INORGANIC NITROGEN METABOLISM IN CERTAIN FUNGI

by

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ABSTRACT

Cell free extracts of Aspergillus wentii, Aspergillus flavus and Penicillium atrovenetum catalyze the oxidation of ammonium to hydroxylamine, nitrite and nitrate. Hydroxylamine and nitrite are oxidized as well by cell free extracts, the oxidations being coupled to the reduction of mammalian cytochrome c as they are in the chemoautotrophic bacteria. In addition to this, nitrate is reduced to nitrite by the cell free extracts.

Some of the characteristics of the system, and the relation between heterotrophic and autotrophic nitrification are discussed.

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INTRODUCTION

It is a well known fact that the majority of soil microorganisms prefer ammonium salts as their source of nitrogen because of its easy assimilation into cell proteins. Other microorganisms possess enzymes which enable them to reduce the more oxidized nitrogen compounds, such as nitrate and nitrite, to ammonium.

The conversion of ammonium to nitrite, involving hydroxylamine as an intermediate, is accomplished by organisms belonging to the genus Nitrosomonas, while those organisms of the genus Nitrobacter can oxidize nitrite to nitrate. Although it has been reported that certain fungi possess the ability to nitrify ammonium salts, little is known of the mechanism of the oxidation. It was highly desirable, therefore, that information be obtained regarding the reactions of heterotrophic ammonium oxidation so a comparison with chemoautotrophic nitrification could be made.

As the substrate undergoes a transformation which involves a net change of 8 electrons (e.g. $\overset{-3}{NH_3} \longrightarrow \overset{+5}{NO_3^-}$)

in both systems, it would be expected that the intermediate products and the coupling enzyme systems would perhaps be of a similar nature. From the point of view of comparative biochemistry, it was therefore thought to be of interest to study the pathway of ammonium oxidation in the heterotrophic fungi.

HISTORICAL

The reactions of nitrification which are carried out by Nitrosomonas and Nitrobacter have received intensive study in recent years. From the time of the first report of the nitrifying bacteria until recent times, little progress had been made on the study of these microorganisms. However, certain major improvements in culturing techniques and methods of studying the organisms have opened the field to more intensive study.

The oxidation of ammonium to nitrite and nitrite to nitrate had been accredited solely to Nitrosomonas and Nitrobacter until it was found that certain fungi were capable of oxidizing ammonium to nitrate. Although the biochemical mechanisms of the chemosynthetic microbial transformations of ammonium and nitrite have been more or less elucidated, the sequence of reactions in the heterotrophic fungal systems has not because of the lack of data on the pathways of heterotrophic nitrification.

Sakaguchi and Wang (1935) observed that cultures of Aspergillus oryzae could produce nitrate in a medium

containing both ammonium and nitrite, but not with either nitrogen source alone; still other aspergilli were able to oxidize nitrite to nitrate.

A more recent investigation by Malavolta et al (1955) indicated that Asp. wentii could produce nitrite and nitrate when grown in a medium containing ammonium. Eylar and Schmidt (1958) made an intensive study of almost 1000 strains of fungi and found that only Penicillium atrovenetum and Asp. flavus were able to produce nitrate from ammonium.

Marshall and Alexander (1962) attempted to determine the mechanism of fungal nitrification using Asp. flavus. They reported that crude cell free extracts of the organism would not oxidize ammonium, hydroxylamine or nitrite, or reduce nitrate to nitrite. However, the extracts were capable of producing nitrite from β-nitropropionic acid in the presence of 10^{-4} molar cerous ions and they proposed that β-nitropropionic acid is a likely intermediate in heterotrophic nitrification.

Any work which could be taken as a precedent has been done on the chemoautotrophs Nitrosomonas and Nitrobacter. The autotrophic metabolism of both organisms has been

studied in some detail.

Hofman and Lees (1953) demonstrated that hydroxylamine was the first product of ammonium oxidation. Since that time, Imshenetskii et al (1954, 1955) reported ammonium and hydroxylamine oxidation by autolysates of Nitrosomonas but because of their failure to eliminate unbroken cells and the excessively long incubation periods used, the reliability of their results is subject to question.

Nicholas and Jones (1960) found that cell free extracts of Nitrosomonas were capable of oxidizing hydroxylamine to nitrite in the presence of suitable electron acceptors. These workers also attempted to demonstrate cell free ammonia oxidation but met with no success. More recently, Delwiche, Burge and Malavolta (1961) reported that Nitrosomonas cell free extracts could couple phosphate esterification to hydroxylamine oxidation.

Recently Aleem et al (1962) discovered the nature of the previously unknown intermediate in nitrification. It was found to be nitrohydroxylamine and its position in the sequence of reactions is as follows:

$$NH_3 \longrightarrow NH_2OH \longrightarrow NO_2 \cdot NHOH \longrightarrow NO_2^-$$

The compound is formed by a condensation reaction involving hydroxylamine and nitrite. Its enzymatic oxidation does not involve cytochromes, flavins or pyridine nucleotides.

Aleem and Lees (1963) have reported the purification and characterization of the enzyme hydroxylamine-cytochrome c reductase from Nitrosomonas. It was stimulated by iron and copper and inhibited by cyanide and atabrine. The atabrine inhibition could be reversed by the addition of either FMN or FAD. The enzyme was very stable, retaining most of its initial activity after several months storage at 4° C.

Aleem and Alexander (1958) obtained for the first time, cell free extracts of Nitrobacter which could rapidly oxidize nitrite to nitrate. The oxidation was stimulated by iron and inhibited by cyanide. In 1959 Aleem and Nason found that Nitrobacter nitrite oxidase was a particulate enzyme, referred to by them as the "cytochrome electron transport particle capable of catalyzing the enzymatic transfer of electrons from nitrite to molecular oxygen". In 1960, the same workers demonstrated the coupling of phosphate esterification to nitrite oxidation by "red particles" from

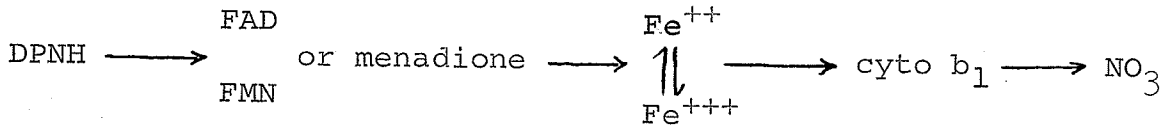
Nitrobacter and in a more recent study (unpublished data) partially purified and characterized a nitrite-cytochrome c reductase from the same organism. The enzyme was inhibited by various metal binding agents.

A large variety of plants and microorganisms possess the ability to reduce nitrate to nitrite. An assimilatory nitrate reductase from Neurospora was first characterized by Nason and Evans (1953). The enzyme had molybdenum as a metal constituent and a flavoprotein prosthetic group. Nicholas and Nason (1954) reported that the flavin and molybdenum function as electron carriers in the nitrate reducing system of Neurospora. They suggested the following pathway of electron transfer:

$$\text{TPNH} \longrightarrow \text{FAD} \longrightarrow \text{Mo} \longrightarrow NO_3^-$$

In addition to assimilation, the reduction of nitrate can have other useful functions. Nitrate may serve as an electron acceptor to facilitate growth of microorganisms under microaerophilic or anaerobic conditions. This phenomonon was first reported by Quastel, Stephenson and Wetham (1925). They found that E. coli would grow under anaerobic conditions if supplied with nitrate.

In 1955, Wainright reported that the adaptive nitrate reductase from E. coli was stimulated by menadione and ferrous ions in the presence of FAD. He also suggested the system might contain a cytochrome component. A few years later, Itagaki and Taniguchi (1959) proposed the following reaction scheme which agreed in essence with Wainright's results:



Still other proposed sequences of electron transport have been published and these may be found in the several reviews on this subject (Virtanen - 1952, McElroy and Glass - 1956, Nicholas - 1958, Fewson - 1961, Nason - 1962).

MATERIALS AND METHODS

Heterotrophic Nitrifiers

The organisms selected for use in the investigation were *Aspergillus wentii*, *Aspergillus flavus* and *Penicillium atrovenetum* as they are known to nitrify ammonium salts (Malavolta, de Carmargo and Haag - 1955, Eylar and Schmidt - 1959).

