washed once with the cacodylate buffer and dehydrated by allowing them to stand at room temperature for 15 minutes each in 50, 70 and 90% ethanol and finally in absolute alcohol for 1 hour with one change of alcohol at 30 minutes interval.

The dehydrated agar cubes were placed in gelatin capsules (Parke-Davis & Co., Canada) - one cube per capsule-containing a few drops of the embedding mixture and allowed to stand at room temperature for at least 2 hours.

The capsules were then filled with the embedding mixture keeping the agar cube in the center with a long needle and the capsules were kept in a vacuum oven at 50°C for 16 to 24 hours to polymerize the resin to yield clear plastic blocks.

Ultra-thin sections (60-90 nm) were cut on an LKB type 4802 A (LKB Productur-Sweden) or a Reichert Om U2 (Reichert, Austria) ultrotome with a glass knife prepared by using an LKB type 7801 A Knife-Maker. The sections, mounted on a carbon-coated copper grids (Ladd Research Corporation, U.S.A.), were stained with a freshly prepared lead citrate solution, air-dried and examined on the electron microscope.

Cell-free extracts. The cell-free extract from fresh N. europaea cells was prepared without the addition of BSA and the ammonia-oxidizing activity was assayed on a Gilson Oxygraph as described previously. There was no oxygen uptake when 1.7 mM of $(NH_4)_2SO_4$ was added to the reaction mixture (Fig. 1) in the Oxygraph cuvette. A control sample (taken as an "inactive extract") of 10 microlitres was withdrawn and was mixed well with 0.19 ml of 0.1 M potassium phosphate buffer (pH 7.5). A tiny drop of this diluted sample was spread as a thin film on carbon coated copper grids (Ladd Research Corporation, U.S.A.) and allowed to stand at room

temperature for 15 minutes. The grids were negatively stained for 10 seconds with a freshly prepared solution of 1% potassium phosphotungstate and allowed to air-dry at room temperature. Ammonia was oxidized rapidly upon the addition of either spermine or BSA (Fig. 1), prior to the addition of the substrate, to the reaction mixture. The oxidation was allowed to proceed for one minute before withdrawing a 10 microlitre sample of the "active extract" which was treated exactly in the same manner as described for the control sample.

The inhibition of active ammonia oxidation was achieved by the addition of 0.3 mM dipyridyl to the reaction mixture. The inhibitor was allowed to react for 3 minutes before the withdrawal of the sample. This sample was also treated in the same manner as the control.

All the grids were air-dried and examined on the electron microscope.

Partially resolved and reconstituted system. The Sepharose fractions were obtained under partially anaerobic conditions, from active cell-free extract (prepared from fresh cells in the presence of BSA) as described earlier. Neither fraction 1 nor fraction 4 plus 6 oxidized ammonia when they were assayed sep-

arately in the presence of spermine or BSA. However, there was a rapid oxidation when all the three fractions were combined together (Table 16). Aliquots of samples from the reaction mixtures (1.5 ml containing either fraction 1 alone, fractions 4 plus 6 or fractions 1 plus 4 plus 6 and 2 mM spermine, 1.7 mM $(NH_4)_2SO_4$ and 0.1 M potassium phosphate buffer of pH 7.5) were withdrawn after incubation for 1 minute and diluted 15 times with 0.1 M potassium phosphate buffer (pH 7.5). The diluted samples were thinly spread on carbon-coated copper grids and negatively stained in exactly the same manner as described for the cell-free extract control sample. The grids were allowed to air dry and examined.

R E S U L T S

RESULTS

PART I. GENERAL ASPECTS OF CELL-FREE AMMONIA AND HYDROXYLAMINE OXIDATIONS

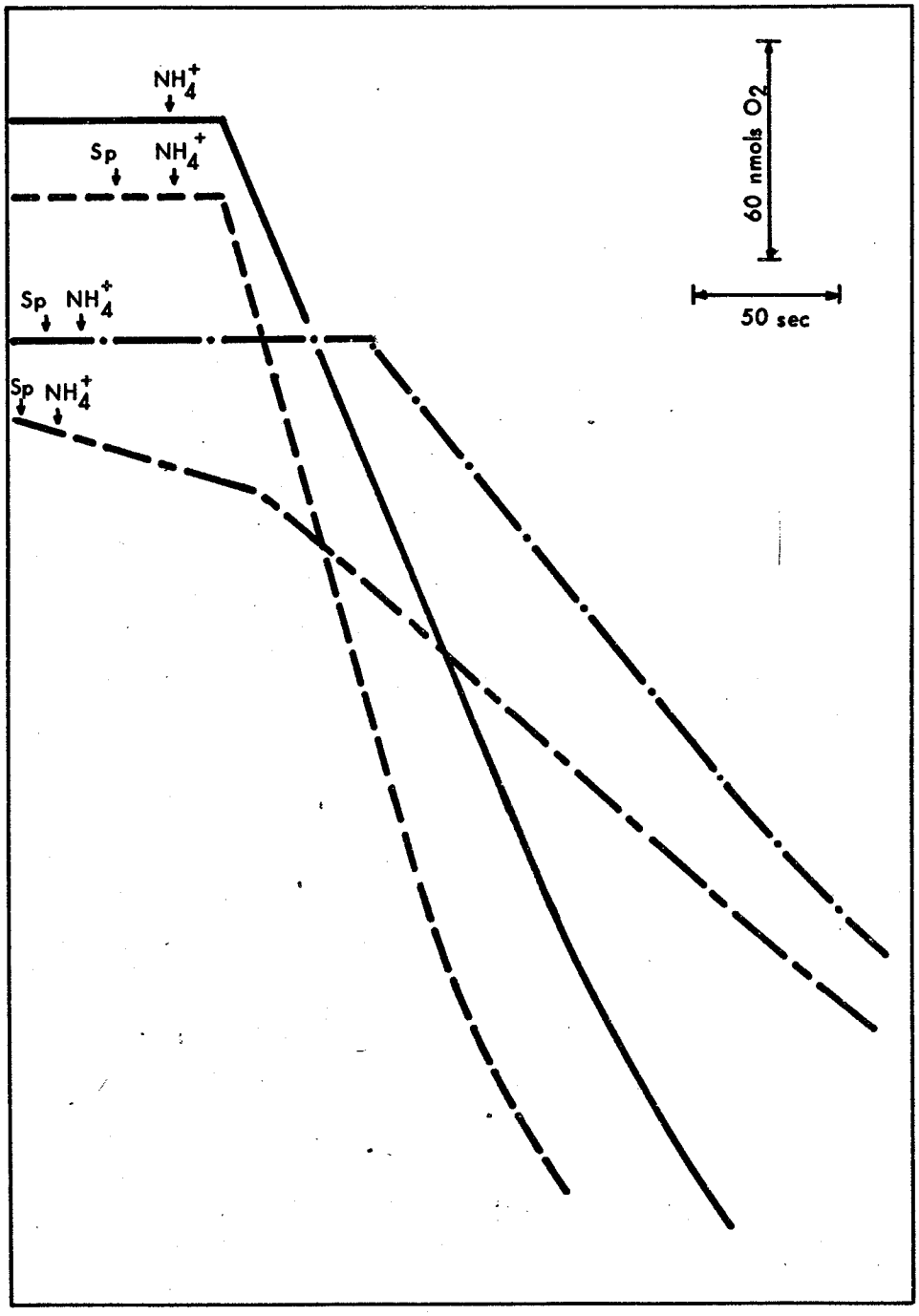
Conditions for the Preparation of Active Ammonia-Oxidizing Extracts

Cell-free extracts of Nitrosomonas europaea oxidized ammonia to nitrite when activated by spermine and/or bovine serum albumin (BSA). The extract was most active when fresh cells were broken in the presence of BSA and the resulting extract was then activated with spermine. However, the activity was much lower in the extracts obtained by breaking the cells under partial N_2 atmosphere (Fig. 1). There was always a lag period of various lengths after the addition of ammonia before the oxygen consumption started.

When the extract was prepared from old cells which were preserved as a 20 mg/ml suspension in 0.1 M potassium phosphate buffer (pH 7.5) at 4°C for 4 to 6 weeks, the ammonia-oxidizing activity was much

Fig. 1. Ammonia oxidation by cell-free extracts of N. europaea. The reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of the extract, 1.7 mM $(NH_4)_2SO_4$ and 0.1 M potassium phosphate buffer (pH 7.5). Spermine (2 mM) when indicated was added before $(NH_4)_2SO_4$ addition. The cell-free extracts were prepared from fresh cells as described in Materials and Methods under the following conditions:

Extract prepared under nitrogen, — - — -; extract prepared without any additions, — . — ; extract prepared in the presence of BSA and assayed without spermine, ——— and with spermine. ——— —


NH_4^+
Sp
NH_4^+
Sp
NH_4^+
Sp
NH_4^+
60 nmols O_2
50 sec

lower even when the extract was activated by spermine and BSA together. The addition of NADH stimulated the activity, but $CuCl_2$ addition in the presence of BSA and NADH was found to be required for maximum activity (Table 1). When $CuCl_2$ was added in the absence of BSA it inhibited ammonia oxidation and the activity could not be restored by NADH. However, BSA did reverse the inhibition although the activity (after the reversal of inhibition) was not as high as it was when $CuCl_2$ was added after BSA followed by NADH (Table 1). The increased rate of 0_2 consumption by the addition of NADH was earlier shown to be due to the increased rate of ammonia oxidation to nitrite, NADH acting catalytically, by a stoichiometric and time course study (39). In the absence of ammonia NADH was oxidized only very slowly as shown later (Fig. 6).

Effect of Dialysis

Almost all the activity was lost when the extract prepared from fresh cells was dialyzed against 0.1 M potassium phosphate buffer (pH 7.5) for 1 hour at $4^{o}C$. The activity could be restored and was further stimulated by the addition of $CuCl_2$ and NADH (Table 2). However, some extracts which

Table 1. Activation of ammonia-oxidation in the cell-free extracts from old cells of *N. europaea*.

Reaction	Additions*	O_2 Uptake (nmoles/min)	Lag (min)
1	Spermine + $(NH_4)_2SO_4$	26	1.5
2	Spermine + $(NH)_2SO_4$ + NADH	40	0
3	BSA + $(NH_4)_2SO_4$	2	-
4	BSA + $(NH_4)_2SO_4$ + $CuCl_2$	2	-
5	BSA + $(NH_4)_2SO_4$ + $CuCl_2$ + NADH	36	0
6	Spermine + $(NH_4)_2SO_4$ + $CuCl_2$	4	-
7	Spermine + $(NH_4)_2SO_4$ + $CuCl_2$ + NADH	4	-
8	Spermine + $(NH_4)_2SO_4$ + $CuCl_2$ + NADH + BSA	28	0
9	Spermine + BSA + $(NH_4)_2SO_4$	28	1.5
10	Spermine + BSA + $(NH_4)_2SO_4$ + NADH	40	0
11	Spermine + BSA + $CuCl_2$ + $(NH_4)_2SO_4$	40	7.5
12	Spermine + BSA + $CuCl_2$ + $(NH_4)_2SO_4$ + NADH (or NH_2OH)	70	0

The cell-free extract was prepared without BSA, from 4 to 6 weeks old cells and the reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of the extract and 0.1 M potassium phosphate buffer (pH 7.5).

*The additions were made in the sequence shown in the table in microlitre volumes. Final conc. Spermine, 2 mM; $(NH_4)_2SO_4$, 1.7 mM; BSA powder, 20 mg/ml; $CuCl_2$, 0.1 mM and NADH, 67 μM.

Table 2. Effect of dialysis on the cell-free extracts of *N. europaea*.

Reaction	Extract	O_2 Uptake (nmoles/min)
1	Control	28
2	Control + $CuCl_2$ (or NADH)	40
3	Control + $CuCl_2$ + NADH	65
4	Dialyzed	2
5	Dialyzed + $CuCl_2$ (or NADH)	38
6	Dialyzed + $CuCl_2$ + NADH	60

The cell-free extract was the same as used in Table 1. The reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The cell-free extract was divided into 2 parts. One was kept at 4°C as control and the other was dialyzed against 0.1 M potassium phosphate buffer (pH 7.5) for one hour with one change of buffer at half an hour interval. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of the extract, 2 mM spermine, 30 mg BSA, 1.7 mM $(NH_4)_2SO_4$ and 0.1 M potassium phosphate buffer (pH 7.5). $CuCl_2$ and/or NADH when indicated, were added in microlitre volumes to obtain a final concentration of 0.1 mM and 67 µM respectively.

were prepared in the presence of BSA and were highly active were not affected by dialysis under the above conditions.

Effect of Freezing

N. europaea cells lost over 70% of their ammonia-oxidizing activity when they were stored frozen at -20°C overnight. When the frozen cells were thawed and broken with BSA, the resultant cell-free extract did not oxidize ammonia and NADH, with or without $CuCl_2$, activated it only slightly (Table 3). The extract from unfrozen cells, however, could be stored frozen overnight without significant loss of the activity.

Effect of Pronase and Phospholipase

The ammonia-oxidizing activity of fresh, intact N. europaea cells was not affected by either pronase or phospholipase A treatment, but there was a considerable decrease in the activity of the cell-free extracts by either treatment (Table 4). These observations indicated the phospholipoprotein nature of the ammonia-oxidizing enzyme complex. However, the attempts to reactivate the phospholipase system by the addition of either lecithin micelles or the micelles of phospholipids extracted from N. europaea

Table 3. Effect of freezing on the ammonia-oxidizing activity of N. europaea cells and extracts prepared from such (frozen) cells.

Reaction No.	System	Additions	O_2 Uptake (nmoles/min)
1	Unfrozen cells	-	220
2	Frozen cells	-	62
3	Extract from unfrozen cells	Spermine (± NADH or $CuCl_2$)	100
4	Extract from frozen cells	Spermine	2
5	Extract from frozen cells	Spermine + NADH	10
6	Extract from frozen cells	Spermine + $CuCl_2$ (± NADH)	10
7	Frozen extract*	Spermine	78
8	Frozen extract*	Spermine + NADH	92
9	Frozen extract*	Spermine + $CuCl_2$ (± NADH)	100

Fresh cell suspension (30 mg/ml) in 0.1 M potassium phosphate buffer (pH 7.5) was divided into 2 parts. One was stored at 4°C and the other at -20°C (frozen) overnight. The cell-free extracts were prepared on the following day and the reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained either 3 mg cells (wet weight) or 0.5 ml of the cell-free extract, 0.1 M potassium phosphate buffer (pH 7.5) and 1.7 mM $(NH_4)_2SO_4$. Addition of 2 mM spermine (before the addition of $(NH_4)_2SO_4$), 67 µM NADH and 0.1 mM $CuCl_2$ were done in microlitre volumes. *An extract from unfrozen cells was frozen and stored overnight at -20°C.

Table 4. Effect of pronase and phospholipase on ammonia-oxidizing activity of *N. europaea* cells and cell-free extracts.

Reaction	System	Treatment		Inhibition
		P-lipase A (µl)	pronase (µg)	%
1	Intact cells	25	-	0
2	Intact cells	-	100	0
3	Intact cells	25	100	0
4	Cell-free extract	25	-	70
5	Cell-free extract	-	50	75
6	Cell-free extract	25	50	96

The treatment of either intact cells (0.6 mg wet cells) or cell-free extract (0.5 ml) was carried out by preincubation with appropriate agent(s) at room temperature (25°C) for 7 minutes. The cell-free extract was prepared from fresh cells without BSA and the reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained either 0.6 mg wet cells or 0.5 ml of cell-free extract and 0.1 M potassium phosphate buffer (pH 7.5). Additions of spermine (2 mM, only in cell-free extract) and $(NH_4)_2SO_4$ (1.7 mM) were done in microlitre volumes.

according to Johnson and Davenport (37) were not successful. There was no effect of pronase and/or phospholipase A on hydroxylamine oxidation in intact cells or cell-free extracts of N. europaea.

Effect of pH on the K_m Values

The optimal pH and temperature for ammonia oxidation in the cell-free extracts were the same as those for ammonia oxidation by intact cells viz. pH 7.7 and 25°C in 0.1 M potassium phosphate buffer agreeing with the results found by Kwok (39). As shown in Fig. 2 and Fig. 3, the rate of ammonia oxidation by N. europaea cells or cell-free extracts was markedly influenced by the pH and the concentration of ammonia. The effect of increasing pH values was to reduce the slope of the double resiprocal rate-concentration plots without affecting the maximal velocity. The K_m values decreased with increasing pH (Table 5).

When the K_m values for ammonia were expressed in the concentration of undissociated form of ammonia (NH_3) rather than the total concentration of ammonia ($NH_4^+ + NH_3$) using a pK value of 9.25, the results shown on the right side of Table 5 were obtained.

Fig. 2. Effect of pH and ammonia concentration on the oxidation of ammonia by N. europaea cells. The reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained 0.6 mg wet cells, 0.1 M potassium phosphate buffer and ammonia as indicated. The initial rate of oxidation (v) was expressed as nmoles O_2 consumed per min.

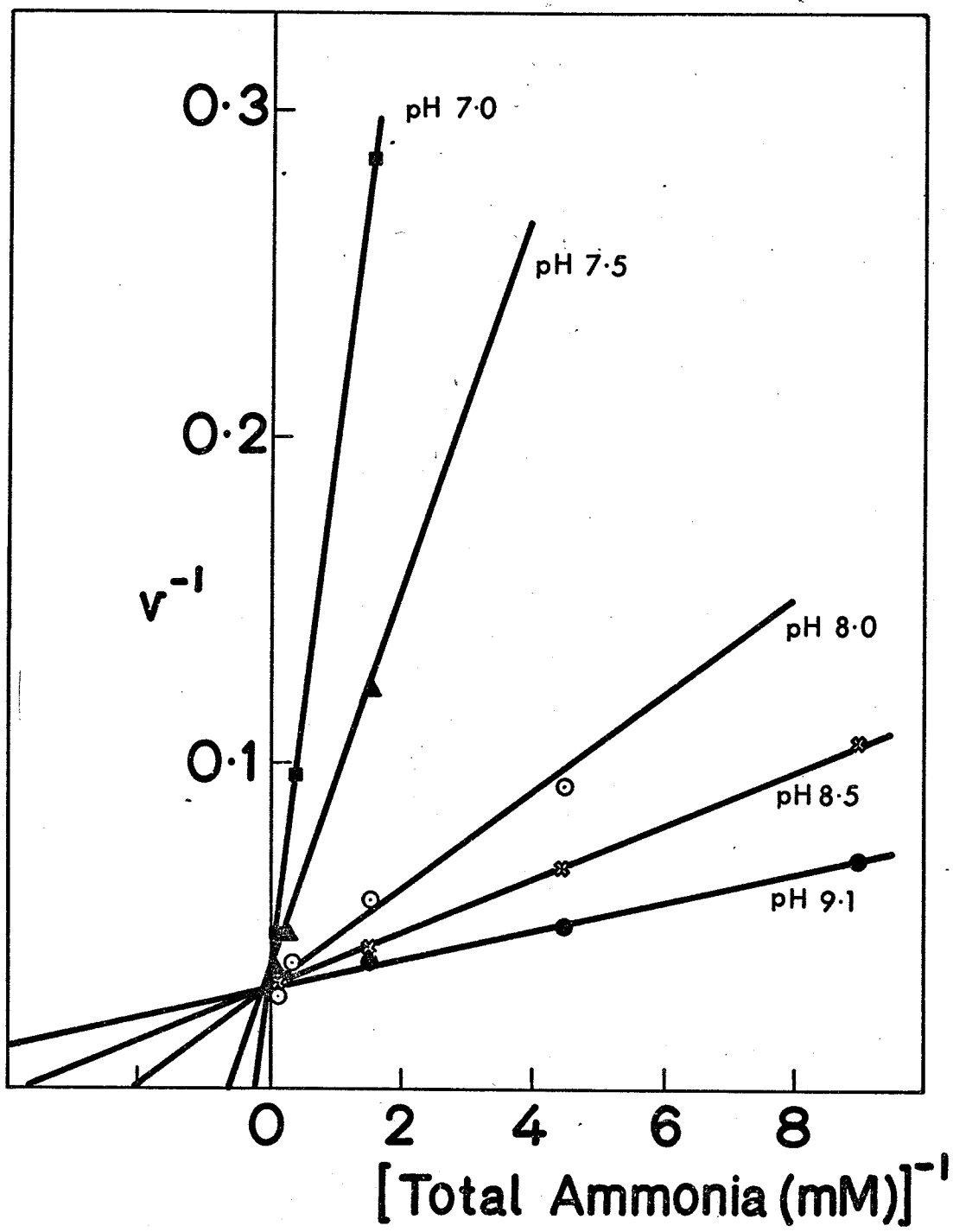

0·3
0·2
0·1
v⁻¹
pH 7·0
pH 7·5
pH 8·0
pH 8·5
pH 9·1
0
2
4
6
8
[Total Ammonia (mM)]⁻¹

Fig. 3. Effect of pH and ammonia concentration on the oxidation of ammonia by the cell-free extracts of N. europaea. The reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of the extract, 2 mM spermine, 0.1 M potassium phosphate buffer and ammonia as indicated. The pH indicated was the value obtained after mixing all the ingredients. The initial rate of oxidation (v) was expressed as nmoles O_2 consumed per min.

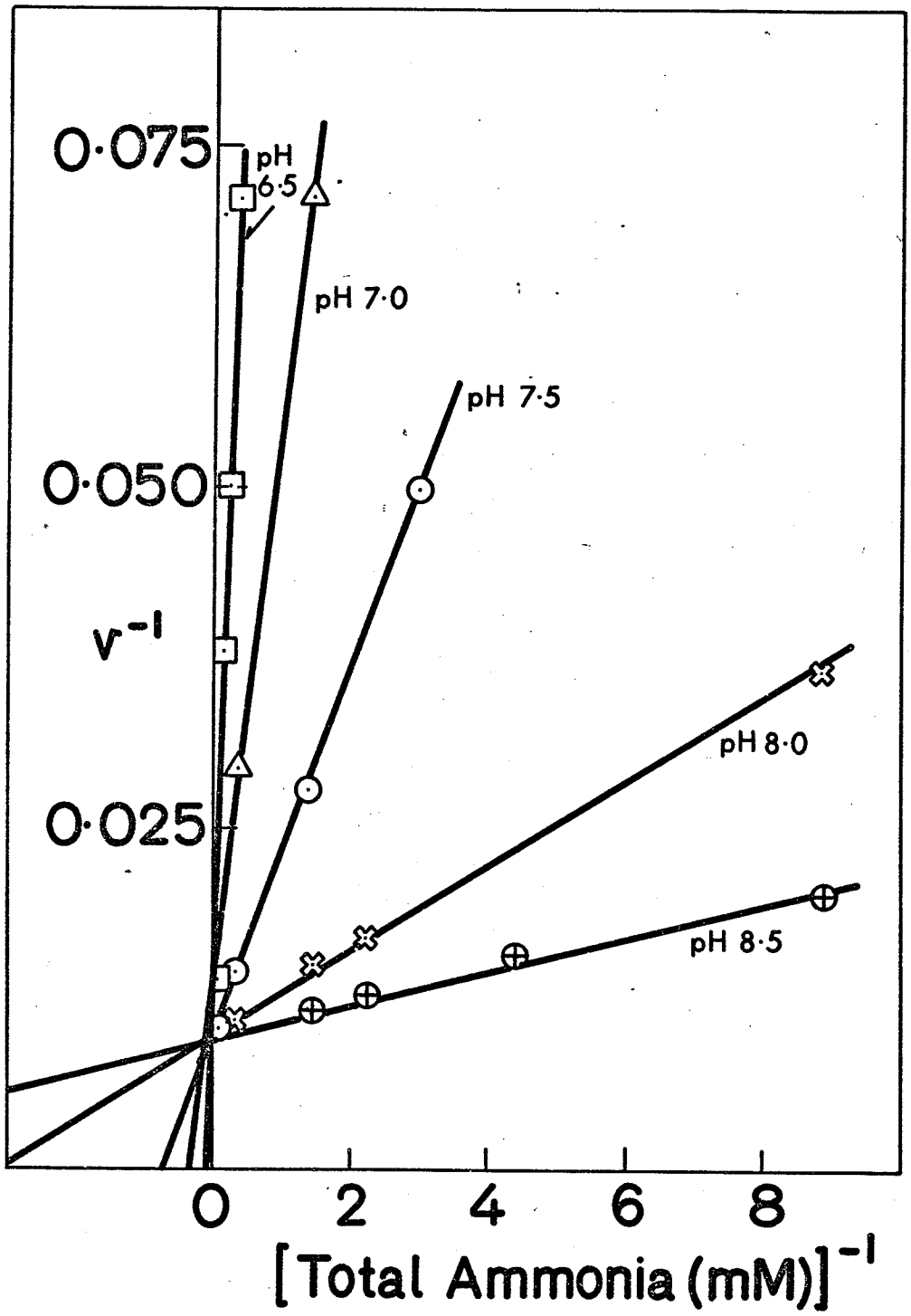

0·075
0·050
0·025
v⁻¹
pH 6·5
pH 7·0
pH 7·5
pH 8·0
pH 8·5
0
2
4
6
8
[Total Ammonia (mM)]⁻¹

Table 5. Effect of pH on the K_m values for ammonia in N. europaea cells and cell-free extracts.

pH	K_m			
	$NH_4^+ + NH_3$ (mM)		NH_3 (μM)	
	A[a]	B[b]	A[a]	B[b]
6.5	-	10.0	-	18
7.0	4.0	4.0	23	23
7.5	1.6	1.3	29	24
8.0	0.48	0.32	26	18
8.5	0.30	0.12	46	20
9.1	0.14	-	58	-

[a]Whole-cell experiments (data obtained from Fig. 2).

[b]Cell-free extract experiments (data obtained from Fig. 3).

The effect of pH on the K_m had virtually disappeared and the values remained, within experimental error, unchanged at various hydrogen ion concentrations.

Effect of Inhibitors

Table 6 shows the effect of various inhibitors on the ammonia-oxidizing activity of the cell-free extracts activated with BSA and spermine. Hooper and Terry (32) have extensively studied the effect of several inhibitors on ammonia oxidation by intact Nitrosomonas cells but not on the cell-free system. They reported that the oxidation of ammonia, but not hydroxylamine, in N. europaea cells was inhibited by metal-binding agents; compounds which interact with heme proteins; uncouplers of oxidative phosphorylation; artificial electron acceptors e.g. PMS; compounds which react with free radicals e.g. methanol or N_2O and also by illumination with 420 lux (5000 foot candles) of light.

In the cell-free system, metal-binding agents such as KCN, diethyldithiocarbamate, α,α'-dipyridyl, 1, 10-orthophenanthroline were potent inhibitors of ammonia oxidation. As mentioned later, $CuCl_2$ activated some extracts for ammonia oxidation whereas

Table 6. Effect of inhibitors on ammonia oxidation by cell-free extracts of N. europaea.

Inhibitor	Concentration (M)	Inhibition (%)
Mercuric chloride	10^{-4}	50
Potassium cyanide	10^{-5}	99
	10^{-6}	54
Sodium azide	10^{-4}	40
TTA	10^{-4}	0
	7.5×10^{-4}	33
CCCP	10^{-4}	50
	5×10^{-4}	66
Dicumarol	10^{-3}	50
Atebrin	10^{-4}	50
Diethyldithiocarbamate	10^{-5}	99
Bathocuproine sulfonate	10^{-4}	12
α,α'-dipyridyl	10^{-4}	99
1, 10 o-phenanthroline	10^{-4}	99
EDTA	10^{-2}	33 (variable)

The cell-free extract was prepared from fresh cells and the reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of the extract, 2 mM spermine, 1.7 mM $(NH_4)_2SO_4$ and 0.1 M potassium phosphate buffer (pH 7.5). Inhibitors were added in the concentrations (final) indicated, in microlitre volumes prior to the additions of spermine and $(NH_4)_2SO_4$.

α,α'-dipyridyl or 1, 10-orthophenanthroline together with $CuCl_2$ activated the rate of hydroxylamine oxidation. $CuCl_2$, $FeCl_3$, $CoCl_2$ and $NiCl_3$ did not reverse the inhibition of ammonia oxidation by these metal chelators. Mercuric chloride was also a potent inhibitor. m-Carbonylcyanidephenylhydrazone (CCCP) and theonyltrifluoroacetone (TTA), uncouplers of oxidative phosphorylation, inhibited ammonia oxidation at high concentrations. The inhibition by 2, 4-dinitrophenol and valinomycin was variable. They were not inhibitory when the extracts (or fresh cells) were highly active in their ammonia oxidative activity. In some extracts (stored frozen), however, the ammonia-oxidizing activity was inhibited up to 60% by 2, 4-dinitrophenol (10^{-3}M concentration) and 90% by valinomycin (10^{-4}M).

Effect of $CuCl_2$ on Ammonia Oxidation

In some cell-free extracts, especially those prepared from either old cells or from the cells which were not very active or in some extracts which were stored frozen, ammonia oxidation could be activated considerably by the addition of 0.1 mM $CuCl_2$

as discussed earlier. On the other hand, the addition of 0.1 mM $FeCl_3$ inhibited ammonia oxidation in those extracts (Fig. 4). Other metal ions such as Ni^{++}, Co^{++}, Mg^{++}, Mn^{++} and Ca^{++} did not replace $CuCl_2$ at the same concentration.

In the cell-free system, the inhibition by valinomycin but not by dipyridyl or o-phenanthroline could be reversed by 0.1 mM $CuCl_2$ whereas in intact cells various metals such as Cu^{++}, Fe^{++} or Co^{++} could reverse the inhibition by dipyridyl or o-phenanthroline. There was no inhibition of ammonia oxidation by valinomycin in intact N. europaea cells.

Effect of $CuCl_2$, Dipyridyl and o-Phenanthroline

(1) Hydroxylamine Oxidation. Hydroxylamine, an intermediate of ammonia oxidation, was slowly oxidized by the cell-free extracts of N. europaea (Fig. 5). Metal-binding compounds α,α'-dipyridyl and o-phenanthroline, in the presence of $CuCl_2$ activated the rate of hydroxylamine oxidation (assayed as O_2 uptake). An activation of 8 and 12 fold was obtained with 0.3 mM of dipyridyl and 0.1 mM $CuCl_2$ and with 0.1 mM each of o-phenanthroline and $CuCl_2$ respectively. Other metal ions such as Fe^{+++}, Co^{++}, Ni^{++}, Ca^{++}, Mg^{++}

Fig. 4. Effect of $CuCl_2$ and $FeCl_3$ on the rate of the oxidation of ammonia by cell-free extracts of N. europaea. The cell-free extract was prepared from 10 days old cells and the reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of the cell-free extract, 0.1 M potassium phosphate buffer (pH 7.5), 2 mM spermine and 1.7 mM $(NH_4)_2SO_4$. Additions were made in microlitre volumes. Control, —·—; 0.1 mM $FeCl_3$, — — — and 0.1 mM $CuCl_2$, ——— .

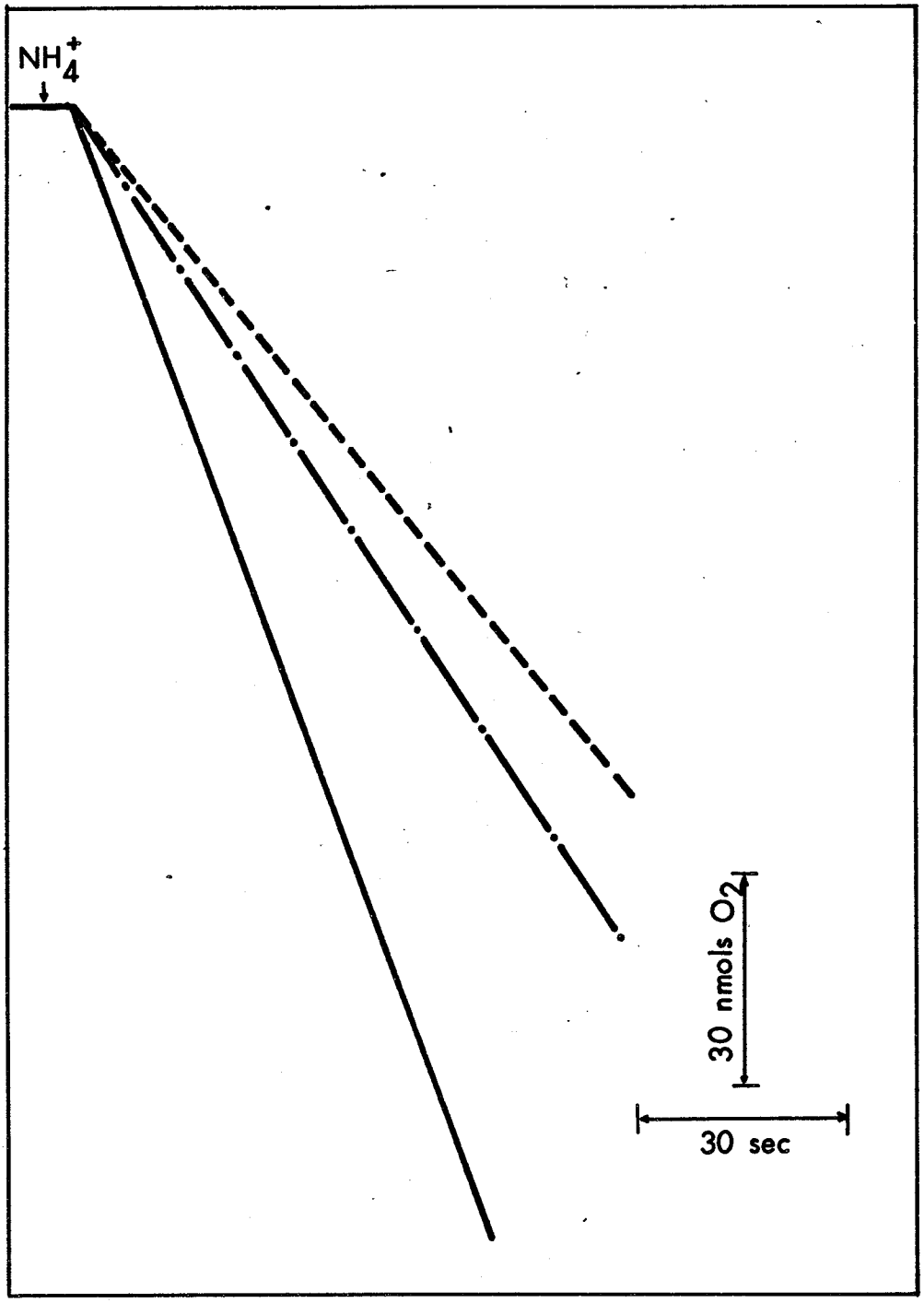
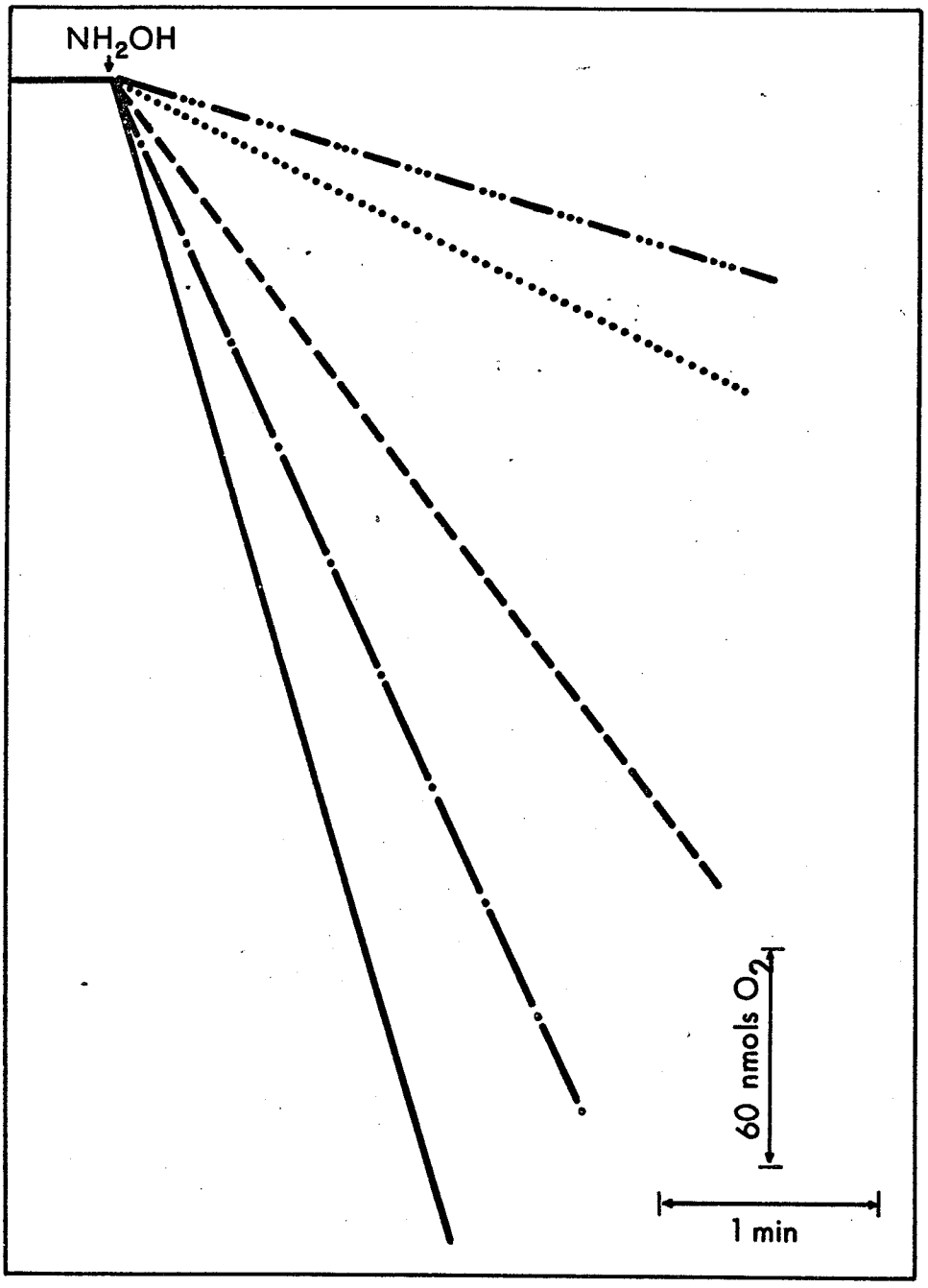

NH_4^+
30 nmols O_2
30 sec

Fig. 5. Effect of $CuCl_2$, dipyridyl and o-phenanthroline on the rate of NH_2OH oxidation by the cell-free extracts of N. europaea. The cell-free extract was prepared from old cells (20 mg/ml) without BSA and the reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of the cell-free extract, 0.1 M potassium phosphate buffer (pH 7.5) and 67 μM NH_2OH. The additions of $CuCl_2$, dipyridyl or o-phenanthroline were made prior to the addition of NH_2OH.

Control, — ··· —; 0.1 mM of either $CuCl_2$, dipyridyl or o-phenanthroline . . . . . ; 0.1 mM each of $CuCl_2$ and dipyridyl, ---- ; 0.1 mM $CuCl_2$ and 0.3 mM dipyridyl — . — ; and 0.1 mM each of $CuCl_2$ and o-phenanthroline ——— .


NH_2OH
60 nmols O_2
1 min

or Mn^{++} did not have similar activating effect when they were tested either singly or together with either dipyridyl or o-phenanthroline.

When the stoichiometry of hydroxylamine oxidation was studied in the presence of metal chelators and $CuCl_2$, the results in Table 7 were obtained. In the control experiment (reaction 1), the ratio of O_2 uptake/NO_2^- formed was 1. Either dipyridyl or o-phenanthroline, with or without $CuCl_2$, increased the total O_2 uptake, but decreased the nitrite formation thus increasing the ratio to almost 2 (Table 7). The total O_2 uptake in the presence of metal chelators and $CuCl_2$ decreased when the amount of extract used was reduced from 0.5 to 0.1 ml (reactions 8 and 10 as compared to reactions 9 and 11) suggesting a possible oxidation of endogenous substrate(s) coupled to hydroxylamine oxidation. The endogenous O_2 uptake rate was much slower than that of hydroxylamine and was not affected by metal chelators and $CuCl_2$ in the absence of hydroxylamine.

The reduced yield of nitrite was possibly due to the oxidation of hydroxylamine to N_2O or NO, reactions known to occur in Nitrosomonas (65). These oxidations, however, should have reduced the total O_2

Table 7. Effect of $CuCl_2$, dipyridyl and o-phenanthroline on NH_2OH oxidation by cell-free extracts of N. europaea.