Growth or Organisms

The medium used for the cultivation of the organisms was a mineral salts medium supplemented with sucrose. It had the following composition: 6.0 g sucrose, 1.0 g K_2HPO_4, 15.0 g $(NH_4)_2HPO_4$, 0.010 g $MnSO_4 \cdot H_2O$, 0.010 g $Na_2MoO_4 \cdot 2H_2O$, 0.010 g $ZnSO_4 \cdot 7H_2O$, 0.010 g $FeSO_4 \cdot 7H_2O$, 0.20 g $MgSO_4 \cdot 7H_2O$ and 0.10 g $CaCl_2 \cdot 2H_2O$. Distilled deionized water was added to make the volume to one liter.

The $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ were autoclaved separately and the $FeSO_4 \cdot 7H_2O$ was sterilized by filtration. These components were added to the rest of the medium after cooling.

The above medium was dispensed in 175 ml amounts into 1 liter flasks. After inoculation the flasks were incubated at 28° C on a rotary action shaker (112 r.p.m.).

Preparation of Cell Free Extracts

After seven days of growth the mycelia were collected by filtration through two layers of cheese cloth, washed 15-20 times with distilled water, and pressed dry between filter paper. The mycelia so obtained were disrupted in a ground glass tissue grinder with 0.1 M Tris buffer pH 8.0 (5.0 ml per g wet weight of mycelia). The resulting extract was centrifuged at 27,000 x g for 30 minutes and the pellet discarded. The cell free supernatant was used in all experiments unless otherwise indicated.

Determination of Hydroxylamine

Hydroxylamine was determined according to Czaky's modification of the Blom procedure (1948) in which hydroxylamine is oxidized to nitrite by iodine, and the nitrite determined colorimetrically by the method of Bratton et al (1939).

The reagents used are 1.3% iodine in glacial acetic acid, 2% sodium arsenite, 1% sulfanilic acid in 20% HCl and 0.12% N-(1-naphthyl)-ethylene diamine dihydrochloride.

To an aliquot of the reaction mixture (0.1 - 0.5 ml) were added 0.5 ml of sulfanilic acid and 0.5 ml of the iodine solution. The mixture was shaken, incubated for 5 minutes and 1.0 ml of sodium arsenite solution was then added. The volume was made up to 7.0 ml with distilled water and to this mixture was added 0.5 ml of the N-(1-naphthyl)-ethylene diamine dihydrochloride solution. After 30 minutes incubation the color intensity was measured at 540 mμ in a Klett-Summerson colorimeter.

Determination of Nitrite

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

Aliquots of sample were brought to a volume of 6.5 ml with distilled water and to this were added 0.5 ml of sulfanilic acid and 0.5 ml of N-(1-naphthyl)-ethylene diamine

dihydrochloride solution. The samples were incubated for 30 minutes to insure maximum color development and the color intensity was measured at 540 mμ in a Klett-Summerson colorimeter.

A standard curve was prepared following the above procedure using known dilutions of a sodium nitrite solution. Under the conditions used, 1 mμ mole of nitrite corresponded to 3.07 Klett units (Δ O.D. = 0.0061).

Determination of Nitrate

Nitrate was determined by two methods, the first being that of Buckett, Duffield and Milton (1955). In this method the reagents used were:

a) phenol disulfonic acid. This was prepared by adding 100 g white crystalline phenol to 600 ml 96% H_2SO_4 and 300 ml of fuming H_2SO_4. The mixture was heated at 105° C for three hours and stored in a tightly stoppered bottle.

b) NaOH-versene. This was prepared by dissolving 480 g NaOH in 600 ml of distilled water and making the volume to 1 liter by adding a solution of 10% ethylene diamine tetraacetic acid (disodium salt).

Samples were made alkaline with NaOH and evaporated to dryness. Enough phenol disulfonic acid was added to cover the dried sample completely on initial contact (approximately 1.0 ml) and it was allowed to react for ten minutes. Then 2.5 ml of distilled water was added followed by 2.0 ml of the NaOH-versene solution. The mixture was allowed to cool and the volume was made up to 7.0 ml with distilled water. The color intensity was read in a Klett-Summerson colorimeter at 420 mμ.

If the samples contained nitrite, it was removed by adding 0.5 ml of 8% oxalic acid and 0.5 ml of 10% urea to the sample and heating at 70° C for ten minutes. After this treatment, the determination was carried out in the usual manner.

The second method used for determining nitrate was that of Montgomery and Dymock (1962). The reagents used were:

a) sulfuric acid 80.5 - 83.3% w/w, sp. gr. 1.733 - 1.762 at 20° C. A volume of 455 ml of the acid (minimum 98%) was mixed with 171 ml of nitrate-free water.

b) 24% ammonium chloride solution (w/v)

c) 2,6-dimethyl phenol. A solution was prepared by dissolving 0.122 g in 50 ml of reagent grade acetic acid to which was added 50 ml of the ammonium chloride solution.

d) sulphamic acid papers. Discs of 5.5 cm Whatman #5 filter paper were cut into sixteen equal segments and soaked in a 50% solution of sulphamic acid. The pieces were allowed to dry and were stored in a stoppered bottle.

To 1.6 ml of the H_2SO_4 (cooled to 0-10° C) were added 0.1 ml of the sample and 0.1 ml of 2,6-dimethyl phenol reagent. After five minutes, 3.0 ml of distilled water was added and the mixture was incubated for fifteen minutes. The optical density was read at 304 mμ in a spectrophotometer using a reagent blank as a zero control. The optical density remains unchanged for 2 hours at room temperature or for 2 days at 4° C.

If nitrite was present in the sample, it was removed by adding a sulphamic acid paper and allowing it to react for 5 minutes. One paper will destroy 0.1 mg nitrite-nitrogen per ml.

Warburg Techniques

The oxidation of hydroxylamine and nitrite was measured by both manometry and colorimetric determinations of the substrates and products of the reaction. The Warburg flasks contained 0.5 ml cell free extract, 1 - 20 μ moles of substrate, 0.01 ml TPN (3 mg/ml), 0.02 ml 2% cytochrome c and 0.1 M Tris buffer (pH 6.5 for hydroxylamine oxidation, pH 7.5 for nitrite oxidation) to make the total volume 2.5 ml. In the centre well was placed 0.2 ml of 20% KOH. All experiments were conducted at 28° C.

Thunberg Techniques

The oxidation of ammonium and nitrite were coupled to dye reduction anaerobically. The reactions were carried out in Thunberg tubes which contained 0.5 ml cell free extract, 10 μ moles of substrate, 0.01 ml TPN (3 mg/ml), 0.02 ml 2% cytochrome c, 0.05 ml of 10^{-3} M dye and 0.1 M Tris buffer (pH 8.0 for ammonium oxidation and pH 7.5 for nitrite oxidation) to obtain a total volume of 2.0 ml. After evacuation and tipping in of the substrate from the side arm, the reaction mixtures were incubated for 3 hours at 24° C and read

visually.

Hydroxylamine-cytochrome c Reductase

Enzyme activity was determined by the measurement of cytochrome c reduction at 550 mμ in a Beckman D.B. Spectrophotometer. The reaction mixtures contained 0.2 ml of 2% cytochrome c, 10 μ moles of $NH_2OH \cdot HCl$, 0.3 ml of cell free extract and 0.1 M Tris buffer pH 6.5 to make the volume 2.0 ml. The control cuvette contained the complete reaction mixture with boiled enzyme substituted for the active enzyme preparation.

Nitrite-cytochrome c Reductase

Nitrite-cytochrome c reductase activity was determined by following the reduction of cytochrome c at 550 mμ in a Beckman D.B. Spectrophotometer. Present in the assay mixture were 0.2 ml of 2% cytochrome c, 20 μ moles of $NaNO_2$, 0.3 ml of cell free enzyme extract and 0.1 M Tris buffer pH 7.5 to make the volume 2.0 ml. Enzyme and cytochrome were added to both cuvettes with substrate absent in the control cuvette.