Reaction	Additions	Concentration mM	O_2 Uptake Rate (nmoles/min)	O_2 Uptake Total (nmoles)	NO_2^- formed (nmoles)
1	None	-	18	76	75
2	$CuCl_2$	0.1	28	76	75
3	Dipyridyl	0.1	16	82	61
4	Dipyridyl	0.3	20	104	48
5	o-Phen.	0.1	30	110	48
6	o-Phen.	0.3	40	112	48
7	$CuCl_2$ and dipyridyl	0.1 each	80	104	61
8*	$CuCl_2$ and dipyridyl	0.1 and 0.3	70	94	55
9	$CuCl_2$ and dipyridyl	0.1 and 0.3	136	120	61
10*	$CuCl_2$ and o-Phen.	0.1 each	72	92	55
11	$CuCl_2$ and o-Phen.	0.1 each	210	120	61
12*	$CuCl_2$ and o-Phen.	0.1 and 0.3	132	106	48

The cell-free extract was the same as in Fig. 5. The reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of the extract (*0.1 ml extract in the reaction numbers 8, 10, and 12) and 0.1 M potassium phosphate buffer (pH 7.5). The reaction was started by the addition of 67 µM NH_2OH and was allowed to proceed until the O_2 uptake levelled off. A sample of 0.5 ml was withdrawn and analyzed for NO_2 formed as described in Materials and Methods. Total O_2 uptake was calculated from the Oxygraph charts. All the additions were made in calculated amounts in microlitre volumes prior to the addition of NH_2OH.

uptake instead of increasing it. Any attempts to show the formation of nitrate failed and also, there was no effect of catalase on the 0_2 uptake indicating that the formation of H_20_2 may not be involved. It is possible, however, that H_20_2, if formed at all, may be immediately broken down by the endogenous peroxidase or catalase (16, 59).

(2) NADH Oxidation. Fig. 6 shows the effects of $CuCl_2$ and/or dipyridyl or o-phenanthroline on the rate of NADH oxidation in the cell-free extracts of N. europaea. The rate of NADH oxidation, measured as a decrease in absorbance at 340 nm, was not affected by 0.1 mM $CuCl_2$ alone, but there was approximately 5 fold activation in the rate by either dipyridyl or o-phenanthroline. The addition of $CuCl_2$ together with dipyridyl or o-phenanthroline resulted in an activation of NADH oxidation by 30 fold (Fig. 6). The rate of 0_2 uptake was stimulated approximately 1.5 fold by 0.3 mM of dipyridyl or o-phenanthroline and 4 to 5 fold in the presence of 0.1 mM $CuCl_2$ and 0.3 mM dipyridyl or o-phenanthroline (Table 8). Total 0_2 uptake remained unaffected agreeing with the stoichiometry expected of an equation:

$$NADH + H^+ + \tfrac{1}{2}\, 0_2 \longrightarrow NAD^+ + H_20$$

Fig. 6. Effect of $CuCl_2$, dipyridyl and o-phenanthroline on NADH oxidation by the cell-free extracts of N. europaea. The same cell-free extract as in Fig. 5 was used. The measurement of change in absorbance at 340 nm (spectrophotometric analysis) was carried out as described in Materials and Methods. The reaction mixture in a total volume of 1 ml contained 0.5 ml of the extract, 0.1 M potassium phosphate buffer (pH 7.5) and 0.2 mM NADH.

Control, ▽—▽; 0.3 mM dipyridyl, ⊙—⊙; 0.3 mM o-phenanthroline, ⊡—⊡; 0.1 mM $CuCl_2$ and 0.3 mM dipyridyl, ⊗—⊗ and 0.1 mM $CuCl_2$ and 0.3 mM o-phenanthroline, △—△.

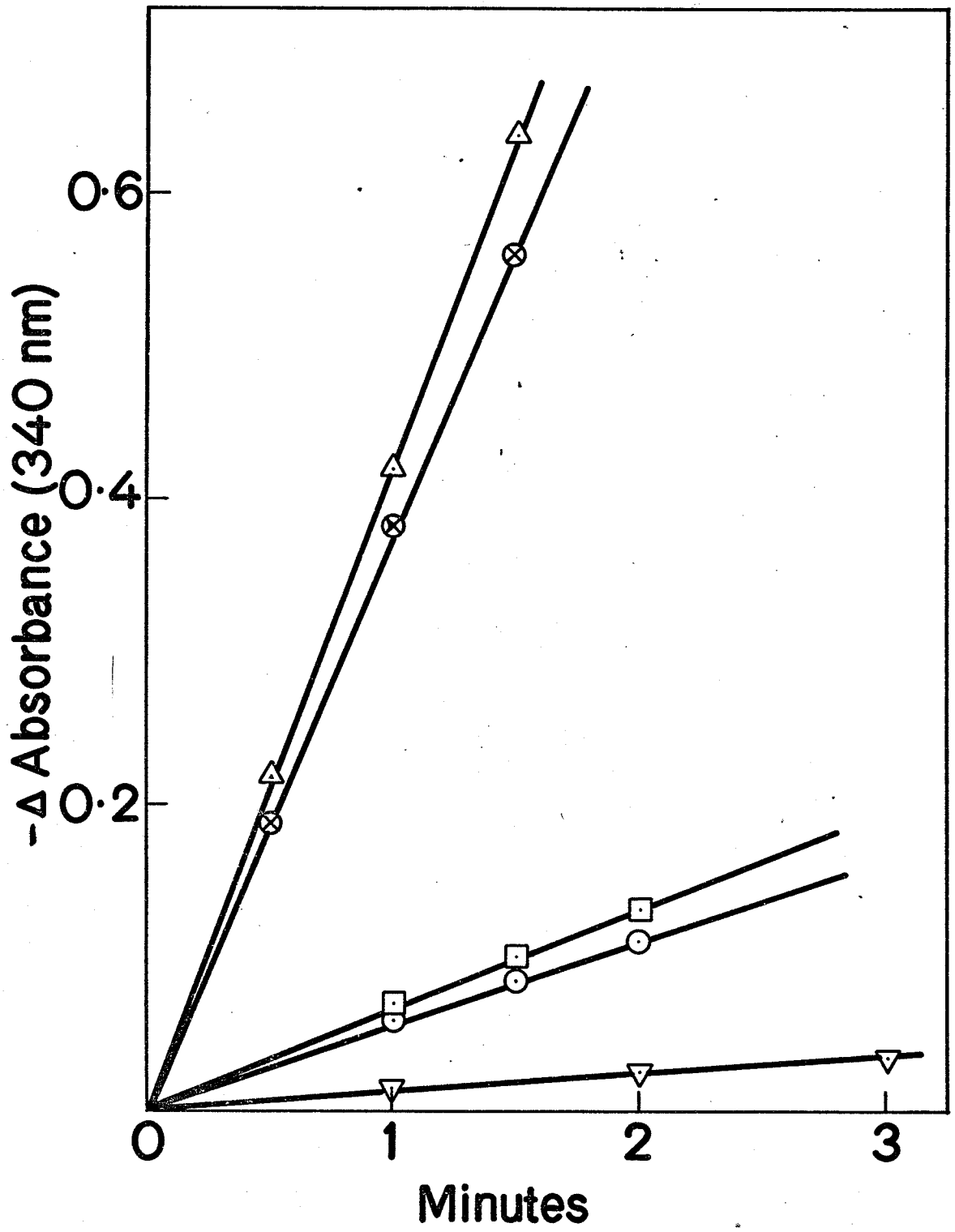

-Δ Absorbance (340 nm)
0·6
0·4
0·2
0
1
2
3
Minutes

Table 8. Effect of $CuCl_2$, dipyridyl and o-phenanthroline on NADH oxidation, measured as O_2 uptake, by the cell-free extracts of N. europaea.

Reaction	Additions	Concentration (mM)	O_2 Uptake (nmoles/min)
1	None	-	8
2	$CuCl_2$	0.1	8
3	Dipyridyl	0.3	12
4	o-Phenanthroline	0.3	14
5	$CuCl_2$ and dipyridyl	0.1 and 0.3	34
6	$CuCl_2$ and o-phenanthroline	0.1 and 0.3	42

The cell-free extract was the same as used for Fig. 5. The reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of the extract, 67 µM NADH and 0.1 M potassium phosphate buffer (pH 7.5). The additions were made in microlitre volume prior to the starting of the reaction with NADH.

(3) Cytochrome c reduction. As shown in Table 9, the rate of cytochrome c (type III, from horse heart) reduction by NADH in the cell-free extract was stimulated 17 fold when both $CuCl_2$ and dipyridyl were present together. However, there was very little activation by either of them when they were added singly.

When NH_2OH was used as a substrate, $CuCl_2$ and dipyridyl (or o-phenanthroline) activated the rate of cytochrome c reduction by 5 to 6 fold (Table 9). This activation was further enhanced (2 fold) when the assay was carried out under anaerobic conditions. Although not shown in Table 9 a partially purified reductase preparation was not activated by $CuCl_2$ and dipyridyl.

There was no effect of $CuCl_2$ and/or dipyridyl (or o-phenanthroline) on cytochrome oxidase activity in the cell-free extract when assayed with reduced horse heart cytochrome c.

Table 9. Effect of $CuCl_2$, dipyridyl and o-phenanthroline on the rate of cytochrome c (mammalian) reduction by NADH or NH_2OH in the cell-free extracts of N. europaea.

Reaction	Additions	Absorbance change at 550 nm/min	
		NADH as a substrate	NH_2OH as a substrate
1	None	0.015	0.18
2	$CuCl_2$	0.023	0.18
3	Dipyridyl	0.023	0.18
4	o-Phenanthroline	-	0.25
5	$CuCl_2$ and dipyridyl	0.25	0.92
6	$CuCl_2$ and o-phenanthroline	-	1.06

The cell-free extract was the same as used for Fig. 5. The change in absorbance at 550 nm was measured as described in Materials and Methods. The reaction mixture in a total volume of 1 ml contained 0.05 mM cytochrome c (type III, from horse heart), 0.01 ml of the extract and 0.1 M potassium phosphate buffer (pH 7.5). The reaction was started by the addition of 0.1 mM of either NADH or NH_2OH and the change in absorbance was recorded immediately. The additions of $CuCl_2$ (0.1 mM), dipyridyl (0.3 mM) and o-phenanthroline (0.3 mM), when indicated, were made prior to the addition of the substrate.

Effect of Phosphate

Phosphate requirement for the growth of N. europaea cells (13) and for the oxidation of ammonia in the cell-free extracts (39) has been reported earlier.

In this study, however, it was observed that both the NH_2OH-cytochrome c reductase and cytochrome oxidase activities were inhibited by 0.1 M potassium phosphate (Table 10). In the crude extracts, the reductase activity was affected more than the oxidase activity whereas the effect was reversed when a partially purified reductase preparation, which also contained some soluble cytochrome oxidase activity, was substituted for the crude extract. The soluble oxidase activity was 40 times faster in 0.01 M than in 0.1 M potassium phosphate buffer (pH 7.5). The particulate oxidase, however, was least affected by the change in phosphate concentration (Table 10).

When NH_2OH oxidation was assayed in the Oxygraph, the results shown in Table 11 were obtained. It was observed that a higher concentration (0.1 M) of phosphate activated NH_2OH oxidation in the absence but inhibited it in the presence of horse heart cytochrome c. In lower phosphate concentration, however, the

Table 10. Effect of phosphate on NH_2OH-cytochrome c reductase and cytochrome oxidase activities.

	Enzyme Activity	Volume of the Preparation used for assay (µl/ml)	Activity* in A 0.01 M phosphate	Activity* in B 0.1 M phosphate	A/B Ratio
Cell-free extract	Reductase[1]	10.0	2.2	0.26	8.5
	Oxidase[2]	50.0	1.0	0.18	5.6
Partially purified frac. 4	Reductase[1]	3.0	0.4	0.1	4.0
	Oxidase[2]	6.0	0.08	0.002	40
100,000 x g Pellet	Oxidase[2]	25.0	0.07	0.035	2.0

[1] NH_2OH-cytochrome c reductase [2] cytochrome oxidase

The cell-free extract, from fresh cells (20 mg/ml, without BSA) and the partially purified preparation, fraction 4, of NH_2OH-cytochrome c reductase were prepared and the reaction was carried out in a Shimadzu Spectrophotometer as described in Materials and Methods. The 100,000 x g pellet, obtained by ultracentrifugation of the cell-free extract, was homogenized, washed and resuspended in 1/10th of the original volume of 0.1 M potassium phosphate buffer (pH 7.5).

Table 10. Continued

Reductase Assay. The reaction mixture in a total volume of 1 ml contained 0.075 mM cytochrome c (type III, from horse heart), the specified volume of the enzyme preparation and either 0.01 or 0.1 M potassium phosphate buffer (pH 7.5). The reaction, started by the addition of 0.1 mM NH_2OH was measured as the change in absorption (at 550 nm) per min.

Oxidase Assay. The reaction mixture in a total volume of 1 ml contained 0.075 mM reduced cytochrome c (type III, from horse heart) and either 0.01 or 0.1 M potassium phosphate buffer (pH 7.5). The reaction was started by the addition of the specified volume of the preparation and was measured as the change in absorption (at 550 nm) per min.

*Expressed as the change in absorption per min at 550 nm.

Table 11. Effect of phosphate on NH_2OH oxidation by the cell-free extracts of N. europaea.

Reaction	Additions	O_2 Uptake (nmoles/min) A 0.01 M phosphate	O_2 Uptake (nmoles/min) B 0.1 M phosphate	A/B ratio
1	-	4	12.0	0.333
2	Cyt. c	28	8.0	3.5

The cell-free extract was the same as in Table 10. The reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained either 0.5 ml (reaction 1) or 0.1 ml (reaction 2) of the extract, 0.1 mM NH_2OH and 0.01 or 0.1 M potassium phosphate (pH 7.5) buffer. The addition of 40 µM mammalian cytochrome c (type III, from horse heart) was done in microlitre volume prior to the addition of NH_2OH.

addition of horse heart cytochrome c activated NH_2OH oxidation considerably.

Inhibition by phosphate of the oxidation of hydroxylamine to nitrite in the presence of mammalian cytochrome c by the crude, sonicated extracts of N. europaea was also reported by Nicholas and Jones (55). They pointed out that phosphate was a competitive inhibitor for mammalian cytochrome c.

PART II. PARTIAL RESOLUTION AND RECONSTITUTION OF THE AMMONIA-OXIDIZING SYSTEM

An attempt was made to resolve partially, the ammonia-oxidizing enzyme complex from the cell-free extracts of N. europaea, by resorting to differential centrifugation and column chromatographic techniques. From the results obtained (Table 12) which are discussed below, it was observed that the differential centrifugation technique was not as effective as the column chromatographic method. At least three separate fractions, which oxidized ammonia only when they were combined together, were obtained by the chromatography of the active cell-free extracts on Sepharose 6B columns. On the other hand, when the active cell-free extracts were ultracentrifuged, the pellet alone had most of the ammonia-oxidizing activity and a further resolution on a Sepharose 6B column was found to be necessary to obtain an inactive membrane fraction, which could be activated by the addition of the supernatant.

Differential Centrifugation

Of the total recovery of 33% activity obtained by the combination of the spinco supernatant and the

pellet, 28% was retained in the pellet alone (Table 12). Partial N_2 atmosphere was found to be protective during the high speed centrifugation, although the extract lost approximately 45% of its activity when stored at 4°C under partial N_2 atmosphere (Table 12). These results indicated that the enzyme complex could not be properly resolved by the differential centrifugation alone. However, when the pellet was passed through a Sepharose 6B column, a membrane fraction (1P) was obtained which could not oxidize ammonia by itself and NADH activated it only slightly. Upon the addition of the supernatant to this 1 P fraction, an active enzyme complex could be reconstituted which oxidized ammonia at 16% of the rate of the original cell-free extract activity (Table 12). The supernatant could be concentrated by ammonium sulfate precipitation followed by dialysis against 0.1 M potassium phosphate buffer (pH 7.5) without much loss in its activity but NADH was consistantly found necessary for the activating effect of such a concentrate on 1 P fraction.

Since N_2 atmosphere was found to be protective during ultracentrifugation, it was thought that it might replace the requirement of BSA during the

Table 12. Partial resolution and reconstitution of ammonia-oxidizing system by differential centrifugation of the cell-free extracts prepared in the presence of BSA.

Reaction	System	Additions	Activity (%)
1	Cell-free extract (control)	-	100
2	Cell-free extract kept under N_2	-	56
3	Pellet	± NADH	28
4	Pellet + Supernatant	± NADH	33
5	1P	± NADH	1.0
6	1P + Supernatant	± NADH	16
7	*1P	± NADH	1.0
8	*1P + $(NH_4)_2SO_4$ concentrated supernatant	-	1.0
9	Same as reaction 8	+ NADH	11

*1P was stored frozen overnight and assayed on the following day.

Table 12. Continued

The cell-free extract was obtained from fresh cells and the reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The extract was divided into 2 parts; one was kept at 4°C under N_2 atmosphere whereas the other was ultracentrifuged at 100,000 x g for 1 hr under N_2 atmosphere as described in Materials and Methods. A portion of the supernatant was saved as control and the rest was concentrated (10 fold) with 85% saturation of $(NH_4)_2SO_4$ followed by dialysis against 0.1 M potassium phosphate buffer (pH 7.5) with 3 changes overnight. 1P was obtained by passing 1.5 ml of the pellet (suspended in 1/10th of 0.1 M potassium phosphate buffer (pH 7.5), through a Sepharose 6B column (aerobic) as described in Materials and Methods. The first fraction was collected in 3 ml volume and was designated as 1P. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of the extract, (or 0.05 ml of pellet or 0.2 ml of 1P), 2 mM spermine, 1.7 mM $(NH_4)_2SO_4$ and 0.1 M potassium phosphate buffer (pH 7.5).

Additions: NADH (67 μM) was added in microlitre volume, supernatant in 0.5 ml volume and concentrated supernatant in 0.05 ml volume prior to the addition of $(NH_4)_2SO_4$.

breakage of the cells. The results obtained in Table 13 show however, that the extract prepared under N_2 atmosphere had only 50% of activity as compared to the extract obtained in the presence of BSA. However, the addition of BSA to the extract prepared under N_2, at the time of assay, did have some stimulating effect (Table 13).

The pellet obtained after the ultracentrifugation of the extract prepared under N_2, had almost all the ammonia-oxidizing activity but it required either BSA or NADH for the oxidation of ammonia. The addition of the supernatant (from the extract prepared under N_2) had an inhibitory effect on the pellet activity (Table 13).

The membrane fraction obtained by passing the pellet (from the extract prepared under N_2) through a Sepharose 6B column did not have any ammonia-oxidizing activity and it could not be significantly activated by BSA, NADH, the supernatant from the extract prepared under N_2 or the supernatant from the extract prepared in the presence of BSA.

Column Chromatography

Sepharose 6B fractionation of the cell-free extracts

Partial resolution of the ammonia-oxidizing

Table 13. Partial resolution and reconstitution of ammonia-oxidizing system obtained by ultracentrifugation of the cell-free extracts prepared under N_2 atmosphere in the absence of BSA.

Reaction	System	Additions	Activity (%)
1	Cell-free extract (control)	-	100
2	Cell-free extract (prepared under N_2)	-	50
3	Same as 2	BSA	70
4	Pellet ± supernatant	-	7.5
5	Pellet	BSA or NADH	47.5
6	Pellet + supernatant	NADH	25
7	Pellet + supernatant	BSA (± NADH)	35
8	1P ± supernatant	-	2.5
9	1P	BSA (± NADH)	7.5
10	1P + supernatant	BSA (± NADH)	7.5
11	1P + BSA - Sup*	(± NADH)	10

*BSA-Sup was the supernatant obtained after the ultracentrifugation of the cell-free extract prepared in the presence of BSA.

Table 13. Continued

The cell suspension (fresh cells) was divided into two equal parts. One part was broken with 20 mg/ml BSA (control) and the other under N_2 atmosphere without BSA. The cells were broken and the reaction was carried out as described in Materials and Methods. The cell-free extract prepared under N_2 was ultracentrifuged at 100,000 x g for 1 hr as described in Materials and Methods. The pellet was resuspended in 1/10 the original volume of 0.1 M potassium phosphate buffer (pH 7.5). 1P was obtained and the assay conditions were the same as described for Table 12.

enzyme complex could be better achieved by passing the cell-free extracts (prepared in the presence of BSA) through a Sepharose 6B column under partially anaerobic conditions as described in Materials and Methods. Approximately 40 to 45% of the cell-free extract activity could be regained upon combining the Sepharose 6B fractions 1, 4 and 6 (Tables 15 and 16). All the three fractions were required for the reconstitution of the ammonia-oxidizing system.