RESULTS

Ammonia Oxidation

1. Effect of electron acceptors (see table 1)

Of the various electron acceptors tried, it was found that TPN was more effective in stimulating ammonium oxidation than DPN. Also, TPN in the presence of cytochrome c was the most effective combination of electron acceptors. Added FMN, FAD or ATP had no effect on the oxidation. The cytochrome c in the reaction mixtures could be partially replaced by methylene blue, 2,6-dichlorophenol indol phenol or phenazine methosulphate.

2. Coupled dye reduction (see Materials and Methods)

Methylene blue, 2,6-dichlorophenol indol phenol and phenazine methosulfate were reduced by reaction mixtures containing cell free enzyme extract, ammonium and TPN. It was not possible to demonstrate the formation of any of the normal oxidation products of ammonium. If any of the components of the system were deleted, no dye reduction

Table 1

The effect of electron carriers on ammonium oxidation by crude cell free extracts of Aspergillus wentii.

cell free extract plus	mμ moles NO_2 formed
ammonium	1.0
" + cytochrome c	5.1
" + DPN	3.1
" + TPN	5.5
" + DPN + cytochrome c	17.4
" + TPN + cytochrome c	22.6

Reaction mixtures all contained 0.5 ml of cell free extract, 10 μ moles of $(NH_4)_2HPO_4$, 0.02 ml of 2% cytochrome c, 0.01 ml of TPN or DPN (3 mg/ml) and 0.1 M Tris buffer pH 8.0 to make the final volume 2.0 ml. The tubes were incubated at 24° C for 4 hours.

occurred.

3. Optimum hydrogen ion concentration

The optimum hydrogen ion concentration for ammonia oxidation by crude cell free extracts was found to be at pH 8.0. There was no difference in enzyme activity in phosphate or Tris buffers.

4. Oxidation products (see table 2)

Cell free fungal extracts were found to oxidize ammonium to hydroxylamine, nitrite and nitrate in the presence of TPN and cytochrome c. Added ethylene diamine tetraacetic acid (disodium salt) was found to stimulate the oxidation. Allyl thiourea in a final concentration of 10^{-3} M was not inhibitory to the system (as it is for Nitrosomonas) and in fact a slight stimulation was noted. The amounts of hydroxylamine and nitrite formed as compared to the amount of nitrate produced would seem to indicate that the figures obtained for the first two are actually an indication of the amount of the compound "in transit" (see Table 1a).

Table 2

The oxidation of ammonium by cell free extracts of *Aspergillus wentii* to produce hydroxylamine, nitrite and nitrate.

reaction mixtures	mμ moles NH_2OH formed	mμ moles NO_2 formed	mμ moles NO_3 formed
complete mixture	11.6	37.6	514.4
- TPN, -cytochrome *c*	3.4	5.8	381.0
- TPN, -cytochrome *c*, -EDTA	0	0	0
+ allylthiourea	21.4	33.2	572.6

Reaction mixtures contained 0.5 ml of cell free extract, 10 μ moles $(NH_4)_2HPO_4$, 0.02 ml 2% cytochrome *c*, 0.01 ml TPN (3 mg/ml), 0.1 ml 10^{-2} M EDTA and 0.1 M Tris buffer pH 8.0 to make the total volume 2.0 ml. Allylthiourea was added to make the final concentration 10^{-3} M. The mixtures were incubated at 24° C for 4 hours.

5. Effect of metals (see table 3)

Of the various metals used in the experiment, ferrous and ferric iron, cobalt and copper gave the most stimulation. Nickel inhibited the oxidation completely while magnesium, manganese, and calcium had no effect. Zinc and molybdate produced evidence of slight stimulation.

6. Time course study of ammonium oxidation (see table 1a)

It was found that over a period of 480 minutes, the concentration of hydroxylamine gradually increased, the concentration of nitrite increased initially and then decreased, while the amount of nitrate present increased steadily during the course of the experiment.

Hydroxylamine Oxidation

1. Effect of electron acceptors (see table 4)

Of the electron acceptors tried in the experiment, TPN in conjunction with cytochrome c was the most effective combination for the stimulation of hydroxylamine oxidation. It was found also that added $KClO_3$ resulted in more nitrite

Table 3

The effect of added metals on cell free ammonium oxidation by extracts of Aspergillus wentii.

treatment	mμ moles NH_2OH formed	mμ moles NO_2 formed	mμ moles NO_3 formed	% stimulation
reaction mixture alone	14.6	21.8	71.0	-
+ Fe^{2+}	15.4	31.5	160.5	93
Mg^{2+}	13.2	21.8	78.8	6
Mn^{2+}	12.6	21.8	75.5	2
MoO_4^{2-}	14.2	24.6	84.0	14
Cu^{2+}	14.2	29.1	91.4	25
Zn^{2+}	15.1	30.2	75.0	12
Ca^{2+}	14.2	23.3	73.5	2
Fe^{3+}	13.8	30.1	145.0	76
Co^{2+}	13.2	32.1	108.1	43
Ni^{2+}	0	0	0	0

Table 3 cont'd

Reaction mixtures contained 0.5 ml of dialysed cell free extract, 20 μ moles of $(NH_4)_2HPO_4$, 0.02 ml of 2% cytochrome c, 0.01 ml TPN (3 mg/ml), 2.5×10^{-4} M metal (final concentration) and 0.1 M phosphate buffer pH 8.0 to make the volume 2.0 ml. The mixtures were incubated for 3 hours at 24° C.

Table 1a

Time course study of ammonium oxidation by cell free extracts of Aspergillus wentii.

time	mμ moles NH_2OH formed	mμ moles NO_2 formed	mμ moles NO_3 formed
0 mins	0	7.0	14.3
120 mins	12.0	41.0	63.0
240 mins	34.0	51.3	100.4
480 mins	41.0	17.1	217.6

Reaction mixtures contained 2.0 ml of cell free extract, 0.20 ml 2% cytochrome c, 0.10 ml TPN (3 mg/ml), 10 μ moles $(NH_4)_2HPO_4$ and 0.1 M Tris buffer pH 8.0 to make the volume 10.0 ml. Samples of 0.5 ml were taken every 120 minutes and tests were made for hydroxylamine, nitrite and nitrate.

Table 4

The effect of electron acceptors on hydroxylamine oxidation by cell free extracts of Aspergillus wentii.

treatments	mμ moles NO_2 formed
complete reaction mixture	48.6
- enzyme	8.9
- NH_2OH	0
+ cytochrome c	66.7
+ TPN	75.2
+ TPN + cytochrome c	82.0
+ TPN + cytochrome c + $KClO_3$	106.7

Reaction mixtures contained 0.1 ml of cell free extract and 4 μ moles NH_2OH. To this was added 0.02 ml of 2% cytochrome c, 0.01 ml TPN (3 mg/ml), 0.1 ml of 0.2 M $KClO_3$ and 0.1 M Tris buffer pH 6.5 to make the volume 2.0 ml. The tubes were incubated for 6 hours at 24° C. Nitrite oxidase activity was removed by heating at 50° C for 5 minutes.

production.

2. Optimum hydrogen ion concentration (see table 5)

It was found that the optimum hydrogen ion concentration for the oxidation of hydroxylamine by crude cell free fungal extracts was at pH 6.5.

3. Effect of metals (see figure 5)

Ferrous iron and copper both stimulated hydroxylamine oxidation, with copper giving the greater degree of stimulation.

4. Nitrite production (see figure 1)

The oxidation of hydroxylamine to form nitrite is shown in figure 1. The production of nitrite plotted against time yields a straight line relationship (zero order reaction) showing saturation of the enzyme by an excess of substrate.

5. Oxygen uptake (see figure 2)

The relationship between hydroxylamine concentration,

Table 5

The effect of pH on hydroxylamine oxidation by cell free extracts of Aspergillus wentii.

pH	mμ moles NO_2 formed
5.5	4.8
6.0	6.2
6.5	7.1
7.0	5.5
7.5	4.1
8.0	1.4

Reaction mixtures contained 0.5 ml of cell free extract, 5 μ moles NH_2OH, 0.02 ml of 2% cytochrome c, 0.01 ml TPN (3 mg/ml) and 0.1 M Tris buffer to make the volume 2.0 ml. The mixtures were incubated for 2 hours at 24° C.