Effect of O_2 tension during the fractionation

When Sepharose 6B fractionation was carried out under aerobic conditions, the reconstituted system (1 + 4 + 6) did not seem to have any ammonia-oxidizing activity when assayed immediately after the collection of fractions. NADH activated the oxidation of ammonia (Table 14). The same fractions, when they were stored (separately) at 4°C for approximately 2½ to 3 hours, had lower O_2 content and at the same time were quite active upon reconstitution. NADH had no effect (Table 14). These results suggested that lower O_2 content was necessary for the active reconstitution of the fractions which were obtained

Table 14. Effect of O_2 tension on the ammonia-oxidizing activity of partially resolved and reconstituted system.

	O_2 content at 25°C		Activity (nmoles O_2/min) of	
	nmoles/1.5 ml	% saturation	1 + 4 + 6	1 + 4 + 6 + NADH
Immediately after collection	260	72	4	32
After 2.5 to 3 hrs at 4°C	220	61	32	32

The cell-free extract and Sepharose fractions 1, 4 and 6 were obtained and the reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The O_2 content was calculated from the Oxygraph charts after mixing the three fractions in the reaction vessel, 360 nmoles of O_2 per 1.5 ml of the assay buffer being taken as 100% O_2 saturation at 25°C. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of each of the fractions, 2 mM spermine, 1.7 mM $(NH_4)_2SO_4$ and 0.1 M potassium phosphate buffer (pH 7.5). NADH (67 µM) was added in microlitre volume.

Table 15. Comparative ammonia-oxidizing activities of the Sepharose fractions obtained under aerobic and anaerobic conditions from the cell-free extracts of N. europaea.

Reaction	Fractions obtained		Activity	Lag
	aerobically	anaerobically	(% cell-free extract)	(min)
1	1 + 4 + 6	-	32	8.5
2	-	1 + 4 + 6	16	3
3	-	1* + 4 + 6	20	5
4	1	4 + 6	42	5
5	4 + 6	1	16	8.5

*Fraction 1 was aerated by gentle stirring before combining it with the other two fractions.
Aerobically obtained fractions, which were assayed after 2½ to 3 hours storage at 4°C, were the same as used in Table 14. Anaerobically obtained fractions were collected by passing the same cell-free extract as in Table 14, through a Sepharose 6B column equilibrated with anaerobic buffer as described in Materials and Methods. All the other experimental conditions were the same as given in Table 14.
The O_2 tension of the combined, aerobically obtained fractions was approximately equivalent to that of anaerobically obtained fractions, viz. 61%, at the time of assay.

Table 16. Ammonia-oxidizing activity of partially resolved and reconstituted system obtained by column chromatography of the cell-free extracts prepared in the presence of BSA.

Reaction	System	Additions	Activity	Lag
			(%)	(min)
1	Cell-free extract	-	100	1
2	1	-	3	-
3	1	NADH	14	-
4	1 + 4	-	3	-
5	1 + 4 + 6	-	40	5
6	1 + 4	NADH	43	0
7	1 + 4	NH_2OH	35	0
8	1 + 6	± NADH	14	-
9	1	S_2 (or reductase)	6	-
10	1	S_2 (or reductase), NADH	35	0
11	1 + 6	S_2 (or reductase)	29	12
12	1 + 6*	S_2	49	4
13	1 + 6	S_2, anaerobic buffer	32	6
14	1	S_2, anaerobic buffer	6	-
15	1	S_1, ± NADH	32	-

Table 16. Continued

The preparations of the cell-free extract, Sepharose fractions 1, 4 and 6, (obtained under partially anaerobic conditions), S_1, S_2 and partially purified NH_2OH-cytochrome c reductase were carried out and the reaction was assayed in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of either the extract or the Sepharose fraction(s) (*except in the reaction 12 where 1.0 ml of fraction 6 was used), 2 mM spermine, 1.7 mM $(NH_4)_2SO_4$ and 0.1 M potassium phosphate buffer (pH 7.5). The anaerobic buffer was obtained by bubbling N_2 gas in the buffer for 5 to 7 min.
The additions, in microlitre volumes, were made as follows: NADH, 67 µM; NH_2OH, 0.33 µM, S_1, 0.05 ml and S_2 (or partially purified reductase), 0.03 ml. The ammonia-oxidizing activity was expressed in terms of nmoles of O_2 consumed per min and the percentage was calculated by taking the cell-free extract activity as 100% and multiplying the fraction(s) activity by the dilution factor of 1.5.

under aerobic conditions. However, the combination of all the three fractions obtained anaerobically, had only half the activity as compared to the activity of the combination of all the three aerobically obtained fractions (Table 15). The highest activity was obtained upon the reconstitution of aerobically obtained fraction 1 and anaerobically obtained fractions 4 and 6. Also, the long lag in aerobically obtained 1 + 4 + 6 combination was shortened when anaerobically obtained 4 and 6 fractions were substituted for aerobic 4 and 6 (Table 15).

When the ammonia-oxidizing enzyme complex was resolved under partially anaerobic columns (i.e. the bubbling of N_2 gas was started after the elution of fraction 1, refer Materials and Methods), the recovery of the activity of the reconstituted system (viz. 1 + 4 + 6) was approximately equivalent to that obtained by combining aerobically obtained fraction 1 with anaerobically obtained fractions 4 and 6 (compare reaction 4, Table 15 and reaction 5, Table 16).

Absorption Spectra and Reconstitution of the Fractions

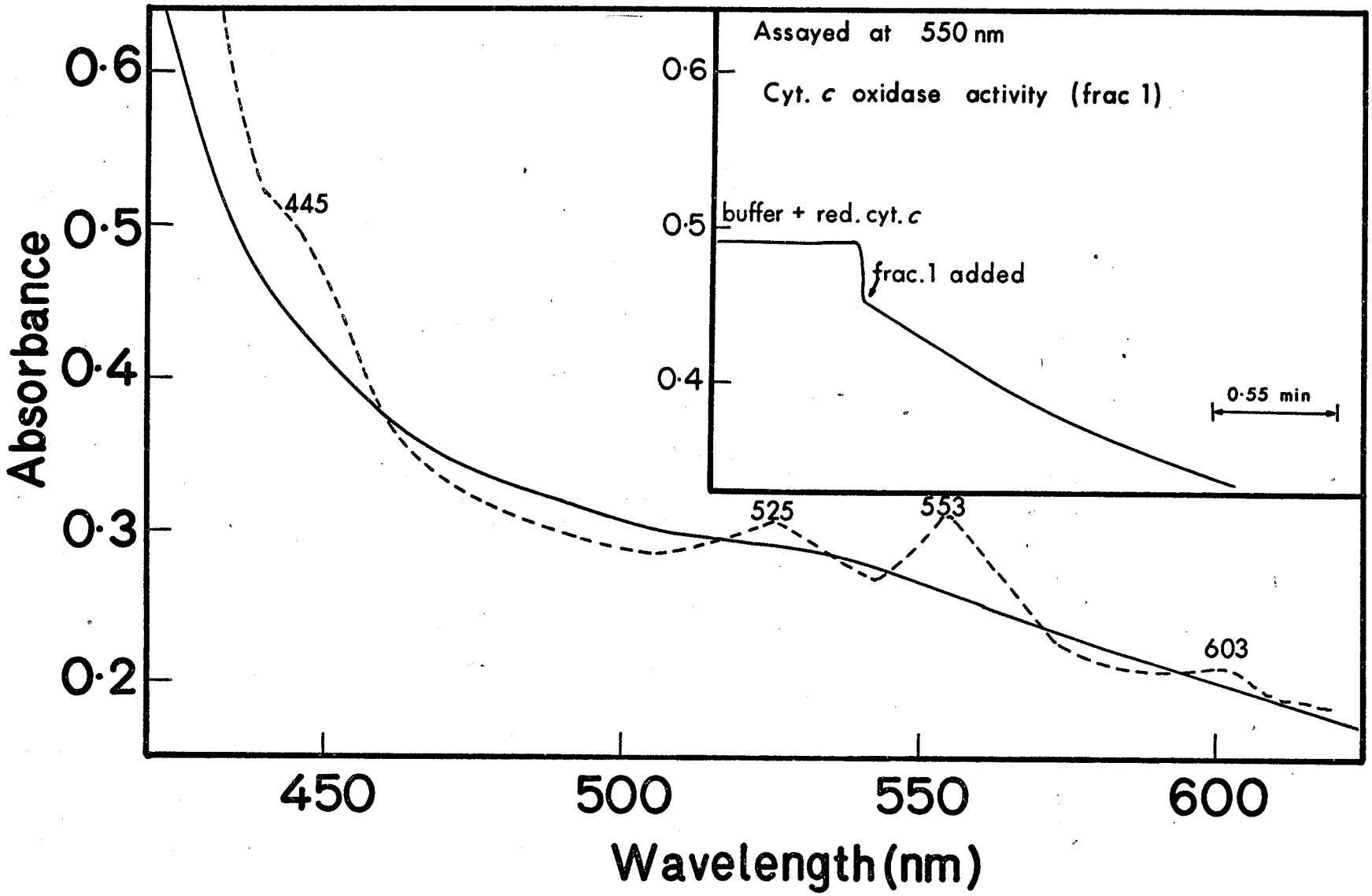
The absorption spectra of hydrosulfite reduced fraction 1, which was high molecular weight membrane fraction excluded from Sepharose 6B, showed the absorption peaks at 603 nm, a shoulder at 445 nm and peaks at 423, 525 and 553 nm indicating the presence of a-type cytochrome oxidase(s) and a small amount of cytochrome c (Fig. 7 a). When assayed with reduced horse heart cytochrome c, this fraction showed a high cytochrome oxidase activity (Fig. 7 a, insert) but it had very little NH_2OH-cytochrome c reductase activity. Thus, the major componant(s) of fraction 1 seemed to be cytochrome oxidase(s). An effort to replace fraction 1 with cytochrome a_1 purified according to Erickson, Hooper and Terry (19) was unsuccessful.

Fraction 4, when reduced with hydrosulfite, had absorption peaks at 423, 525 and 553 nm; shoulders at 530 and 560 nm; and a peak at 462 nm thus indicating the presence of cytochromes of b-, c- and P-460 types. This fraction could not oxidize mammalian cytochrome c, but had a very high NH_2OH-cytochrome c reductase activity when assayed in 0.1 M potassium phosphate buffer (pH 7.5) with horse heart cytochrome c and

Fig. 7 (a, b and c). Oxidized and reduced spectra of fractions 1, 4 and 6 (Sepharose 6B). The fractions were the same as in Table 16. Absolute oxidized spectrum of 1 ml of each fraction was recorded (using 1 ml cuvette) against a reference cuvette containing 1 ml of 0.1 M potassium phosphate buffer (pH 7.5) as described in Materials and Methods. The reduced spectrum of each fraction was recorded after the addition of a few crystals of sodium dithionite to the oxidized sample and 5 minutes incubation to effect complete reduction.

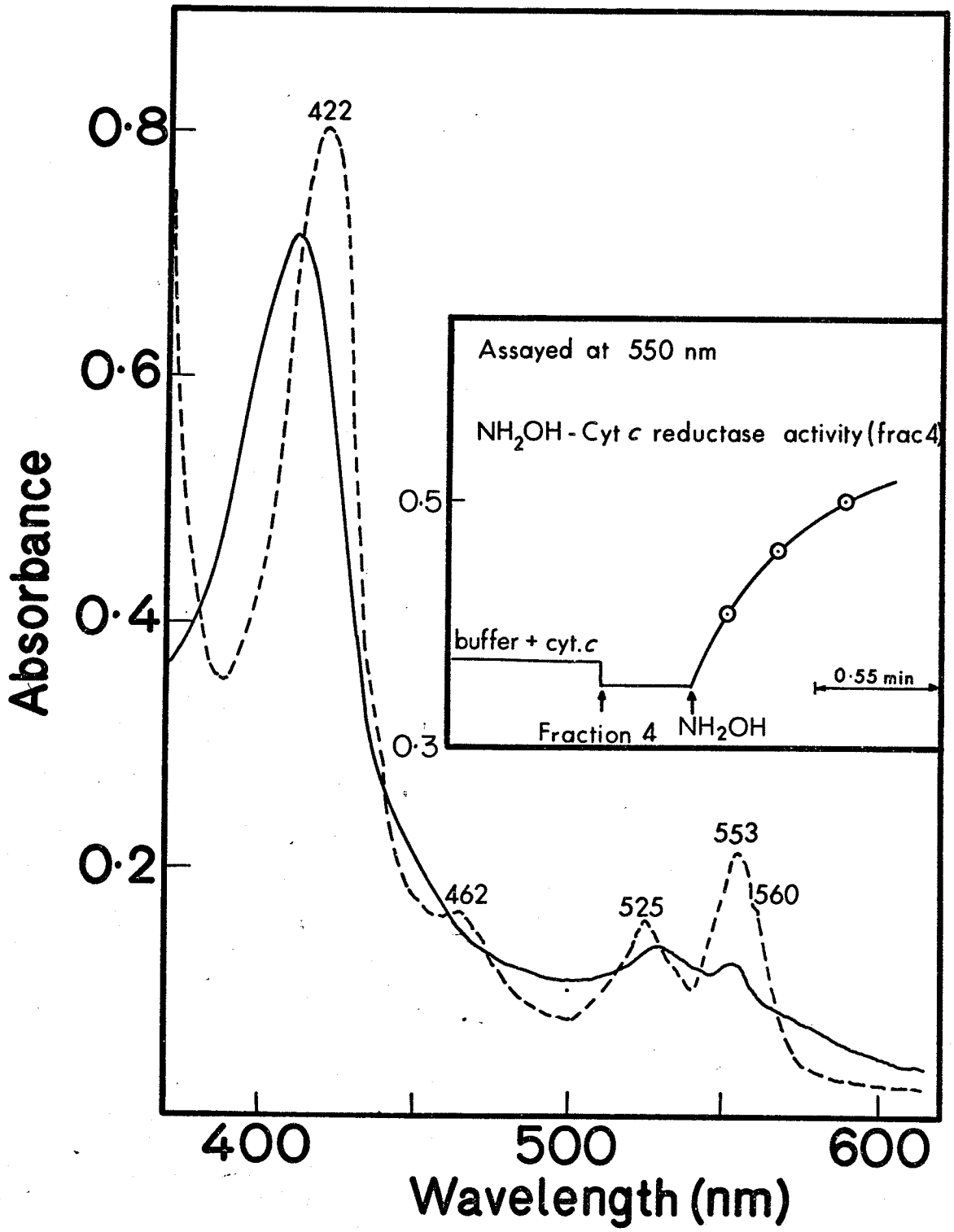
Fig. 7a. Oxidized (———) and reduced (-----) spectra of fraction 1.

Insert: Cytochrome oxidase activity of fraction 1. The reaction mixture in a total volume of 1 ml containing 50 μM reduced cytochrome c (type III, from horse heart) and 0.1 M potassium phosphate buffer (pH 7.5). Fraction 1 (0.1 ml, 0.14 mg protein) was added at the time indicated and the decrease in absorbance at 550 nm was recorded as described in Materials and Methods.


Absorbance
0·6
0·5
0·4
0·3
0·2
445
525
553
603
450
500
550
600
Wavelength (nm)
Assayed at 550 nm
Cyt. c oxidase activity (frac 1)
buffer + red. cyt. c
frac. 1 added
0·55 min

NH_2OH as substrates (Fig. 7 b). These observations suggested that fraction 4 was a complex mixture of several proteins including cytochromes b, c and P-460 and also the enzyme NH_2OH-cytochrome c reductase. Indeed, this fraction 4 could be replaced as shown in Table 16 by a partially purified preparation of the reductase and also by a preparation S_2 (ref. Materials and Methods) which was obtained during the purification of cytochrome a_1 according to the method of Erickson, Hooper, and Terry (19). Both of these preparations (partially purified reductase and S_2) were found to contain cytochromes b and c and also a considerably high NH_2OH-cytochrome c reductase activity, but neither of them could oxidize reduced horse heart cytochrome c when assayed in 0.1 M potassium phosphate buffer (pH 7.5). The amounts of S_2 or reductase preparations to be used in the reconstitution experiments were critical. If the volumes of these preparations were increased above those used in Table 16, the ammonia-oxidizing activity of the system was inhibited. Various fractions with measurable cytochrome oxidase activity (in 0.1 M potassium phosphate buffer, pH 7.5) obtained during the purification of cytochrome a_1 (19)

Fig. 7b. Oxidized (——) and reduced (----) spectra of fraction 4. The reaction mixture in a total volume of 1 ml contained 50 μM cytochrome c (type III, from horse heart) and 0.1 M potassium phosphate buffer (pH 7.5). Fraction 4 (0.1 ml, 0.16 mg protein) and NH_2OH were added at the times indicated and the increase in absorbance at 550 nm was recorded as described in Materials and Methods.

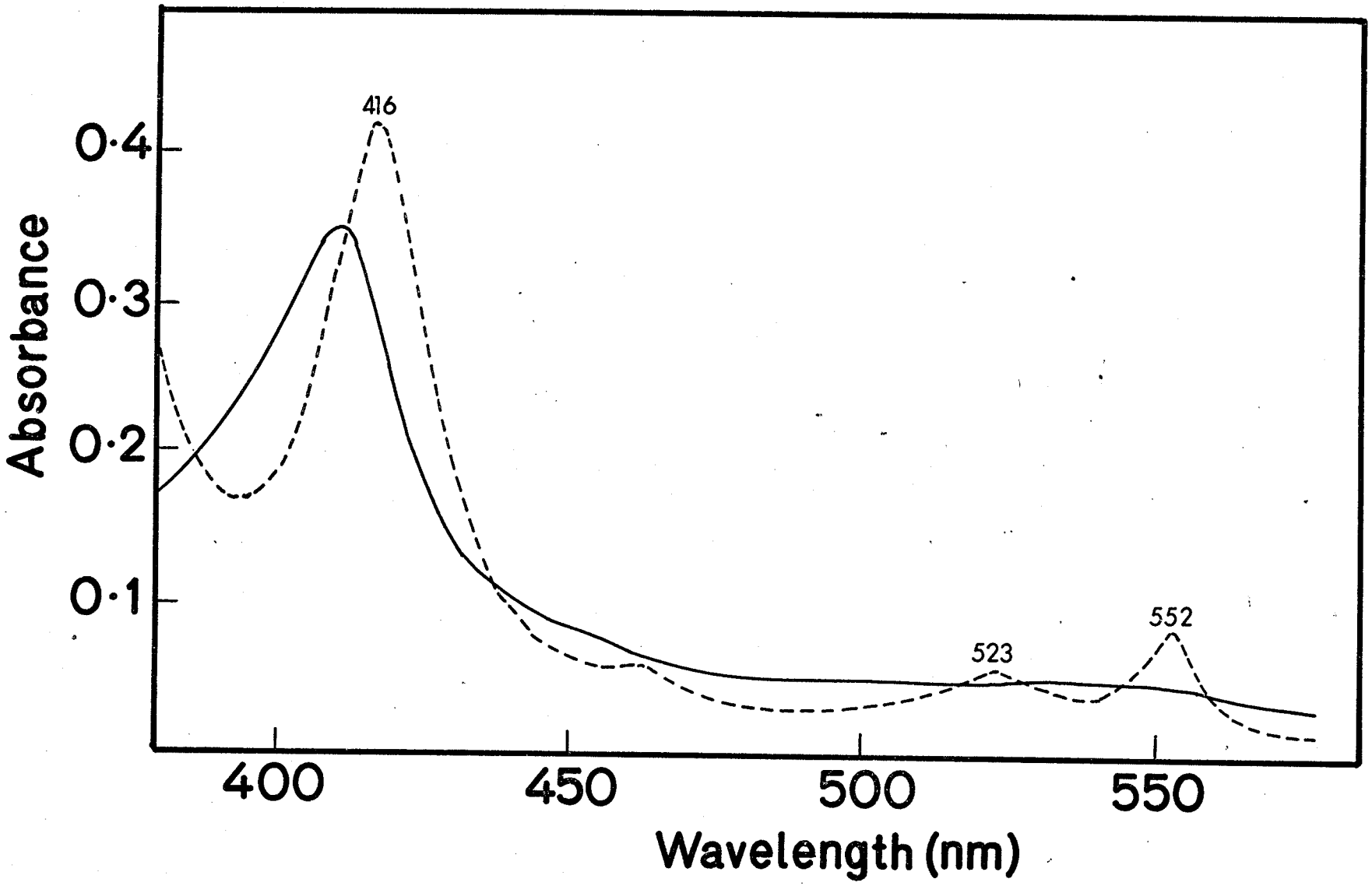

0·8
0·6
0·4
0·2
Absorbance
422
462
525
553
560
400
500
600
Wavelength (nm)
Assayed at 550 nm
NH2OH - Cyt c reductase activity (frac4)
0·5
0·3
buffer + cyt.c
Fraction 4
NH2OH
0·55 min

were not only unable to replace fraction 1 but were also found to be inhibitory when added to a complete reconstituted system.