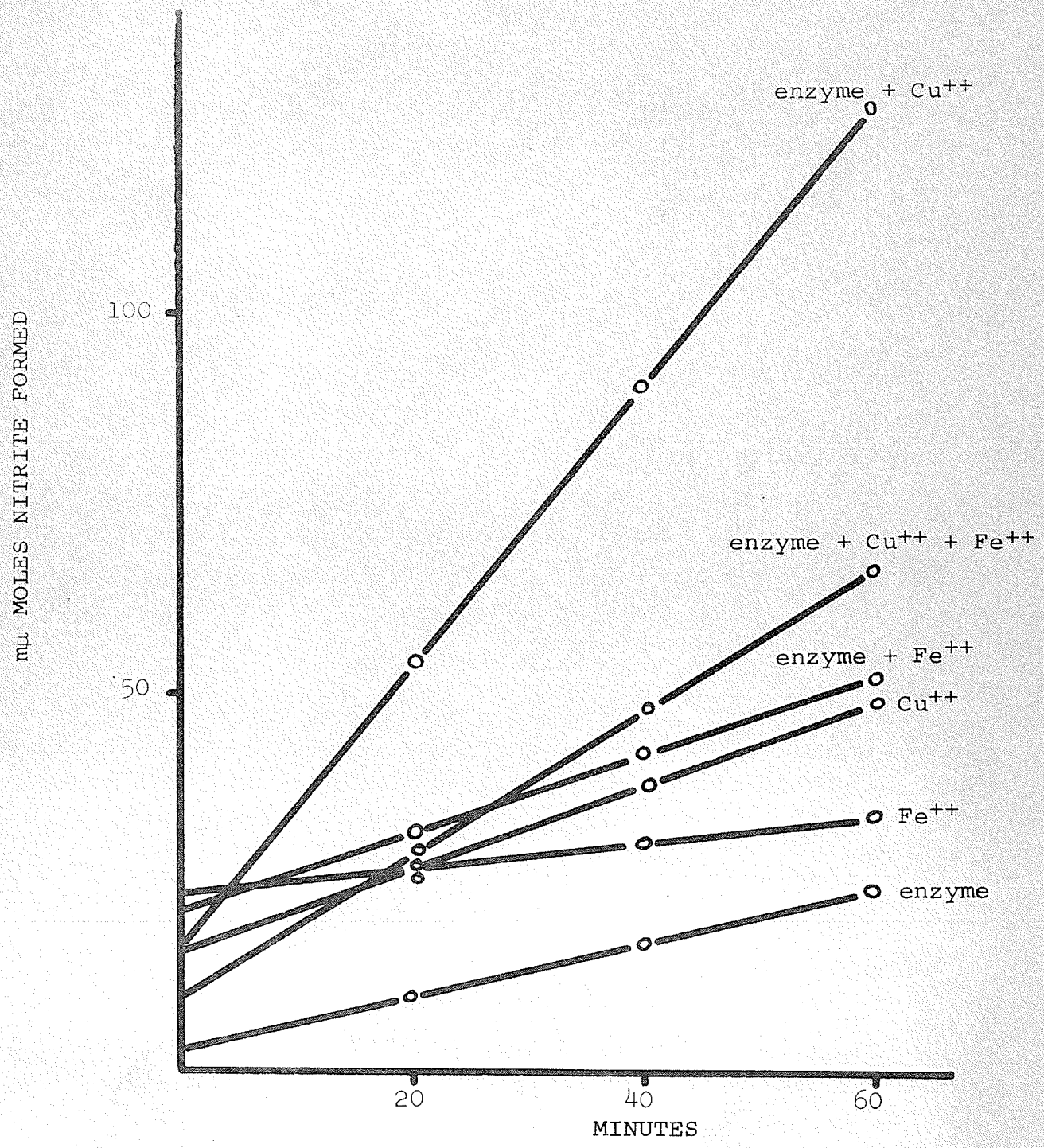


Figure 5. Effect of copper and ferrous iron on hydroxylamine oxidation by Aspergillus wentii.

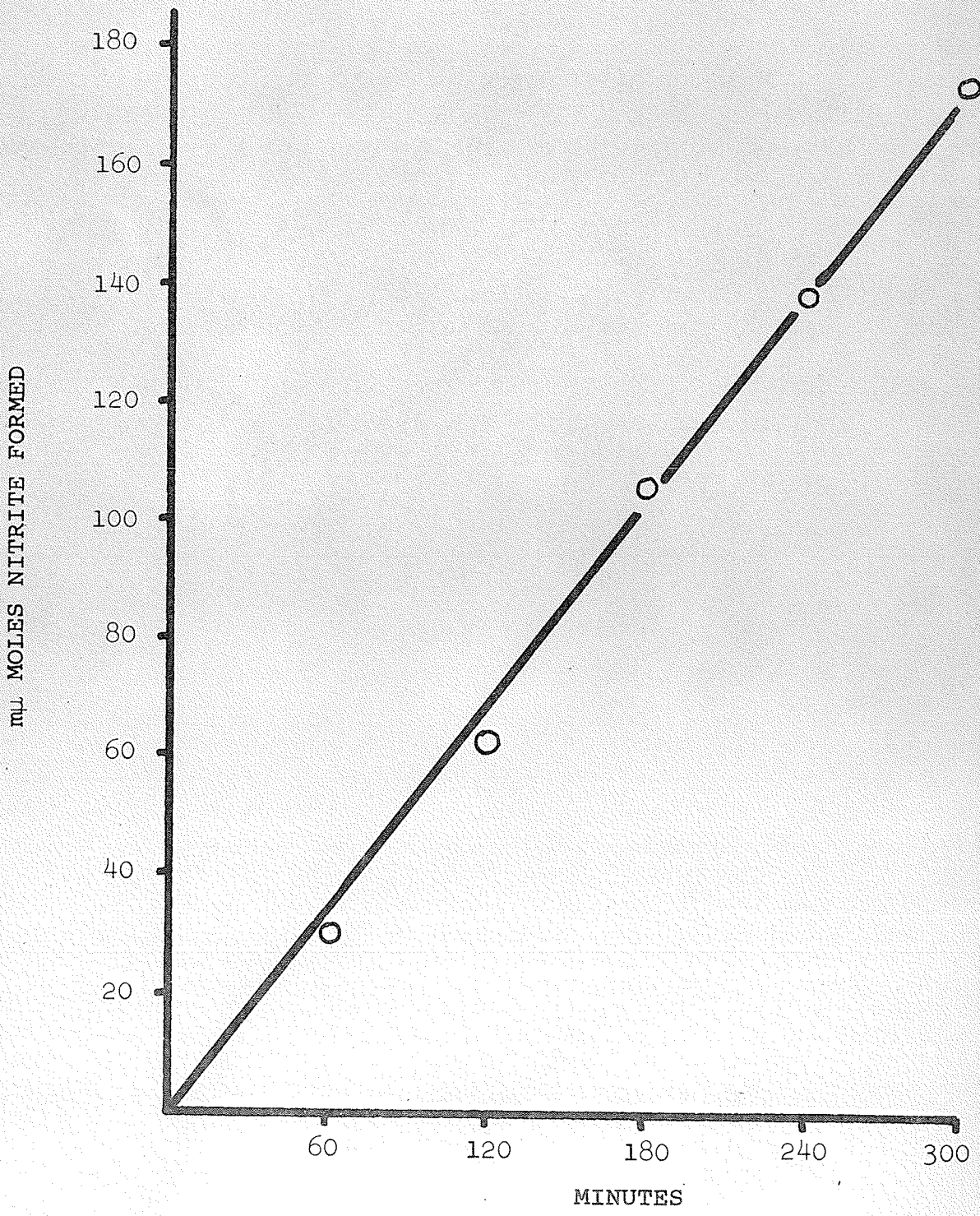


Figure 1. Nitrite production from hydroxylamine oxidation by Aspergillus wentii.