Fraction 6 could sometimes be replaced partially by either NADH or NH_2OH (Table 14), but not by NAD^+. However, fraction 6 had no absorption peak at 340 nm and any attempts to detect the presence of NAD^+ in this fraction by reduction with alcohol dehydrogenase plus ethanol or hydrosulfite failed. As shown in Table 16, the ammonia-oxidizing activity of the reconstituted system increased from 30% to 49% when the volume of fraction 6 was doubled (reactions 11 and 12). This increase in the activity was not due to the reduction of 0_2 content since the addition of anaerobic buffer equal to the volume of fraction 6 did not increase the activity (reaction 13). However, the long lag was shortened by the addition of anaerobic buffer (reactions 11 and 13).

The absorption spectra of the hydrosulfite-reduced fraction 6 showed the peaks at 416, 523 and 552 as well as 462 nms (Fig. 7 c), indicating the presence of a cytochrome c which perhaps was different from that present in fraction 4 and some cytochrome

Fig. 7c. Oxidized (———) and reduced (----) spectra of fraction 6.


Absorbance
0·4
0·3
0·2
0·1
416
523
552
400
450
500
550
Wavelength (nm)

P-460 which was sometimes absent in this fraction. Neither the NH_2OH-cytochrome c reductase nor cytochrome oxidase activity could be detected in fraction 6 when it was assayed with either oxidized or reduced horse heart cytochrome c in 0.1 M potassium phosphate buffer (pH 7.5). From these results, it was believed that fraction 6, which eluted as a pink band whenever the column was sufficiently anaerobic and turned brown upon exposure to air, contained a smaller molecular weight protein(s) including a cytochrome of c-type and sometimes a small amount of cytochrome P-460.

Effect of BSA, $CuCl_2$ and NADH

BSA, with or without $CuCl_2$ together with NADH could not replace fraction 4. NADH, but not BSA and/or $CuCl_2$ could sometimes replace fraction 6. There was a slight activating effect on the ammonia-oxidizing activity of the fractions 1 + 4, when BSA and $CuCl_2$ were added prior to the addition of NADH (Table 17).

Table 17. Effect of BSA, $CuCl_2$ and NADH on partially resolved and reconstituted system (1 + 4 + 6) obtained from the cell-free extracts of N. europaea.

Reaction	Additions	O_2 Uptake (nmoles/min)		
		1	1 + 4	1 + 4 + 6
1	None	2	4	18
2	NADH	4	18	18
3	NADH, BSA	4	18	18
4	NADH, BSA, $CuCl_2$	2	18	18
5	BSA, $CuCl_2$, NADH	4	22	18

The cell-free extract and the Sepharose fractions 1, 4 and 6 (partially anaerobic column fractionation) were prepared and the reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of fraction(s), 2 mM spermine, 1.7 mM $(NH_4)_2SO_4$ and 0.1 M potassium phosphate buffer (pH 7.5). The additions were made, in the order shown, in microlitre volumes as follows: BSA powder, 20 mg/ml; $CuCl_2$, 0.1 mM and NADH, 67 μM.

Effect of Concentration

(1) Ammonium sulfate fractionation. Almost all the activity of fractions 4 and 6 was lost when they were concentrated (either separately or together) by fractionation with ammonium sulfate (80% saturation) followed by dialysis against 0.1 M potassium phosphate buffer (pH 7.5).

(2) Concentration with sucrose. Fractions 4 and 6 (separately or combined) could be concentrated by this method and could be stored (in either dilute or concentrated form) at -20°C for at least 2 weeks with approximately 90% retention of their ammonia-oxidizing activity (assayed with freshly obtained fraction 1). NADH was not required for the activation (Table 18).

(3) Concentration by membrane filtration (Aminco membrane concentrator). By this method, the combined fractions 4 and 6 could be concentrated approximately 20 fold with 66% retention of their ammonia-oxidizing activity. However, NADH was required for the activation and this requirement was not eliminated by the addition of the filtrate. These results suggested a possibility that small molecular weight componants were either lost or inactivated during the filtration.

Table 18. Effect of sucrose concentration on the ammonia-oxidizing activity of fractions 4 and 6.

Reaction	System		Additions	Activity (%)
	Fractions	Condition		
1	1 + 4 + 6	All fresh	-	100
2	1	Fresh	-	7
3	1	Fresh	NADH	20
4	1 + (4 + 6)	Fresh Stored (control)	± NADH	93
5	1 + (4 + 6)	Fresh Stored (Su-conc.)	± NADH	80

The cell-free extract and Sepharose 6B fractions (partially anaerobic column) were obtained and the reaction was carried out as described in Materials and Methods. Fraction 4 and 6 were combined and divided into 2 parts: one was stored without concentration (control) whereas the other was concentrated 5 fold (approximately) by powdered sucrose as described in Materials and Methods. Both control and concentrated (4 + 6) fractions were stored at -20°C for 2 weeks.

Fresh fractions were obtained (under partially anaerobic conditions) from fresh extract and the reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml fraction(s), 2 mM spermine, 1.7 mM $(NH_4)_2SO_4$ and 0.1 M potassium phosphate buffer (pH 7.5). Concentrated fraction (4 + 6) was added in 0.2 ml volume instead of 1 ml used for the control (4 + 6). NADH (67 µM) was added in microlitre volume.

Effect of Storage

(1) Storage of the fractions. The data presented in Table 19 show that anaerobically obtained fractions could be stored frozen (at -20°C) overnight without any loss in their ammonia-oxidizing activity. On the other hand, aerobically obtained fractions not only lost approximately 20 to 25% activity upon storage overnight at -20°C but they also required NADH for the activation of ammonia oxidation. Moreover, these results also indicated that it was the aerobically obtained fraction 1 which underwent some change during storage (overnight) which resulted in NADH, requirement for ammonia oxidation (reactions 6 and 7). Longer periods of storage of either aerobically or anaerobically obtained fraction 1 resulted in a total loss of its ammonia-oxidizing activity.

The fact that partially purified reductase or S_2 preparations, either of which could replace fraction 4, could be stored at -20°C for several months without an appreciable loss of the activity suggested that fraction 4 was fairly stable under these conditions.

Table 19. Effect of storage on the ammonia-oxidizing activity of Sepharose fractions.

Reaction	Fractions obtained		Additions	Activity (nmoles O_2/min)	% Control**
	aerobically	anaerobically			
1	1 + 4 + 6	-	-	2	7
2	1 + 4 + 6	-	NADH	24	75
3	-	1 + 4 + 6	± NADH	16	100
4	-	1* + 4 + 6	± NADH	20	100
5	1	4 + 6	-	2	5
6	1	4 + 6	NADH	34	81
7	4 + 6	1	± NADH	16	100

*Fraction 1 was aerated by gentle stirring before combining it with the other two fractions.

**Control activity was taken from the similar assay system for each reaction in Table 15.

All the fractions obtained in Table 15 experiment were stored frozen (-20°C) in small stoppered tubes overnight and assayed on the following day under the identical experimental conditions to those used in Table 15.

Fraction 6, which was obtained anaerobically could be stored at -20°C for one week with almost 90% retention of its activity whereas the same fraction, when it was obtained aerobically and stored under the same conditions, lost over 60% of its activity, when assayed with freshly obtained fractions 1 and 4.

Any attempts to store either or both of the two fractions 1 and 6 under a partial or total N_2 atmosphere were unsuccessful.

(2) Storage of the intact N. europaea cells. Since the cell-free extracts obtained from old N. europaea cells (stored at 4°C for 4 to 6 weeks) oxidized ammonia very slowly or not at all, it was believed that the aging of the cells might have affected the structural organization of the cell-membranes, thus resulting in an inactivation of fraction 1 (the membrane fraction). The fractionation of both the active and inactive extracts (obtained from fresh and old cells respectively) was therefore carried out. The results of this experiment are presented in Table 20. As shown here, the fractions obtained from the active extract oxidized ammonia rapidly without NADH activation whereas those obtained from inactive extracts did not oxidize ammonia and NADH could activate only slightly.

Table 20. Ammonia-oxidizing activities of the Sepharose fractions obtained from active and inactive cell-free extracts of *N. europaea*.

Reaction	Fractions from		NADH	Activity
	Active extract	Inactive extract		(nmoles O_2/ min)
1	1 + 4 + 6	-	±	30
2	-	1 + 4 + 6	-	0
3	-	1 + 4 + 6	+	4
4	1	4 + 6	±	28
5	4 + 6	1	-	0
6	4 + 6	1	+	4

The active extract (from fresh cells) and the inactive extract (from old cells) were prepared and the Sepharose fractions from each extract were obtained (under partially anaerobic conditions) as described in Materials and Methods. The ammonia-oxidizing activities of the active and inactive extracts were 140 and 16 nmoles O_2 consumed per min respectively.

All the assay conditions were the same as given in Table 16.

That it indeed was fraction 1 which was sensitive to the aging of the cells, was suggested by the observations that this fraction, when it was obtained from the inactive extract, could not be successfully reconstituted with fractions 4 and 6 from the active extracts. On the other hand, fraction 1, which was obtained from active extracts, could be recombined with fractions 4 and 6 from inactive extracts with 93% retention of the ammonia-oxidizing activity - as compared to the activity of 1 + 4 + 6 (all from active extracts) activity - (Table 20).

Effect of Phospholipase A and Lysozyme

As shown in Table 21, the ammonia-oxidizing activity of the reconstituted system was inhibited 75% when fraction 1 but not fractions 4 plus 6 were preincubated with phospholipase A.

The inhibition of fraction 1 activity by lysozyme was completely reversed by the addition of NADH, whereas there was no significant inhibition when fractions 4 plus 6 were preincubated with lysozyme. These results indicated that fraction 1

Table 21. Effect of phospholipase A and lysozyme on Sepharose 6B fractions.

Treatment	Preincubation with	NADH	Inhibition %
Phospholipase A	Fraction 1	±	75
	Fractions 4 plus 6	±	10
Lysozyme	Fraction 1	-	60
		+	0
	Fractions 4 plus 6	±	10

Sepharose 6B fractions were obtained (partially anaerobic column) and the reaction was carried out in a Gilson Oxygraph as described in Materials and Methods.

The fractions were preincubated with either 10 µl of phospholipase A or 150 µg of lysozyme for 15 minutes at room temperature (25°C).

The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of each of the fractions, 2 mM spermine, 1.7 mM $(NH_4)_2SO_4$ and 0.1 M potassium phosphate buffer (pH 7.5). There was no loss in the activity when the untreated fractions were preincubated at room temperature for 15 minutes.

contained phospholipids which played an important role in ammonia oxidation. The oxidation of succinate and NADH in submitochondrial particles was also reported to be inhibited by phospholipase A (9).

Resolution of S_1 on DEAE-cellulose Columns

The preparation S_1 (Materials and Methods) the components of which appeared to be similar to those of S_2 (Materials and Methods) could replace both the fractions 4 and 6 in the reconstituted system (Table 22). Since the active components in fractions 4 and 6 seemed to be concentrated in S_1, S_1 was chromatographed on DEAE-cellulose column for further resolution, as described in Materials and Methods. It could be resolved into two separate fractions viz. AS_1 and AS_2.

Reconstitution of the ammonia-oxidizing system from AS_1, AS_2 and the Sepharose fractions.

A pinkish fraction AS_1 which was excluded with the equilibrating buffer (0.1 M potassium phosphate, pH 7.5), showed a spectrum of cytochrome c when it was reduced with hydrosulfite. The spectrum was

similar to that of fraction 6 except that cytochrome P-460 was absent.

A dark brown portion which remained on the top of the DEAE column, could be eluted as fraction AS_2 with 0.25 M potassium phosphate buffer (pH 7.5). The hydrosulfite reduced spectra of AS_2 revealed the presence of similar components to those found in fraction 4, viz. the cytochromes of b-, c- and P-460 types and a high NH_2OH-cytochrome c reductase activity.

As shown in Table 21, the substitution of AS_1 in the place of Sepharose fraction 6, resulted in a significant increase (from 47 to 70%) in the recovery of the ammonia-oxidizing activity of the reconstituted system (reactions 2 and 3). The substitution of both AS_2 and AS_1 in the places of fractions 4 and 6 respectively further increased the recovery of the activity to 75% (reaction 5). Although AS_2 could replace fraction 4 in the reconstituted system, it was not found to be more effective (reaction 12).

Either an increase in the amount of AS_2 (reaction 11) or the addition of both AS_2 and fraction 4 (reaction 10) resulted in an inhibition of the ammonia-oxidizing activity in the presence of NADH as an activator.

Table 22. Reconstitution of the ammonia-oxidizing system from the Sepharose, AS_1 and AS_2 fractions.

Reaction	System	NADH addition	Activity (%)*
1	1 + S_1	±	36
2	1 + 4 + 6	±	47
3	1 + 4 + AS_1	±	70
4	1 + 4	-(+)	2 (44)
5	1 + AS_2 + AS_1	±	75
6	1 + 6 + AS_1	±	17
7	1 + 6	±	14
8	1 + AS_1	±	17
9	1 + AS_2	-(+)	5 (35)
10	1 + 4 + AS_2	-(+)	5 (11)
11	1 + AS_2	-(+)	5 (10)
12	1 + AS_2 + 6	±	41

*The percentage activity of the reconstituted system was calculated on the basis of the original ammonia-oxidizing activity of the cell-free extract being taken as 100%. The extract, Sepharose fractions (under partial anaerobic conditions) and S_1 were obtained and the reaction was carried out in a Gilson Oxygraph as described in Materials and Methods. AS_2 and AS_1 were obtained by DEAE-cellulose column fractionation of S_1 as described in Materials and Methods. The reaction mixture in a total volume of 1.5 ml contained 0.5 ml of either the cell-free extract or fraction(s) (except in reaction 11 where 1.0 ml of AS_2 was used), 2 mM spermine, 1.7 mM $(NH_4)_2SO_4$ and 0.1 M potassium phosphate buffer (pH 7.5). NADH (67 µM) was added after the reaction was started with $(NH_4)_2SO_4$ addition.

PART III. STRUCTURAL STUDIES

Intact Cells and Cell-free Extracts

A comparison of active and inactive cells of Nitrosomonas europaea is shown in Plates 1 and 2 respectively.

Thin sections of cells active in ammonia oxidation resembled those observed by Murray and Watson (54). General intracellular structure of the active cell appeared contracted and there was considerable amount of darkly stained particulate matter within the cell and also in between the membrane layers (Plate 1). Moreover, the membrane layers were closely associated and the outer layer was drawn taut over them. In contrast, the structure of inactive cells was strickingly different (Plate 2). In this case, the cell appeared relaxed and somewhat swollen. The darkly stained particulate matter within the cell and in between the membrane layers was almost entirely absent. In agreement with an observation by Hooper, Erickson and Terry (31) that the release of proteins by freezing and thawing was

accompanied by the disappearance of particulate matter from between the closely associated membranes, the above results also suggest that this particulate material may be involved in ammonia oxidation. The morphological organization of the particulate matter present between the membrane layers may possibly represent the cytochromes and the electron transport components (31). If so, their close physical interaction with membrane-bound components such as a-type cytochromes would be essential for ammonia oxidation. This concept was supported by the evidence obtained in the electron-micrographs of negatively stained preparation of cell-free extracts.

As observed in Plate 3, the extract which did not oxidize ammonia contained membrane fragments and vesicle-like structures of various sizes scattered in an irregular manner. When activated with spermine, this extract oxidized ammonia slowly (Fig. 1). Electron micrographs of the extract activated by spermine revealed that there was a formation of membrane continum which appeared to have resulted from the joining of several membrane fragments (Plate 4). Addition of BSA to the inactive extract resulted in very active ammonia oxidation (Fig. 1) and at the same time in considerable structural change.

In this case, the vesicle-like structure were observed to be attached to the membranes thus forming clusters of aggregated structures in an organized manner (Plate 5). When this extract, which was actively oxidizing ammonia, was inactivated with dipyridyl and was examined after negative staining, however, the organized structure of the membranes and vesicle-like structure seemed to have been disrupted resulting into irregularly scattered fragments (Plate 6).

Partially Resolved and Reconstituted System

The membrane fraction (fraction 1 from the Sepharose 6B column) which was not active either by itself or together with spermine and/or BSA, appeared somewhat similar to the inactive extract in that there were many scattered fragments present throughout the field (Plate 7). However, rather large membranous folds or rings were also present (arrow in Plate 7) which seemed to have settled one on top of the other. Plate 8 presents a lower magnification view of the fraction 1.

The examination of a mixture of fractions 4 plus 6 (from the sepharose 6B column) and spermine revealed no such membrane folds or rings, but globular structures of various sizes were seen to be joined together in a ring-like manner (Plate 9). These ring-like structures were held together by tiny fragments and globules (arrow in Plate 9). Plate 10 is a general view of fractions 4 + 6 at lower magnification.

Upon the combination of the membrane fraction 1 with fractions 4 + 6 and spermine, a highly active, reconstituted system was obtained. This reconstituted system contained an organized aggregation of large membrane folds with smaller vesicle-like structures. This aggregation was very striking in almost all the areas observed of the reconstituted 1 + 4 + 6 fractions (Plate 11). General pattern of the reconstituted system is shown in Plate 12.

Plates 13 and 14 are the control pictures of BSA and BSA plus spermine respectively. The pictures of spermine alone could not be obtained.