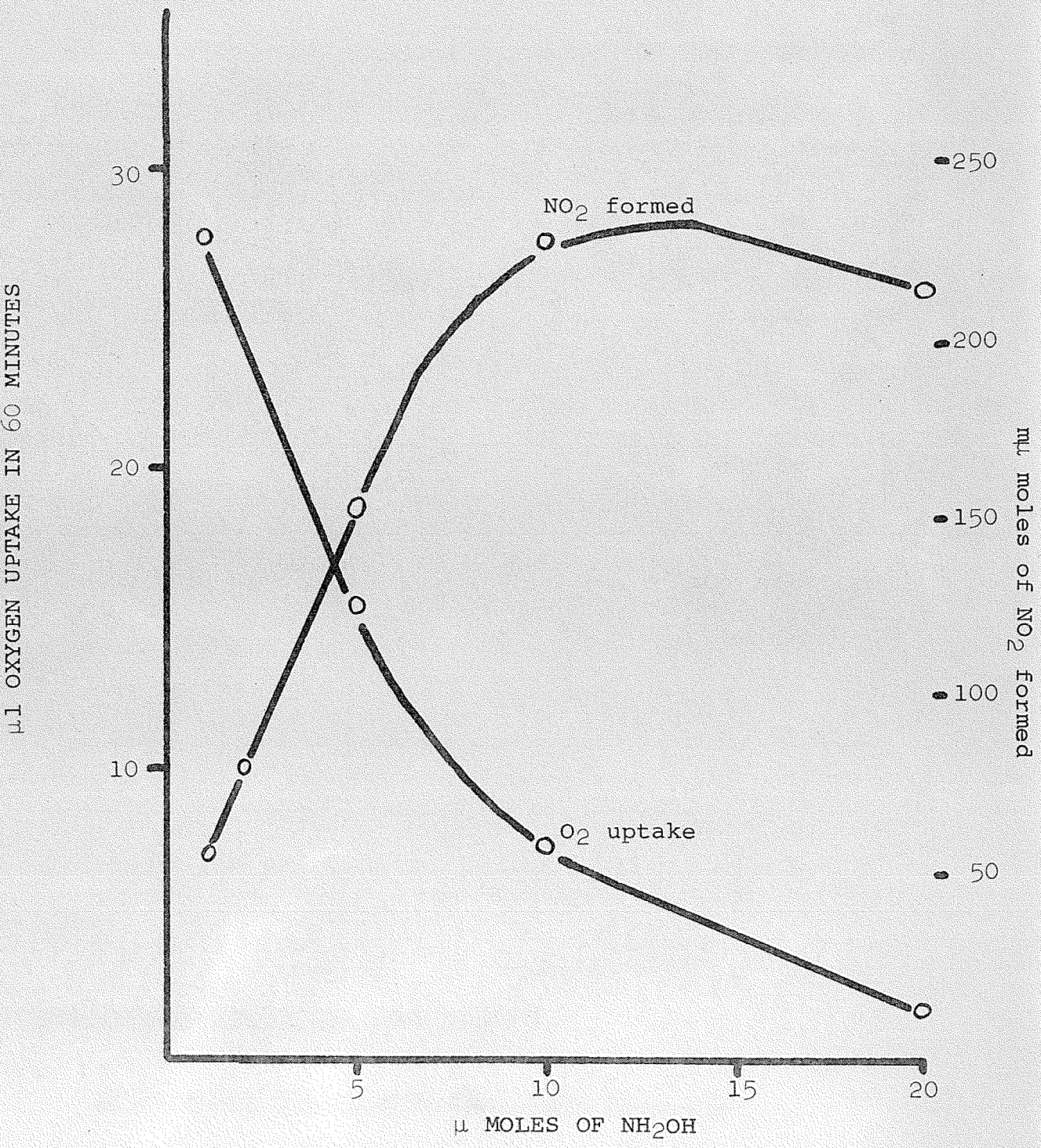


Figure 2. Hydroxylamine oxidation by Aspergillus wentii. Oxygen uptake and concomitant nitrite production.

nitrite production and oxygen uptake is demonstrated graphically in figure 2. A poor correlation was obtained possibly because of the toxic nature of the substrate.

6. Oxidation products (see table 6)

Hydroxylamine was oxidized to nitrite and nitrate by cell free fungal extracts in the presence of added TPN and cytochrome c. As in the cell free ammonium oxidation, the majority of the nitrogen can be found in the form of nitrate with the amount of nitrite present representing nitrite "in transit".

Nitrite Oxidation

1. Effect of electron acceptors

Only cytochrome c stimulated the oxidation of nitrite. Added flavins and pyridine nucleotides had no effect on the reaction.

2. Coupled dye reduction (see table 7)

In Thunberg tubes, both methylene blue and 2,6-dichlorophenol indol phenol were reduced in the presence

Table 6

Oxidation of hydroxylamine by cell free extracts of *Aspergillus wentii* to produce nitrite and nitrate.

treatment	μ moles NO_2 formed	μ moles NO_3 formed (I)	μ moles NO_3 formed (II)
complete mixture	0.171	0.373	0.42
- enzyme	0	0	0
- NH_2OH	0	0	0

Reaction mixtures contained 0.5 ml cell free extract, 10 μ moles NH_2OH, 0.02 ml of 2% cytochrome *c*, 0.01 ml TPN (3 mg/ml) and 0.1 M Tris buffer pH 6.5 to make the volume 1.5 ml. The tubes were incubated for 5 hours at 24° C.

I - phenol disulfonic acid determination

II - 2,6-dimethyl phenol determination

Table 7

Dye reduction coupled to nitrite oxidation by cell free extracts of Aspergillus flavus.

treatment	reduction
+ methylene blue	nil
+ methylene blue + NO_2	complete
+ methylene blue, - enzyme	nil
+ 2,6-DCPIP	nil
+ 2,6-DCPIP + NO_2	complete
+ 2,6-DCPIP - enzyme	nil

Reaction mixtures contained 0.5 ml of cell free extract, 20 μ moles $NaNO_2$, 0.01 ml of 2% cytochrome c, 0.05 ml of 10^{-3} M dye (methylene blue or 2,6-dichlorophenol indol phenol) and 0.1 M Tris buffer pH 7.5 to make the volume 2.0 ml. The experiment was performed in Thunberg tubes and after evacuation and mixing of the components, the tubes were incubated for 4 hours at 24° C.

of enzyme, nitrite and cytochrome c. If any of the components of the reaction mixture were omitted, no reduction occurred.

3. Oxygen uptake (see figure 7)

The relationship between substrate concentration and oxygen uptake in Warburg vessels is shown. The optimum nitrite concentration for maximum oxygen uptake was found to be 5×10^{-3} M. Higher concentrations inhibited the oxygen uptake considerably.

4. Oxidation products

Although there is no doubt that nitrite is oxidized to nitrate, it was difficult to obtain figures which would represent a good nitrogen balance. The reason for this was the presence in the crude cell free extracts of a nitrate reductase (see Results - Nitrate Reduction and Discussion p. 51.

Nitrate Reduction

The enzyme nitrate reductase could be detected in cultures growing with nitrate as the sole nitrogen source and

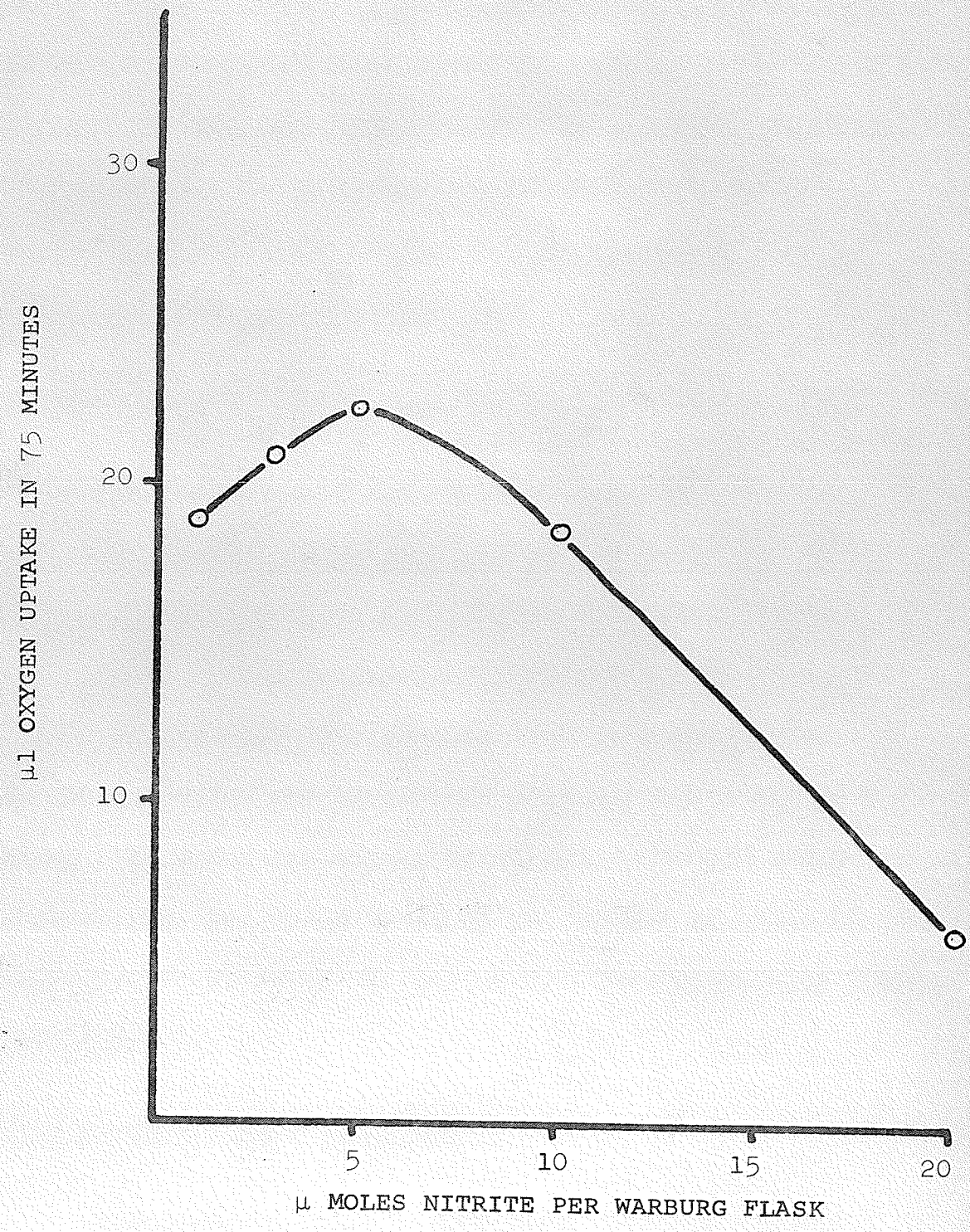


Figure 7. Effect of nitrite concentration on oxygen uptake in Warburg vessels.

also in those cultures with ammonium as the sole nitrogen source (only in cultures in which the concentration of ammonium had decreased sufficiently to allow the formation of nitrate reductase). The enzyme was stimulated by DPNH and FAD (see table 8). Added iron or molybdenum had no detectable effect on the reaction.

Hydroxylamine-cytochrome c Reductase

The enzyme was found to be present in all three organisms used in the investigation. In crude cell free extracts, the enzyme was stable over long periods of time. The activity of the enzyme increased during the first few days of storage and then remained fairly constant (see figure 6). Under the conditions used for the assay of the enzyme (see Materials and Methods) the optimum hydrogen ion concentration was found to be at pH 6.5 for Penicillium atrovenetum (see figure 3) and pH 6.0 for Aspergillus flavus (see figure 4).

Nitrite-cytochrome c Reductase

Nitrite-cytochrome c reductase activity was found in

Table 8

The reduction of nitrate to nitrite by cell free extracts of Aspergillus wentii.

treatment	mμ moles NO_2 formed
enzyme + NO_3	34.2
enzyme + NO_3 + DPNH	40.4
enzyme + NO_3 + DPNH + FAD	62.6

Reaction mixtures contained 0.5 ml of cell free extract, 10 μ moles $NaNO_3$, 0.01 ml 10^{-2} M DPNH, 0.1 ml 10^{-4} M FAD and 0.1 M Tris buffer pH 7.4 to make the volume 1.0 ml. The mixtures were incubated at 24° C for 90 minutes.

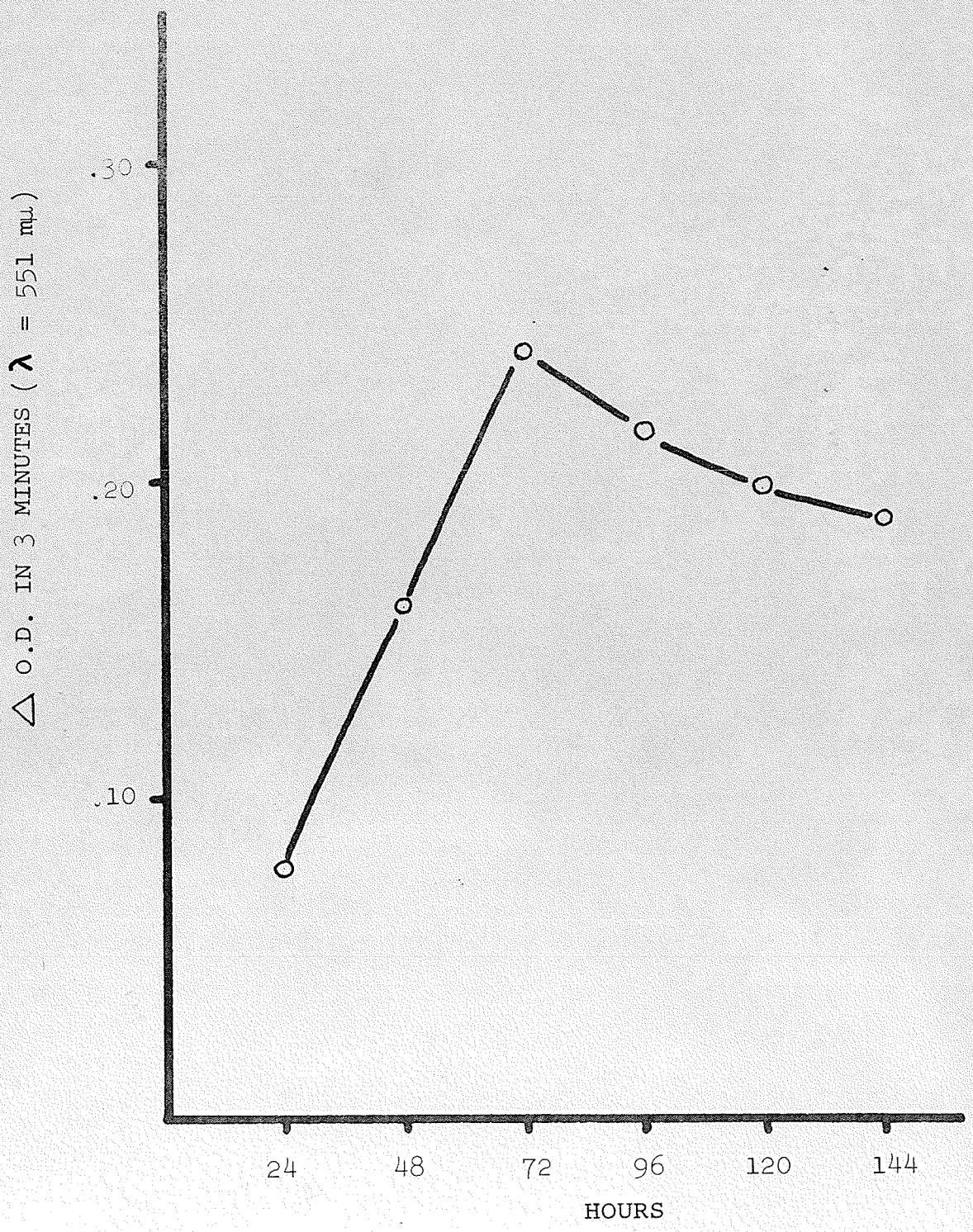


Figure 6. Activity of hydroxylamine-cytochrome c reductase during storage at 4° C.