Plate 1. Active cells of *N. europaea*.
Final Magnification, x 150,000
(approximately)

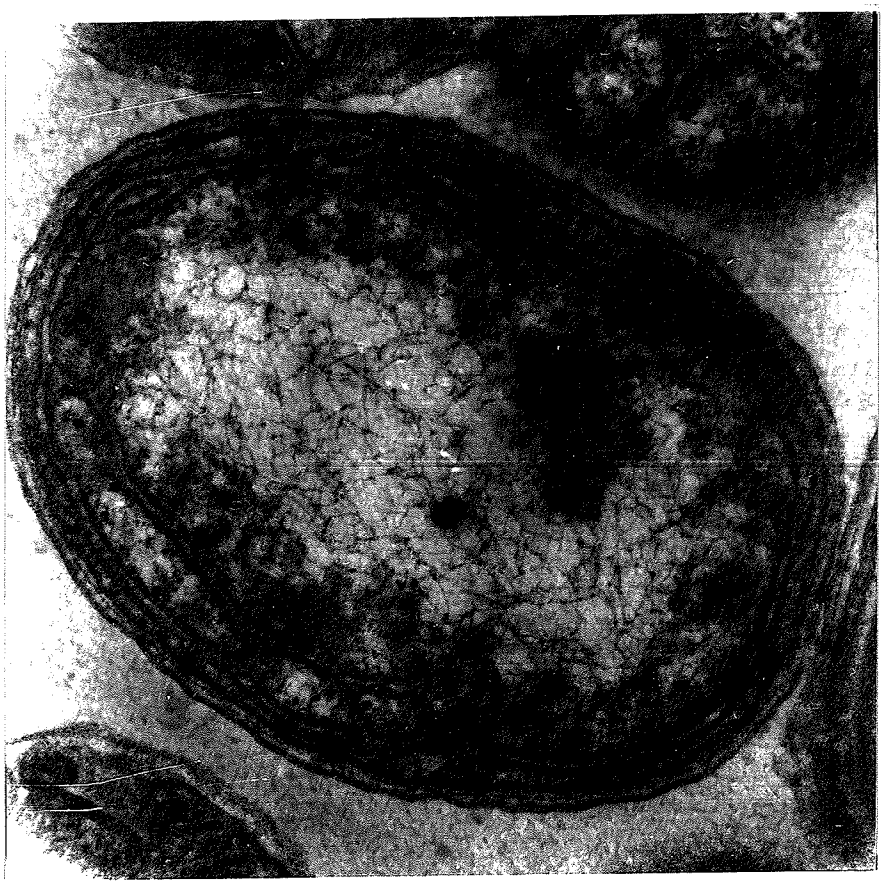


Plate 1a. Lower magnification view of Plate 1.
Final Magnification, x 30,000
(approximately)

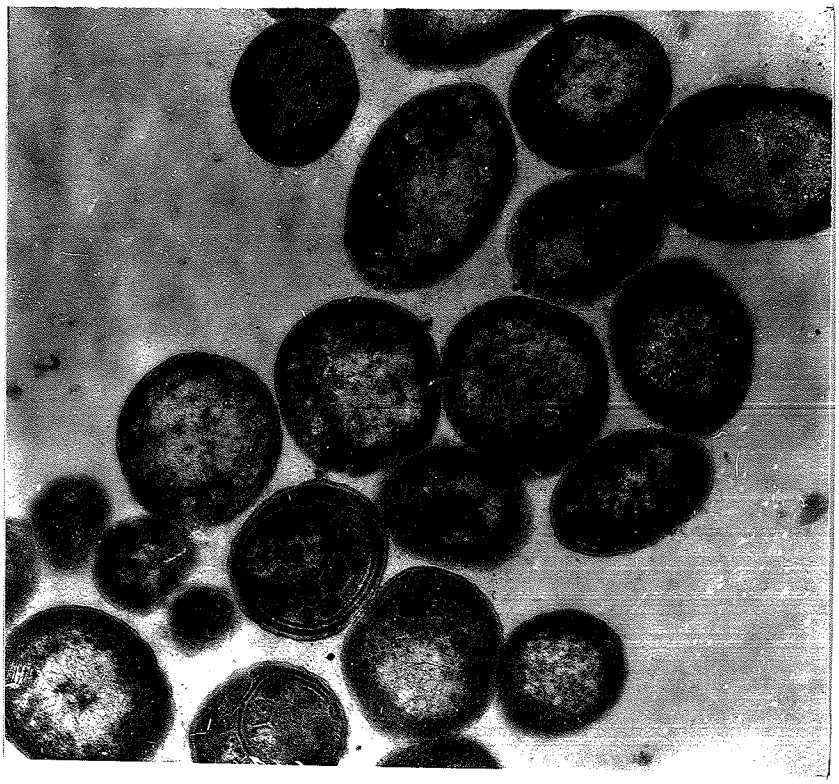


Plate 2. Inactive cells of N. europaea.
Final Magnification, x 150,000
(approximately)

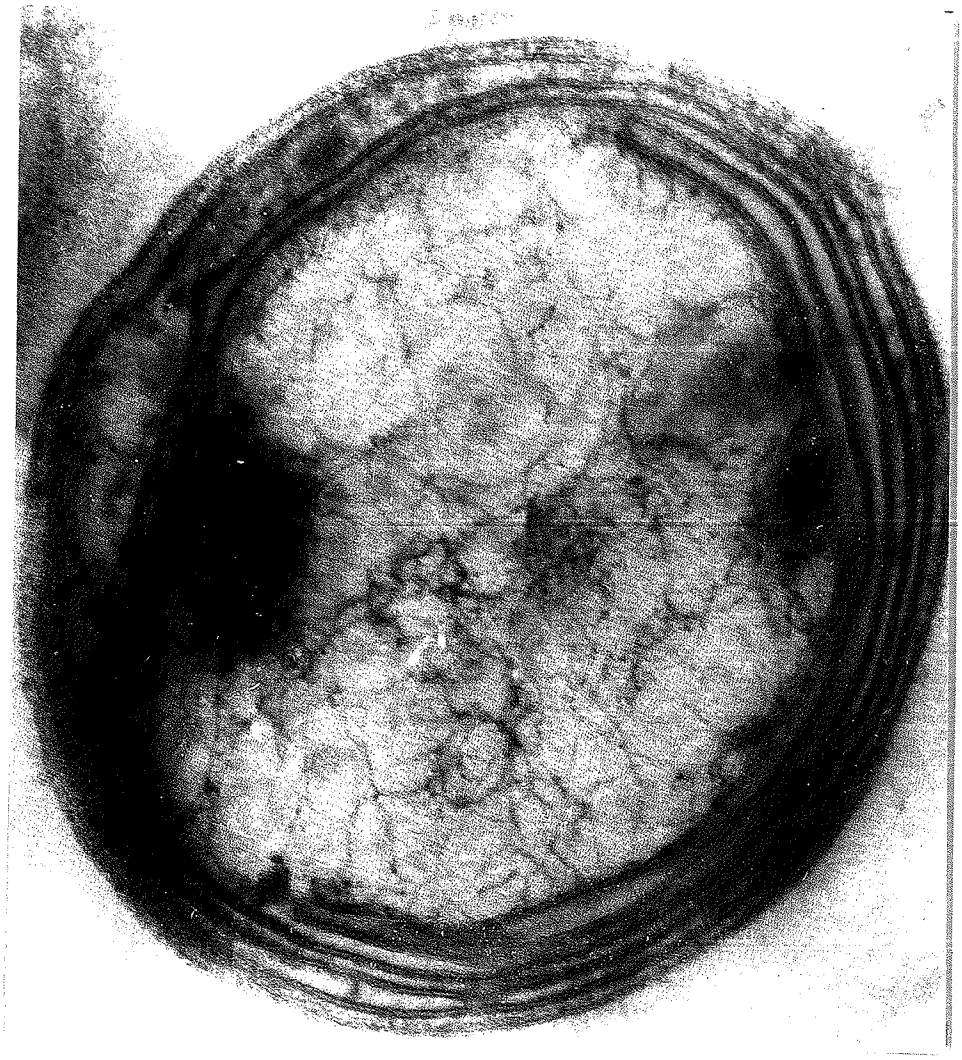


Plate 2a. Lower magnification view of Plate 2.

Final Magnification, x 30,000

(approximately)

Plate 3. Cell-free extract inactive in ammonia oxidation. Final Magnification, x 30,000 (approximately)

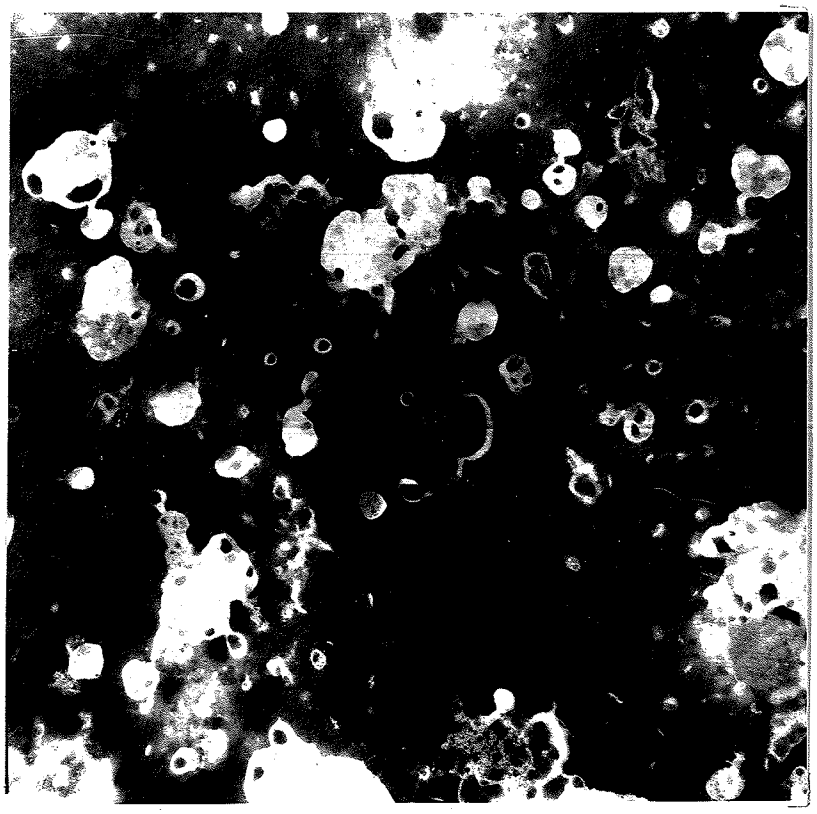


Plate 4. Cell-free extract activated by spermine.
Final Magnification, x 30,000
(approximately)

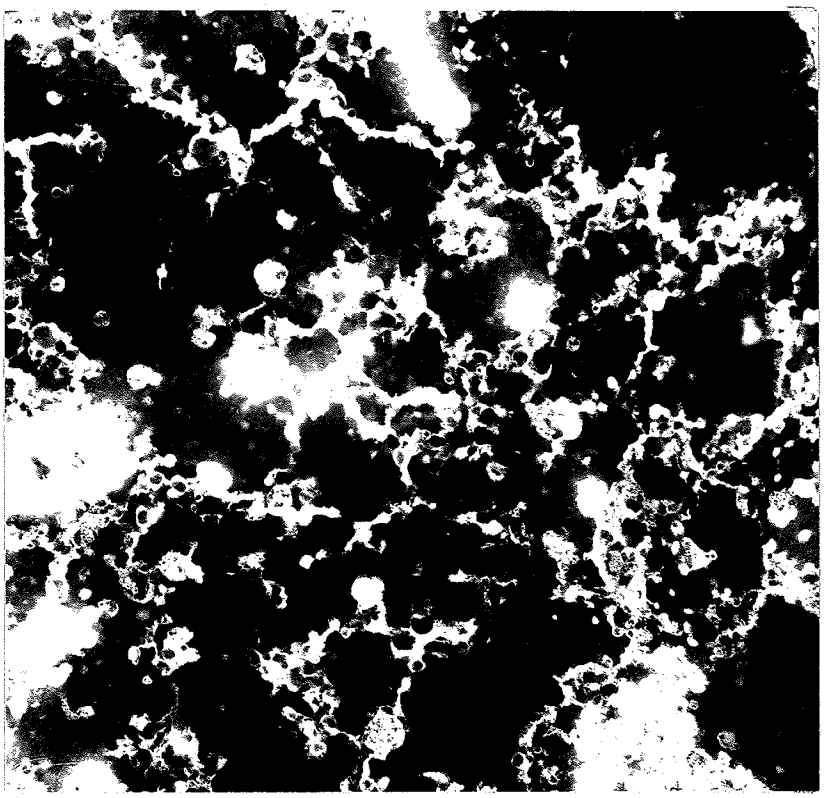


Plate 5. Cell-free extract activated by BSA.
Final Magnification, x 30,000
(approximately)

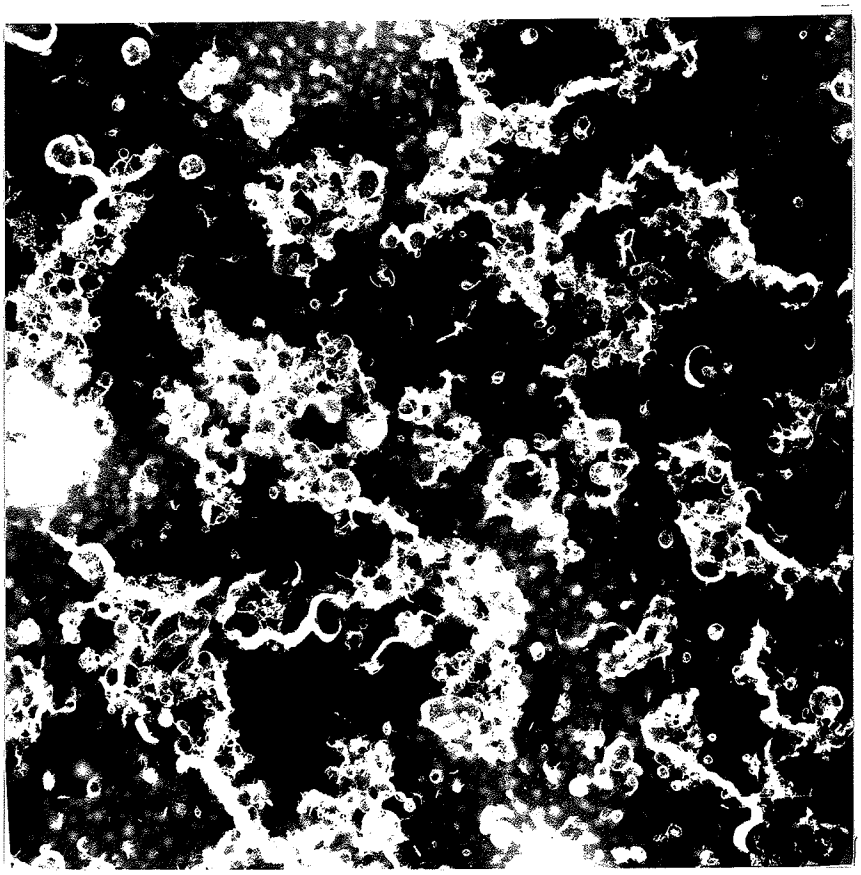


Plate 6. Cell-free extract inactivated by dipyridyl. Final Magnification, x 30,000 (approximately)

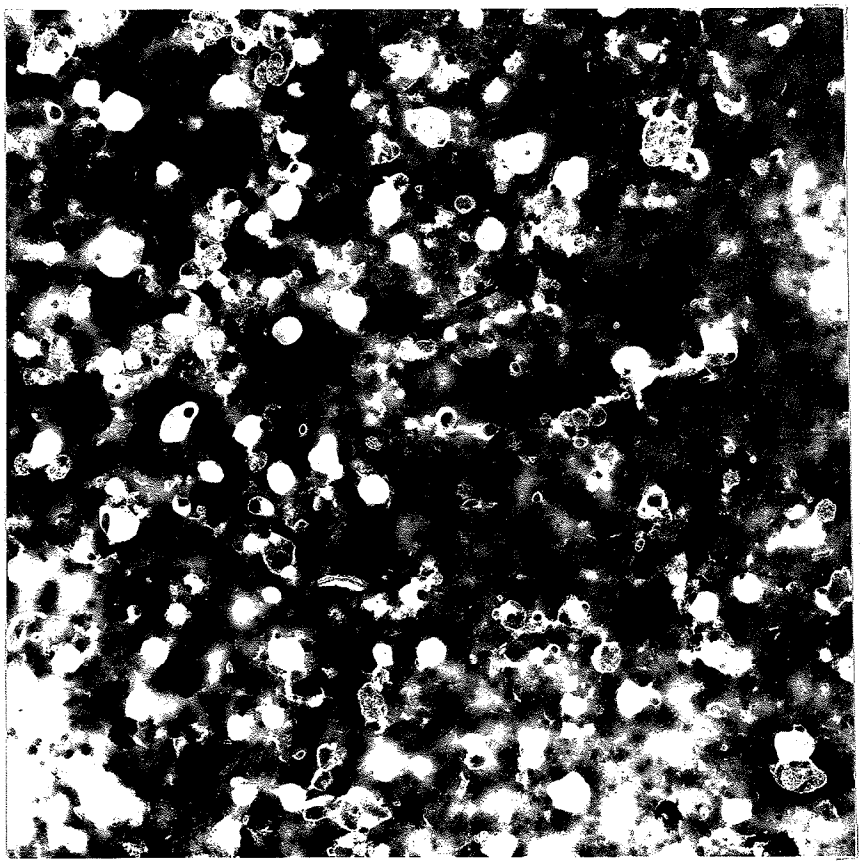


Plate 7. Fraction 1 at higher magnification.
Final Magnification, x 45,000
(approximately)

Plate 8. General pattern of fraction 1 at lower magnification. Final Magnification, x 20,000 (approximately)

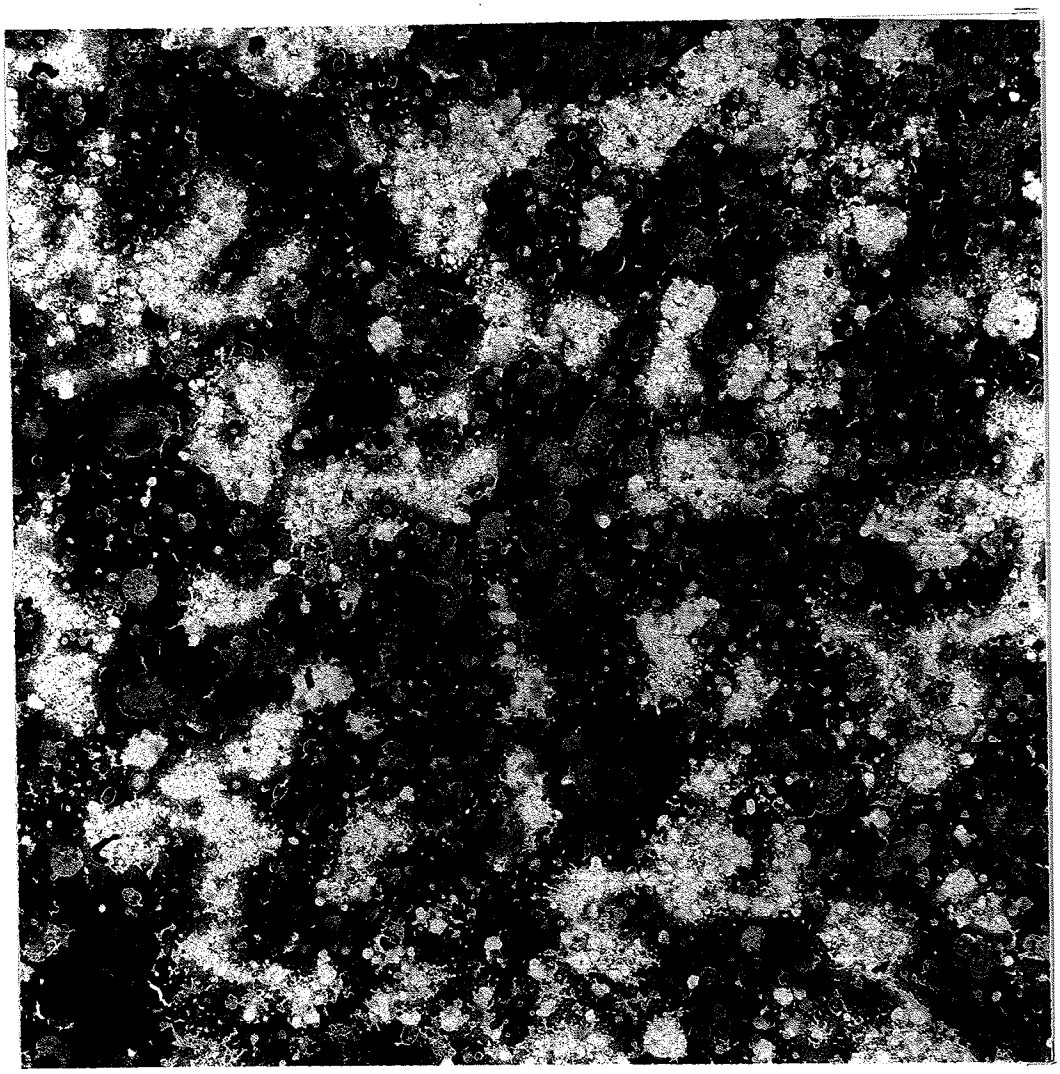


Plate 9. Fractions 4 plus 6 at higher magnification. Final Magnification, x 45,000 (approximately)