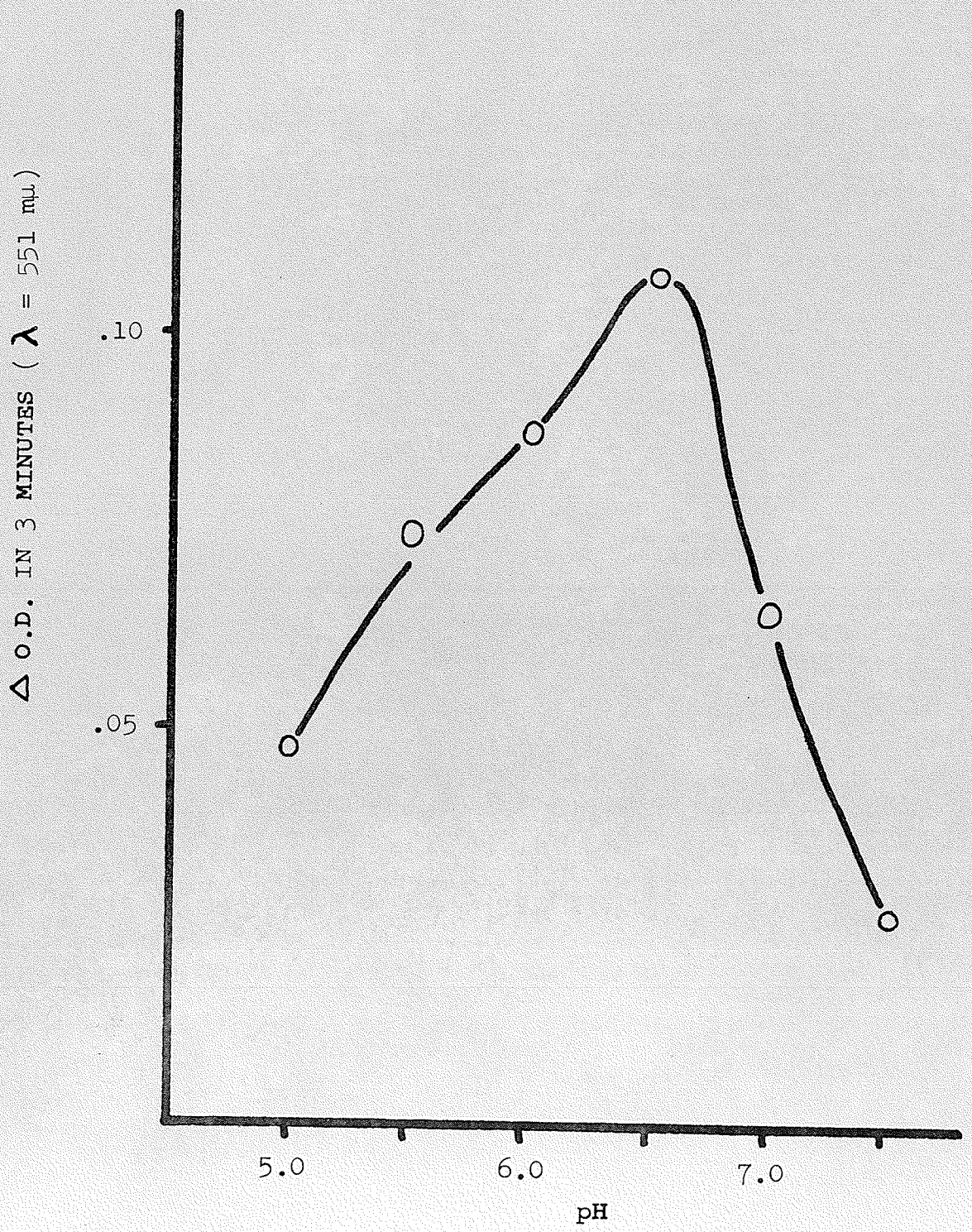


Figure 3. Effect of hydrogen ion concentration on hydroxylamine-cytochrome c reductase from Penicillium atrovenetum.

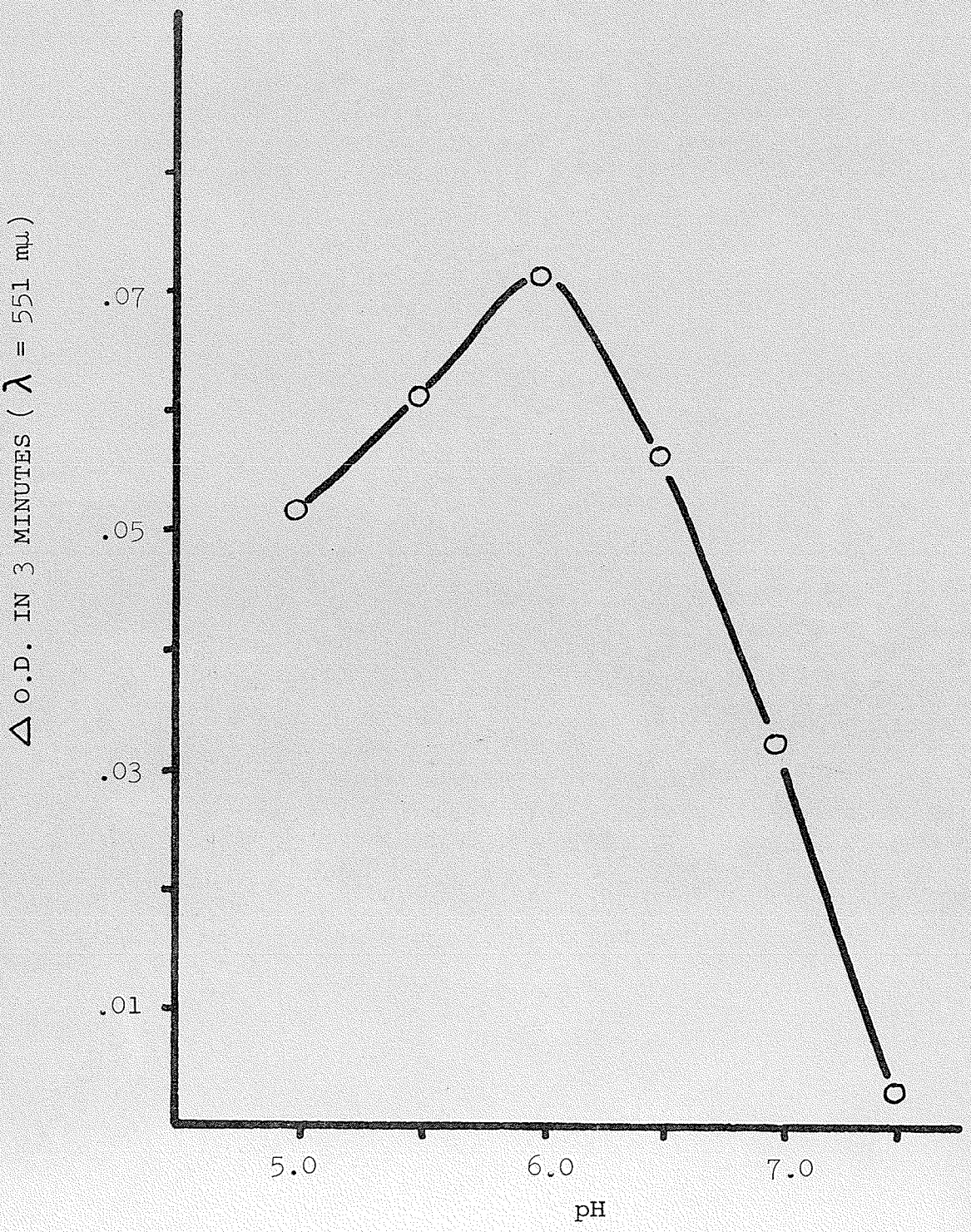


Figure 4. Effect of hydrogen ion concentration on hydroxylamine-cytochrome c reductase from Aspergillus flavus.

extracts of all the organisms used. The enzyme was not stable, being inactivated by heating at 50° C for 5 minutes and losing all of its activity during storage at -17° C for 7 days. The optimum hydrogen ion concentration was found to be at pH 7.0 (see figures 8 and 9). The optimum substrate and cytochrome c concentrations were found to be 1.0×10^{-2} M and 1.62×10^{-4} M respectively (see figures 10 and 11).