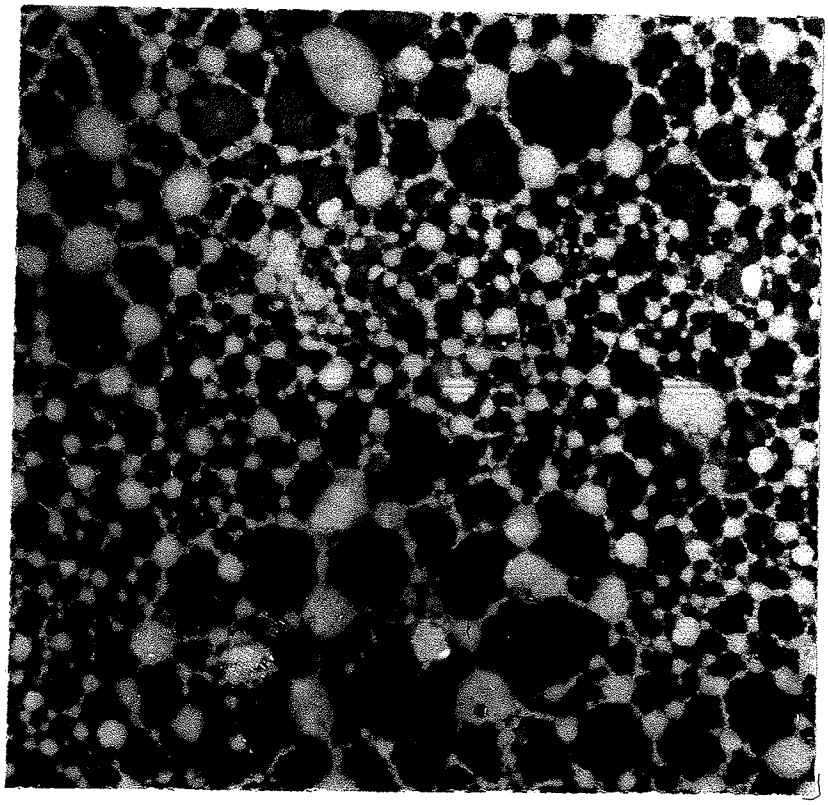


Plate 10. General pattern of fractions 4 plus 6 at lower magnification.
Final Magnification, x 20,000
(approximately)

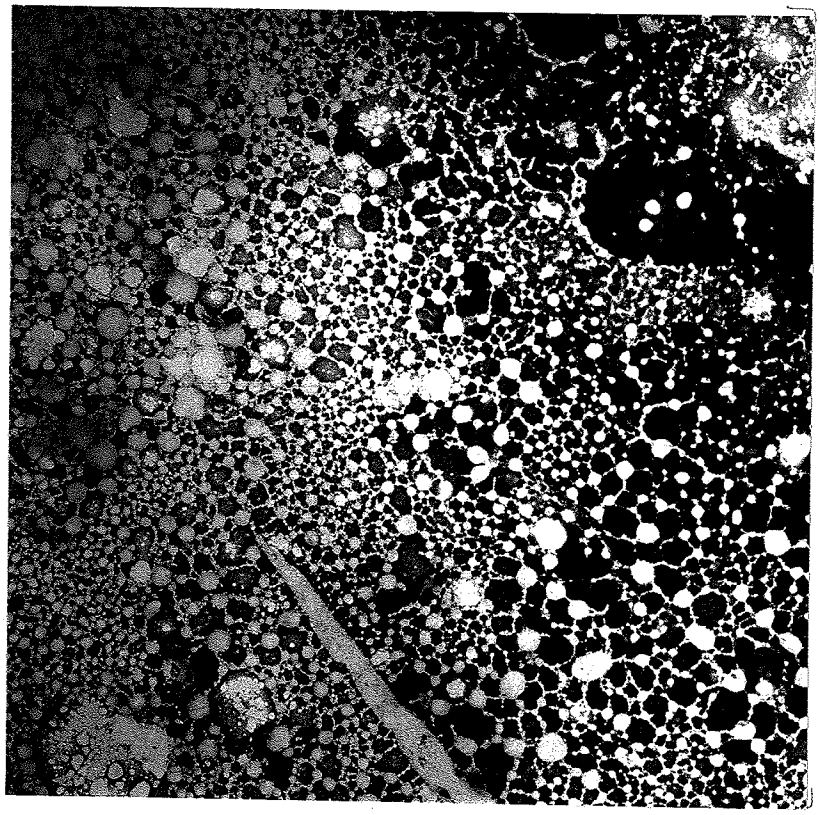


Plate 11. Reconstituted system 1 + 4 + 6 at higher magnification.

Final Magnification, x 60,000 (approximately)

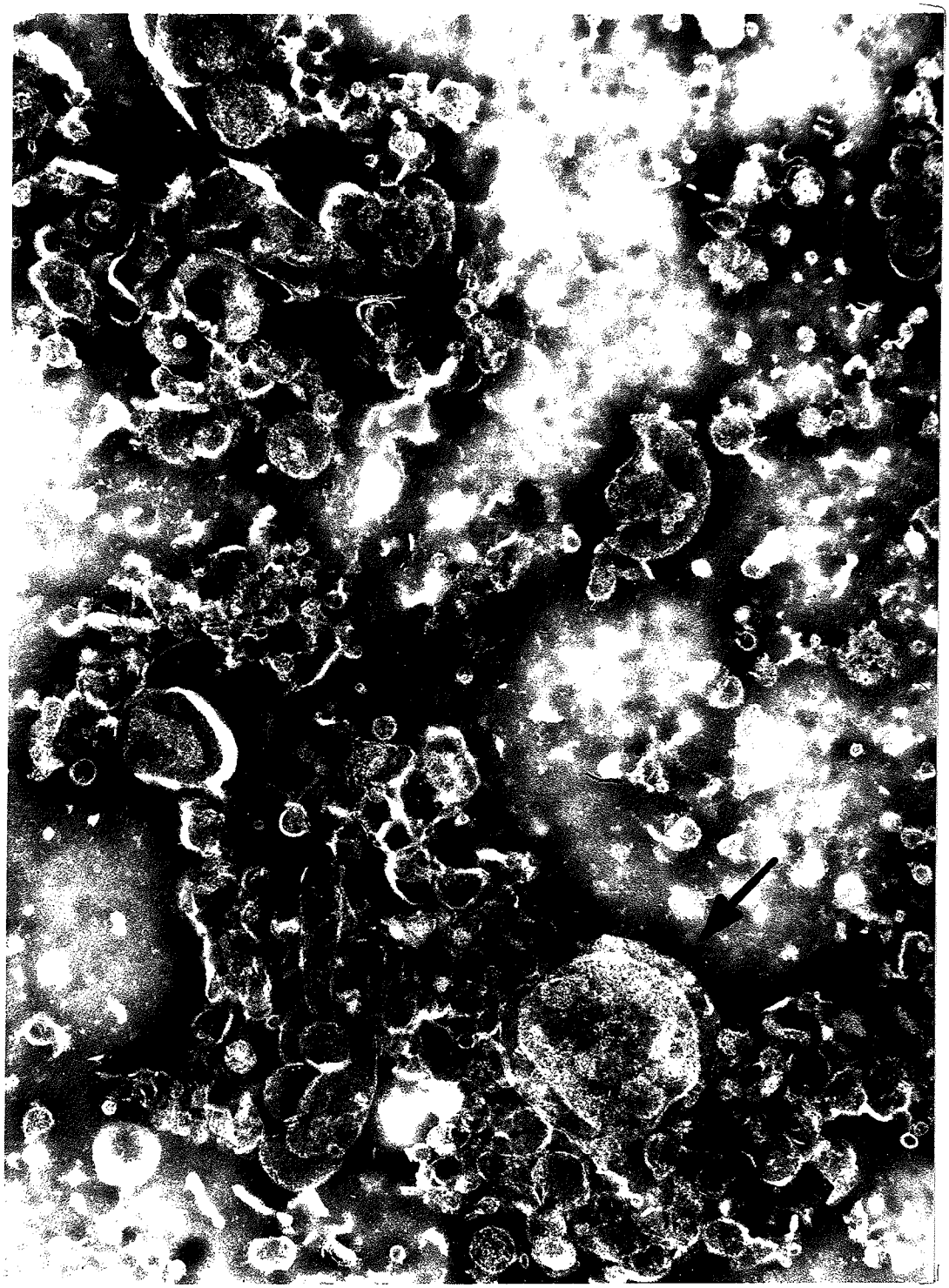


Plate 12. General pattern of the reconstituted (1 + 4 + 6) system at lower magnification. Final Magnification, x 20,000 (approximately)

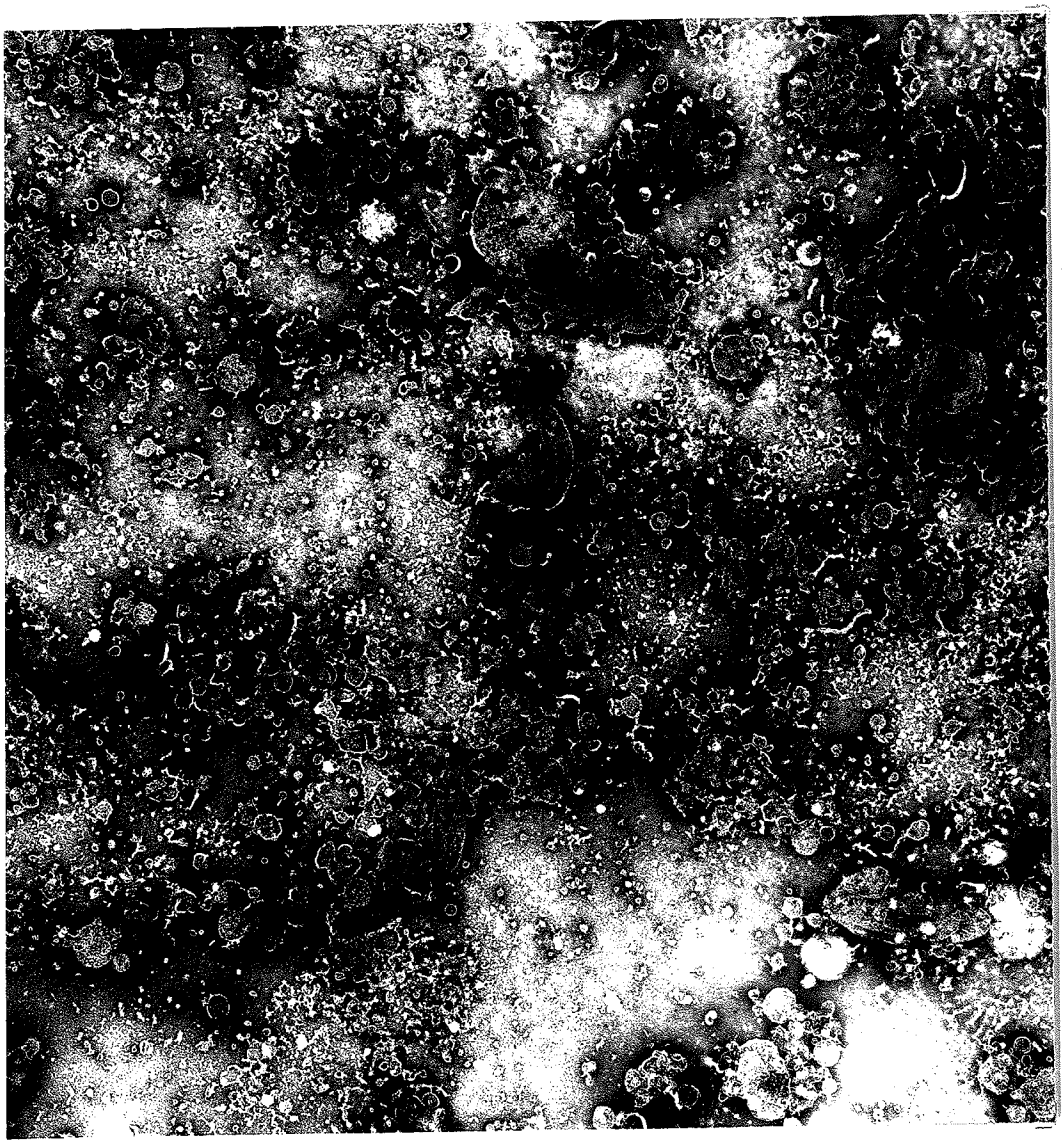


Plate 13. BSA only.

Final Magnification,

x 45,000 (approximately)

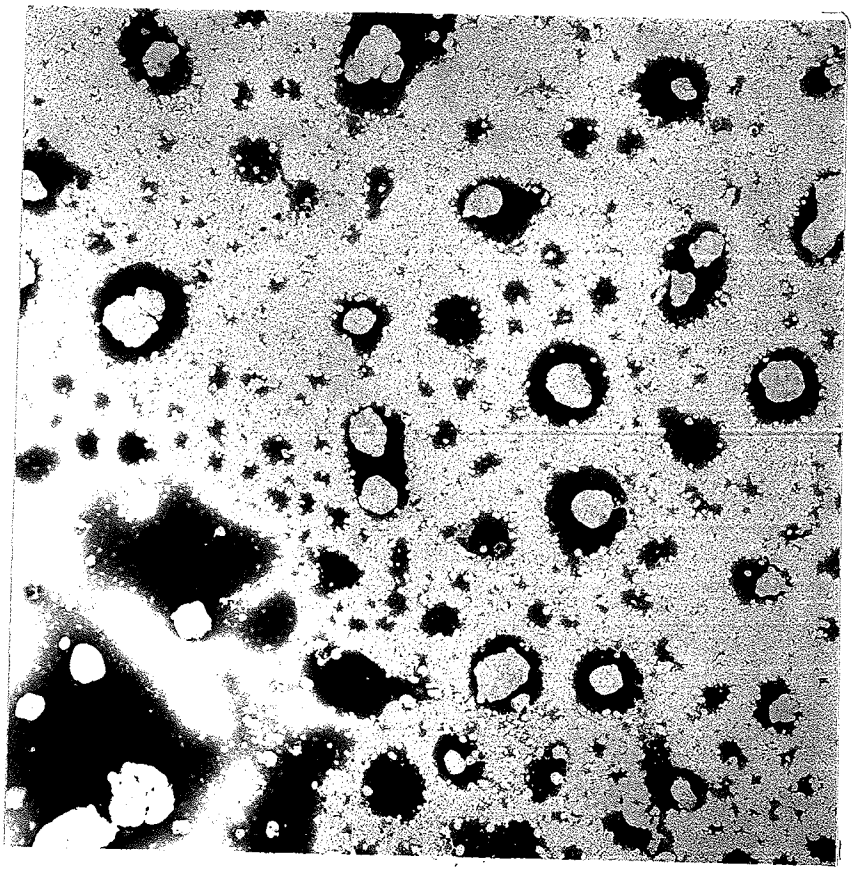
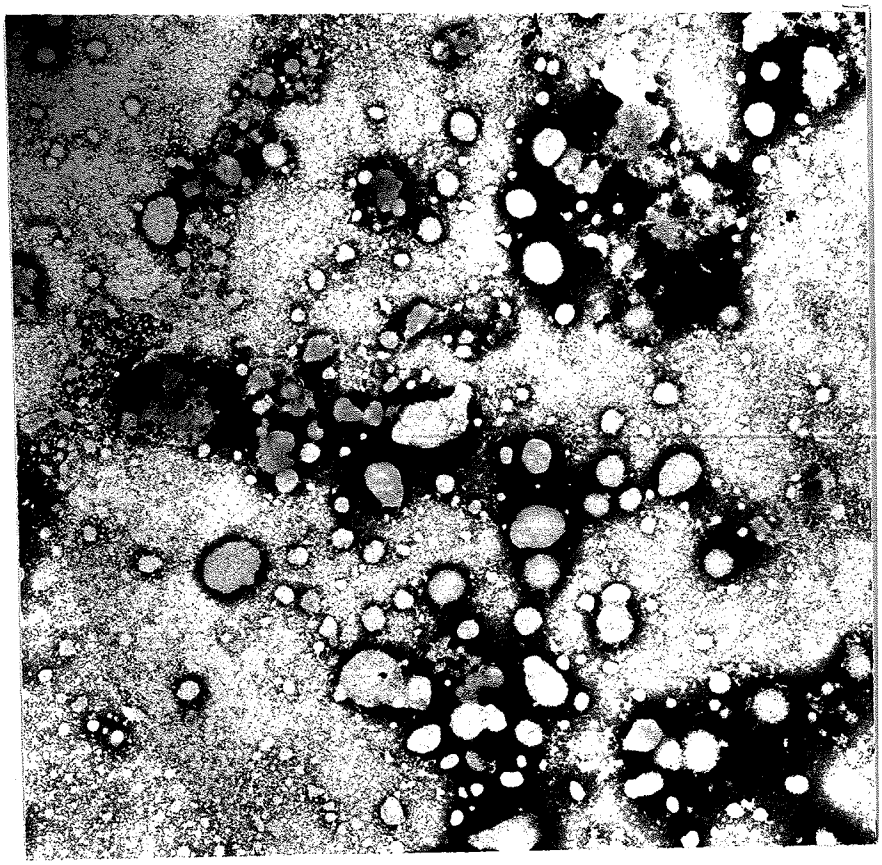


Plate 14. BSA plus spermine.
Final Magnification,
x 45,000 (approximately)



D I S C U S S I O N

DISCUSSION

Part I. General Aspects of Cell-free Ammonia and Hydroxylamine Oxidations

It has been established that the cell-free extracts prepared from N. europaea can oxidize ammonia when activated by Mg^{++}, spermine or BSA (71). As shown in Fig. 1, the presence of BSA and spermine was required for obtaining highly active extracts.

Polyamines are well-known for their roles in neutralizing and stabilizing cellular polyanions including nucleic acids, polysaccharides and phospholipids (76). Stabilizing effect on bacterial protoplasts and spheroplasts-an effect that must occur at the level of cell-membrane - of spermine and spermidine was reported by several workers (24, 48, 75). An anti-oxidant effect of the polyamines had also been observed for unsaturated fatty acids (74). From his results, Harold (24) concluded that the stabilization of Streptococcus protoplasts resulted from ionic binding of the cation to acidic sites on the external

surface of the plasma membrane thus conferring upon it an additional mechanical strength while not altering its permeability to extracellular solutes. The role and effects of polyamines on membrane structure and permeability was studied by Silver et al in 1970 (68). Bachrach (7) reviewed the metabolism and function of spermine and related polyamines.

In the Nitrosomonas system, Mg^{++} could replace spermine (71). Spermine was also reported to replace Mg^{++} in holding together the 30 S and 50 S ribosomes in protein synthesis (57, 76, 77). In view of these observations, it seemed possible that spermine might be acting as a stabilizer of the membrane structure and protecting the active sites for ammonia oxidation in Nitrosomonas. Protective and activating effect of BSA is discussed in the later part of this chapter.

Engel and Alexander (15) made an important observation that N. europaea cells lost their ammonia-oxidizing ability upon "aging". They also proposed that ammonia was oxidized at the cell surface. Lees (43) suggested that during aging the structure of the cell was modified with consequent impairment of the ammonia-oxidizing system. The results presented in Table 1 indicated that the extracts prepared from aged cells oxidized ammonia slowly in the presence

of spermine and NADH activated it further, but a highly active system could be obtained when BSA and $CuCl_2$ were included along with spermine and NADH. Moreover, an active cell-free extract lost all the activity upon dialysis against the assay buffer (0.1 M potassium phosphate, pH 7.5) which could be completely restored by the addition of $CuCl_2$. It could be inferred from these results that an active component either leaked out during aging of the cells or it was somehow inactivated during storage. The addition of external copper, complexed with BSA, replaced this required component. Also, the order of addition was important. BSA is known to form a complex with cupric ion (47). Whenever $CuCl_2$ was added in the absence of BSA, it was found to be inhibitory to the ammonia-oxidizing activity of some of the extracts (Table 1). Further, the addition of $CuCl_2$ along with BSA introduced a long lag (reaction 11, Table 1) which was completely eliminated by NADH or NH_2OH. The effect of NH_2OH in diminishing the lag observed during ammonia oxidation by Nitrosomonas cells has also been reported by Hooper (30). Suzuki and Kwok (70) pointed out that the ammonia-oxidizing activity of inactive spheroplasts could be restored by NH_2OH. From these observations

it was suggested that the copper containing component needed to be reduced before it became active and the reducing power could be supplied by either NADH or NH_2OH or was generated during the long lag period observed in the absence of such added electron donors as NADH or NH_2OH. This suggestion was further supported by a previous report made by Kwok (39) that the oxidation of ammonia was initiated by the priming effect of cytochromes (553 and 603 nm) which were first reduced (upon the addition of ammonia) before oxygen consumption started. It is possible that either one or both of these cytochromes are membrane bound and that at least one of them (603 nm, a-type cytochrome) contains copper. Falcone, Shug and Nicholas (22) reported that N. europaea particles contained a copper protein and that the metal was involved in the hydroxylamine oxidase system. The results obtained in Table 14 also supported the above argument since the Sepharose fractions which were obtained under complete aerobic conditions required either the preincubation or the addition of NADH in order to recombine into an active ammonia-oxidizing complex. Also, during this preincubation period, the O_2 tension in these fractions was found to have decreased indicating the occurrence of endogenous electron flow during this period.

However, complete anaerobic conditions created by chemical agents such as mercaptoethanol or dithiothreitol and also by total N_2 atmosphere were found to destroy the ammonia-oxidizing activity completely. Therefore, it could be deduced that a certain level of O_2 tension was required in order to facilitate the functionally active reconstitution of the ammonia-oxidizing enzyme complex. The protection provided by BSA might be due to the creation of a hydrophobic environment around the enzyme complex, thus preventing the harmful effect of high oxygen tension and at the same time preserving the structural integrity of the membranes. It might be of interest here to point out that a solution of BSA (20 mg per ml in 0.1 M potassium phosphate buffer of pH 7.5) which was kept exposed to air for 45 to 50 minutes at room temperature was found to have absorbed atmospheric O_2. When this BSA solution was used in the reaction mixture instead of BSA powder, it was found to inhibit the ammonia-oxidizing activity of the pellet and supernatant obtained by differential centrifugation technique.

In keeping with the previous observations made by Engel and Alexander (17), the results obtained in Table 3 showed that freezing of the intact

N. europaea cells resulted in considerable loss of their ammonia-oxidizing activity. Moreover, the extracts obtained from such (frozen and thawed) cells could not be activated by the addition of $CuCl_2$ and NADH. On the other hand, some of the very active extracts prepared from fresh cells in the presence of BSA could be stored frozen without much loss in their ammonia-oxidizing activity. However, in some cases, the addition of $CuCl_2$ and NADH was necessary for the activation. Here again, it was indicated that the structural integrity of the cellular membrane might have been disrupted during freezing and thawing of the cells resulting into an inactive extract upon breakage of the cells. This structural integrity was somehow protected by BSA in the extract prepared in its presence from the unfrozen cells and these extracts did not entirely lose their ammonia-oxidizing activity upon freezing. Structural evidence to this effect of BSA, presented in the electron micrographs is discussed in the later part of this chapter.

Meyerhof (52) suggested that although NH_4^+ cannot easily penetrate the cell wall of Nitrosomonas the uncharged NH_3 can and does. Engel (14) believed that ammonia oxidation in Nitrosomonas took place on

the cell-surface (using NH_4^+ as a substrate) and not within the cytoplasm. In this study however, the evidence was presented that it is the uncharged NH_3 which is the actual form of substrate. Similar situation seemed to exist in Nitrobacter agilis, where HNO_2 rather than NO_2^- was suggested to be the substrate for nitrite oxidase system (58).

Phosphate requirement for the growth of Nitrosomonas cells (13); in the hydroxylamine oxidation in crude extracts (55) and in the oxidation of ammonia by the cell-free extracts of N. europaea (39) has been known for a number of years. The fact that phosphate could be replaced by sulfate (39) suggested the ionic influence on the physico-chemical structure of the ammonia-oxidizing system. It was observed in this study that the phosphate concentration used for assaying the ammonia-oxidizing activity (viz: 0.1 M) inhibited both the NH_2OH-cytochrome c reductase and cytochrome oxidase activities when assayed with mammalian cytochrome c (Table 10), while hydroxylamine oxidation by the extracts in the absence of mammalian cytochrome c was faster in 0.1 M than in 0.01 M phosphate (Table 11). These results suggested that phosphate played an important role also in balancing the oxidation-reduction levels of the ammonia-oxidizing system.

Pertinent to this were the observations that an addition of an excess of either reductase (partially purified preparation) or any fraction containing cytochrome oxidase in reconstitution experiments, resulted in an inhibition of the ammonia-oxidizing activity of the reconstituted system. A delicate balance between the oxidation and reduction steps must therefore exist.

Purification, properties and function in NH_2OH oxidation of cytochromes c-552 and c-554 derived from N. europaea were reported by Yamanaka and Shinra in 1974 (88). They reported that NH_2OH-cytochrome c reductase did not react with cytochrome c-552, while it reduced this cytochrome with hydroxylamine in the presence of cytochrome c-554, which was autooxidizable. From their results, Yamanaka and Shinra concluded that electron transfer from hydroxylamine to oxygen in N. europaea occurred as follows:

$NH_2OH \longrightarrow$ NH_2OH-cytochrome c reductase $\longrightarrow$ cytochrome c-554 $\longrightarrow$ cytochrome c-552 $\longrightarrow$ cytochrome oxidase $\longrightarrow$ oxygen.

The oxidation of ammonia by N. europaea extracts was completely inhibited by 10^{-4}M α,α'-dipyridyl or o-phenanthroline (Table 6). The results in Fig. 5, Fig. 6 and Table 7 showed, however, that these metal chelators did not inhibit but activated the rates of NH_2OH and

NADH oxidation by crude cell-free extracts of N. europaea especially in the presence of $CuCl_2$. Nitrite formation from NH_2OH was however, found to be decreased under these conditions (Table 7). Also, the rate of cytochrome c (type III, from horse heart) reduction by NH_2OH was considerably faster when the crude cell-free extracts of N. europaea but not a partially purified preparation of NH_2OH-cytochrome c reductase (27) was treated with either of the chelators and $CuCl_2$.

Nitrite-utilizing enzyme from N. europaea, the properties of which were found to be similar to the copper-requiring "denitrifying enzyme" from Pseudomonas (36) was characterized by Hopper in 1968 (29). He suggested a possibility that the Nitrosomonas nitrite reductase and terminal oxidase were two separate enzymes competing with each other for the electrons originating from NH_2OH. He further pointed out that the terminal oxidase was rate-limiting for NH_2OH oxidation and that the reduction of nitrite was greater than that of O_2. It was also proposed that NH_2OH oxidation involved the removal of two electrons by two separate carriers with each carrier being re-oxidized by one of the two separate oxidases and only the second oxidase was able to function as nitrite reductase (28, 29). α,α'- Dipyridyl however, was found to be a potent inhibitor of the enzyme nitrite

reductase purified by Hooper (28, 29).

In view of the above observations, it seemed possible that two separate reductases and also two different electron acceptors, e.g. cytochromes of c-type, might be involved in the two-step electron transfer from NH_2OH to NO_2^-. One of these reductases was activated by $CuCl_2$ and dipyridyl whereas the other was not affected.

Thus, the oxidation of NH_2OH to NO_2^- might occur according to the following reactions:

(1) $NH_2OH + 2$ cyt. c-554 $(Fe^{+3}) \xrightarrow{NH_2OH\text{-cyt. c reductase}}$ $(NOH) + 2$ cyt.c-554 $(Fe^{+2}) + 2H^+$

(2) $(NOH) + 2$ cyt.c-552 $(Fe^{+3}) + H_2O \xrightarrow{\text{nitrite reductase}} HNO_2 + 2$ cyt.c-552 $(Fe^{+2}) + 2H^+$

(3) 2 cyt.c-554 $(Fe^{+2}) + 2$ cyt.c-552 $(Fe^{+3}) \longleftrightarrow$ 2 cyt.c-554 $(Fe^{+3}) + 2$ cyt. c-552 (Fe^{+2})

or

2 cyt.c-554 $(Fe^{+2}) + O_2 \xrightarrow{\text{auto oxidation}}$ 2 cyt.c-554 (Fe^{+3})

(4) 2 cyt.c-552 $(Fe^{+2}) + \frac{1}{2} O_2 + 2H^+ \xrightarrow{\text{cyt. c oxidase}}$ 2 cyt.c-552 $(Fe^{+3}) + H_2O$

The assignment of cyt. c-554 and cyt. c-552 in reactions 1 and 2 are partly based on Yamanaka and Shinra's (88) results that the former reacted with NH_2OH-cytochrome c reductase and the latter with the Pseudomonas nitrite reductase (87). Although not established in this thesis, the $CuCl_2$-dipyridyl mixture might have stimulated the reaction 2 since the partially purified NH_2OH-cytochrome c reductase activity was not stimulated. Reaction 3 is simply an electron transfer between the two cytochrome c s and it is tempting to speculate its activation by or requirement for Cu^{++}. It will agree with the suggestion by Hooper (29) that NH_2OH-HNO_2 oxido-reductase requires Cu^{++}. The system studied by them may be a combination of reactions 1, 2 and 3 (reaction 2 going from right to left).

Cytochrome c-554 may be auto-oxidized with oxygen directly or oxidized by reaction 3 followed by cytochrome oxidase (reaction 4).

An increased oxygen uptake (Table 7) and a decreased nitrite yield, without nitrate formation, observed in the hydroxylamine oxidation by cell-free extracts in the presence of $CuCl_2$ and dipyridyl (Table 7) cannot be explained at the moment. Since the $CuCl_2$-dipyridyl combination stimulated also the oxidation of NADH, it is possible that somehow the oxidation of unknown endogenous substrate(s) may be

activated during hydroxylamine oxidation under these conditions using either oxygen (increasing O_2 uptake) or nitrite (decreasing nitrite yield) as terminal electron acceptor.

Further investigation into the roles of various cytochromes, the nature of the endogenous substrate(s) and its coupling to hydroxylamine oxidation is required in order to explain satisfactorily the mechanism of hydroxylamine oxidation as well as the effect of $CuCl_2$ plus the chelator.

Part II. Partially Resolved and Reconstituted System

Although a partial nitrogen atmosphere was not helpful during the breakage of the cells (Fig. 1), it was necessary during the ultra-centrifugation of the cell-free extracts in order to obtain an active recombination of membrane (pellet) and soluble (supernatant) fractions. Sometimes, when the centrifugation was carried out aerobically, the combination of membrane and soluble fraction was active only when $CuCl_2$ and NADH were included in addition to spermine and BSA. The same requirements were observed in the extracts prepared from old cells (Table 1). These observations provided

further support to the argument in the earlier part of this chapter that some essential component was sensitive to higher oxygen tension and it had to be reduced before an active ammonia-oxidizing complex was reconstituted.

The pellet contained some soluble enzymes which were removed when it was chromatographed on Sepharose 6B column and the ammonia-oxidizing system could be reconstituted by a combination of this purified membrane fraction (fraction 1P, Table 13) with the soluble supernatant (Table 13). The supernatant could be concentrated by precipitation with ammonium sulfate followed by dialysis, but NADH was consistently required as an activator suggesting either the loss of a small molecular weight component or a harmful effect of oxygen during dialysis.

Column chromatography (with Sepharose 6B) for the resolution of the ammonia-oxidizing enzyme complex was found to be a better method since the complex could be resolved in at least three distinct fractions which could be reconstituted upon combination.

The membrane fraction (fraction 1, Seharose 6B) could be successfully reconstituted with either fraction 4 plus 6 (from the same column) or freeze-thaw supernatant or S_1 or S_2 preparations. All of

these fractions (4 + 6, S_1, S_2 or freeze-thaw supernatant) contained a complex mixture of cytochromes of b-, c- and P-460 types plus the enzyme NH_2OH-cytochrome c reductase but no measurable (in 0.1 M potassium phosphate buffer) mammalian cytochrome c oxidase activity. Either S_1 or S_2 could be resolved further into two separate fractions AS_2 and AS_1 which were likely the same as Sepharose fractions 4 and 6 respectively but more concentrated.

Sometimes, very active extracts could be resolved into only 2 rather than 3 fractions (on Sepharose 6B column) indicating a tightly bound ammonia-oxidizing system in such extracts.

Although fractions 4 plus 6 could be successfully reconstituted with fraction 1 (membrane fraction), any attempts either to further resolve the membrane fraction or to replace it with a partially purified (19) preparation of cytochrome a_1 (oxidase) resulted into complete inactivation of the ammonia-oxidizing system. Aerobic conditions during the separation on Sepharose columns seemed to be helpful for obtaining active fraction 1, although the subsequent storage of aerobically obtained fraction 1 resulted in the requirement for NADH whereas that of the anaerobically obtained fraction 1 did not (Table 19). These results

suggested that the membrane fraction underwent some structural change during storage, probably the oxidation of some active site or component, and an electron donor such as NADH or NH_2OH was required for the activation. It is possible that the essential component discussed earlier was present in the membrane fraction and a reduced state of this component was required for its active reconstitution with fractions 4 plus 6. A longer period (more than 24 hours) of storage (at $-20^{o}C$) almost always resulted in an irreversible (seemingly) inactivation which could not be reversed by either NADH or NH_2OH.

On the other hand, fraction 4, which contained several cytochromes (b-, c- and P-460 types) plus the enzyme NH_2OH-chtochrome c reductase was found to be quite stable when stored at $-20^{o}C$ for several months.

Fraction 6, which seemed to be a smaller molecular weight protein mostly containing cytochrome c and sometimes also a small amount of cytochrome P-460, could usually be replaced either by NADH or NH_2OH in the reconstituted system (Table 16). There was also an indication that fraction 6 was sometimes rate-limiting in the oxidation of ammonia (compare reactions 11 and 12, Table 16). It seemed possible that this fraction was an important electron mediator and possibly a

partially reduced state of this fraction was indeed a necessity for the active reconstitution of the resolved system. A further work with purified cytochromes of N. europaea may clarify the role of this fraction in ammonia oxidation.

The phospholipids of the membrane fraction (fraction 1) appeared to play an important role in the oxidation of ammonia as was shown by the results that phospholipase A-treated fraction 1, but not fractions 4 plus 6, lost approximately 75% of its ammonia-oxidizing activity (Table 21).

That the fraction 1 was indeed the sensitive one to inactivation during the storage of N. europaea cells was clearly indicated by the results in Table 20. Fractions 4 and 6 obtained from inactive extracts (for ammonia oxidation) from old cells could be successfully reconstituted with fraction 1 from an active extract. However, fraction 1 which was obtained from the same inactive extract could not be reconstituted with fractions 4 and 6 from either active or inactive extracts. Here again, the sensitivity of structural integrity of the membrane fraction (fraction 1) and its role in ammonia oxidation was implicated.

Suzuki (72) proposed the following scheme for the oxidation of ammonia in N. europaea:

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

(2) NH_2OH + 2 cyt. c (Fe^{+3}) ⟶ (NOH) + 2 H^+ + 2 cyt. c (Fe^{+2})

(3) 2 cyt. c (Fe^{+2}) + ½ O_2 + 2 H^+ ⟶ 2 cyt. c (Fe^{+3}) + H_2O

(4) (NOH) + A + H_2O ⟶ NO_2^- + AH_2 + H^+

Sum: NH_3 + 1½ O_2 ⟶ NO_2^- + H_2O + H^+

All the four reactions must proceed at the same rate during steady-state oxidation of ammonia. The addition of NADH or NH_2OH eliminated the initial lag period during which AH_2 accumulated and initiated reaction 1. In the absence of NADH or NH_2OH addition, AH_2 is possibly formed by the oxidation of endogenous substrate(s). The membrane fraction (fraction 1) contained cytochrome oxidase (reaction 3) and possibly an enzyme catalyzing the reaction 1. The fraction 4 catalyzed reaction 2 and possibly also reaction 4.

If the scheme for NH_2OH oxidation discussed previously is substantially correct, then cytochrome c here is cytochrome c-554 and A is possibly cytochrome c-552. The fraction 6 could then be cytochrome c-552. Since the assigned roles of the two cytochrome c s is very tentative, the active component in fraction 6 could be either cytochrome c-554 or cytochrome c-552.

Cytochrome P-460 seems to be involved in an unknown way, but possibly in the coupling of reactions 1 and 4. An electron transfer between the two cytochrome c s is also required for this scheme to work.

The mechanism of ammonia oxidation is still far from being completely solved and the schemes discussed are necessarily rather tentative and represent working hypotheses for future investigations.

Part III. Structural Studies

The object of undertaking the ultra structural study of Nitrosomonas intact cells, cell-free system and partially resolved and reconstituted system was only to examine the comparative structural changes occurring during the active and inactive ammonia-oxidizing states. No attempt was made to solubilize or to reconstitute the membrane proteins or lipids in a classical sense. Actually, any attempts to solubilize proteins from membrane by chemical methods such as treatment with detergents or by mechanical means such as sonication or to extract lipids by organic solvents always resulted in inactivation of the ammonia-oxidizing system.

The oxidation of ammonia to nitrite, an energy-yielding activity, is believed to take place in at least three coupled steps (44). Murray (53) pointed out that this sort of grouping of energetic reactions is most often membrane-linked, and, consequently, the elaborate membranes and their arrangements in Nitrosomonas appear to be quite appropriate (53). A supportive evidence to this argument might be observed in the comparative structures of active and inactive states of Nitrosomonas cells (Plates 1 & 2). It appeared that in general, N. europaea cells active in ammonia-oxidation had closely associated and tightly packed cell membranes in circumferential arrangement (31, 54) The darkly stained material which was present in between the membrane layers and also inside the cells seemed to be important in retaining the activity of the cells, for upon aging (Plate 2) or freezing and thawing (31) this material appeared to have become solubilized and lost (Plate 2). Also, upon inactivation by such a procedure, the cells became somewhat swollen and relaxed.

Upon breakage of N. europaea cells by a French Pressure Cell, the cell-free extract did not oxidize ammonia and in this inactive state, it appeared to have hardly any organized state at all (Plate 3).

However, when either spermine or BSA was added to this extract (Plates 4 & 5) ammonia was oxidized and at the same time some noticeable change also occurred in the ultrastructure, i.e. the membranes and vesicles appeared to be in a highly organized structure. This effect was especially pronounced with the addition of bovine serum albumin (Plate 5) where the aggregation was quite remarkable and at the same time the increase in ammonia-oxidizing activity was also almost doubled as compared to spermine activated extracts (Fig. 1). Both *in vivo* and *in vitro* aggregation of ribosomal subunits by polyamines have been reported by several workers (11, 12, 67).

The addition of metal chelating agents such as dipyridyl which inhibited ammonia oxidation appeared to disrupt the organization of the structure to a considerable extent (Plate 6). It is possible that metals especially copper and/or iron are involved in the system as an integral part of the membrane organization and chelation disrupts this organization by removing the metal ions. The role of $CuCl_2$ in the oxidation of ammonia as well as hydroxylamine has been discussed earlier.

The inclusion of BSA was invariably essential for obtaining not only active ammonia-oxidizing extracts but also active fractions for reconstitution during the Sepharose 6B column chromatography. This strongly supported the implication of the protective role of BSA in the maintenance of membrane organization. Ritchie and Nicholas (65) also observed that the addition of BSA to the cell suspension protected hydroxylamine oxidase during the disruption of cells to the extent that measurable activity was obtained without the use of an external electron acceptor. After further enzyme purification however, the addition of PMS was essential for both the oxidation of hydroxylamine and the reduction of nitrite. They further suggested that the effect of BSA might be interpreted as a protection of a physical association between hydroxylamine oxidase and cytochrome oxidase.

Partial restoration of Ca^{++} translocation by BSA in phospholipase c treated mitochondria has been reported by Burstein, Loyter and Racker (10). They pointed out that albumin might perhaps have a non-specific stabilizing effect on mitochondria. Requirements for BSA in the reconstitution experiments with silicotungstate treated submitochondrial and sub-

chloroplast particles have been reported earlier (45, 60). Also, Weinbach, Sheffield and Garbus (84) showed that BSA effected a "passive" contraction of the mitochondria.

When an active extract was resolved by Sepharose 6B column chromatography, the membrane fraction, a high molecular weight complex containing most of the cytochrome oxidase activity, was observed to comprise of membrane particles and vesicle-like structures of various sizes (Plate 7). This fraction did not oxidize ammonia by itself and it could not be activated by either spermine, BSA, $CuCl_2$ or NADH. On the other hand, fractions 4 and 6 were comparatively smaller molecular weight fractions and are depicted in Plate 9 as amorphous clumps which appear to be joined together by smaller globular structures in a circular fashion. These two fractions also were not active in ammonia oxidation when assayed together. However, the combination of all the three fractions (1, 4 and 6) oxidized ammonia rapidly (Table 16) and the ultrastructure of the combined fractions during active ammonia-oxidizing state appeared quite different (Plate 11, 12). In this case, large complexes of vesicle-like structures around membranous folds are clearly observed. In Plate 11, a membranous fold (arrow) appears very similar in size and shape to a purified

preparation of cytochrome oxidase from beef-heart mitochondria by Wakabayashi *et al* (79).

Regardless of the obvious difficulties in correlating structure and function of the ammonia-oxidizing system of *N. europaea*, the visible transition from disrupted and disorganized state of the inactive cell-free extract and also partially resolved fractions to that of relative structurally aggregated order of the active system seems both remarkable and noteworthy.

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