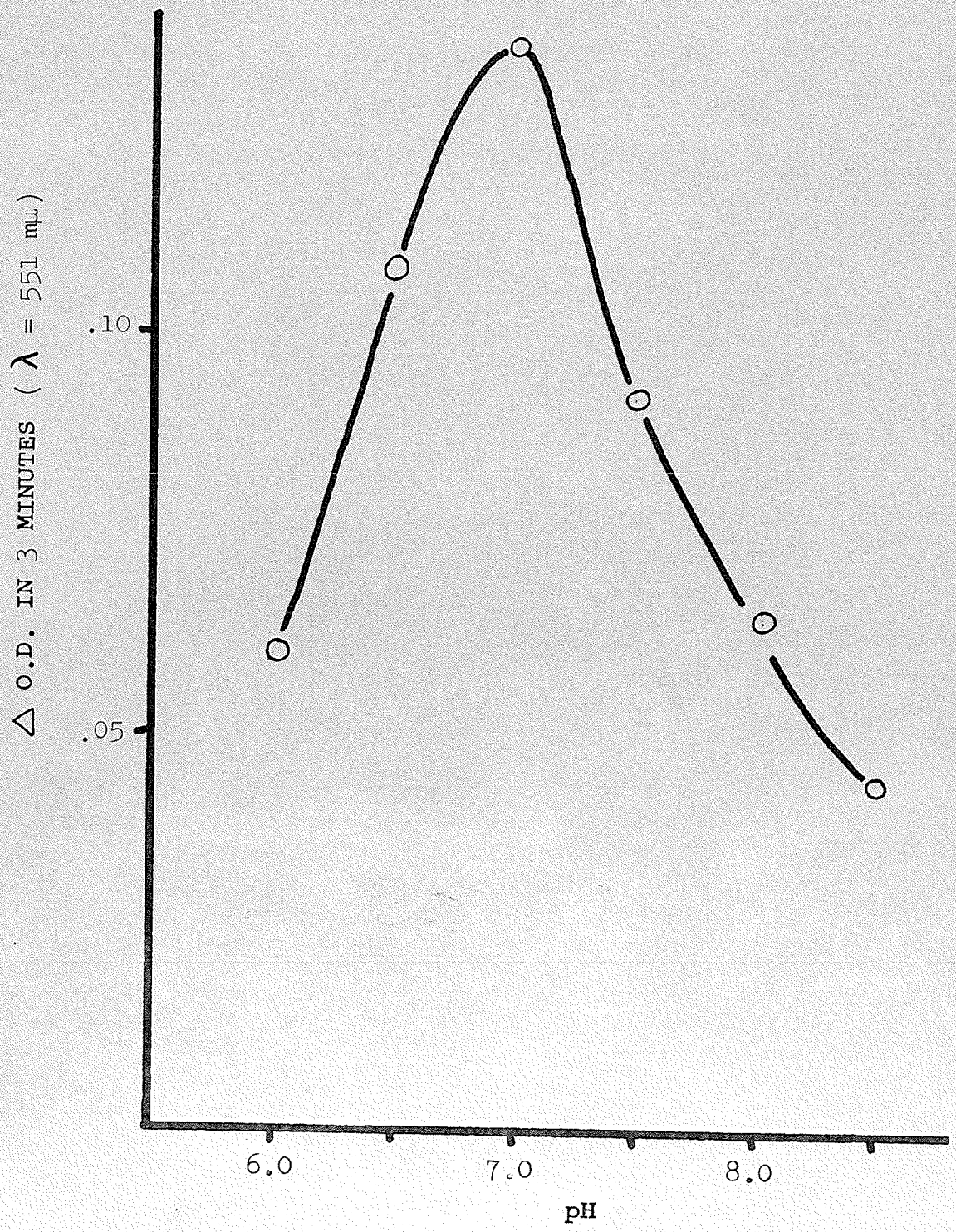


Figure 8. Effect of hydrogen ion concentration on nitrite-cytochrome c reductase from Penicillium atrovenetum.

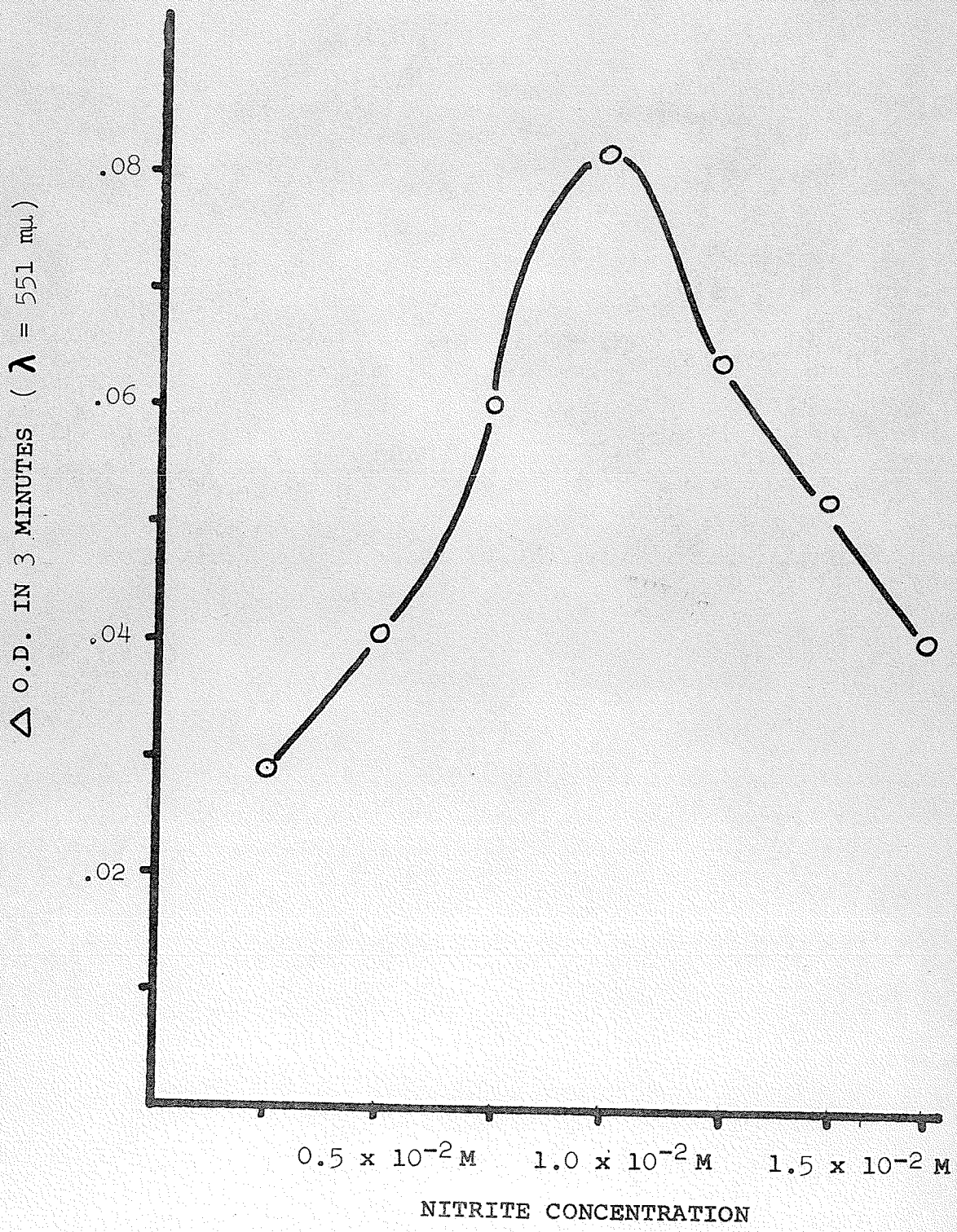


Figure 10. Effect of nitrite concentration on nitrite-cytochrome c reductase from Penicillium atrovenetum.

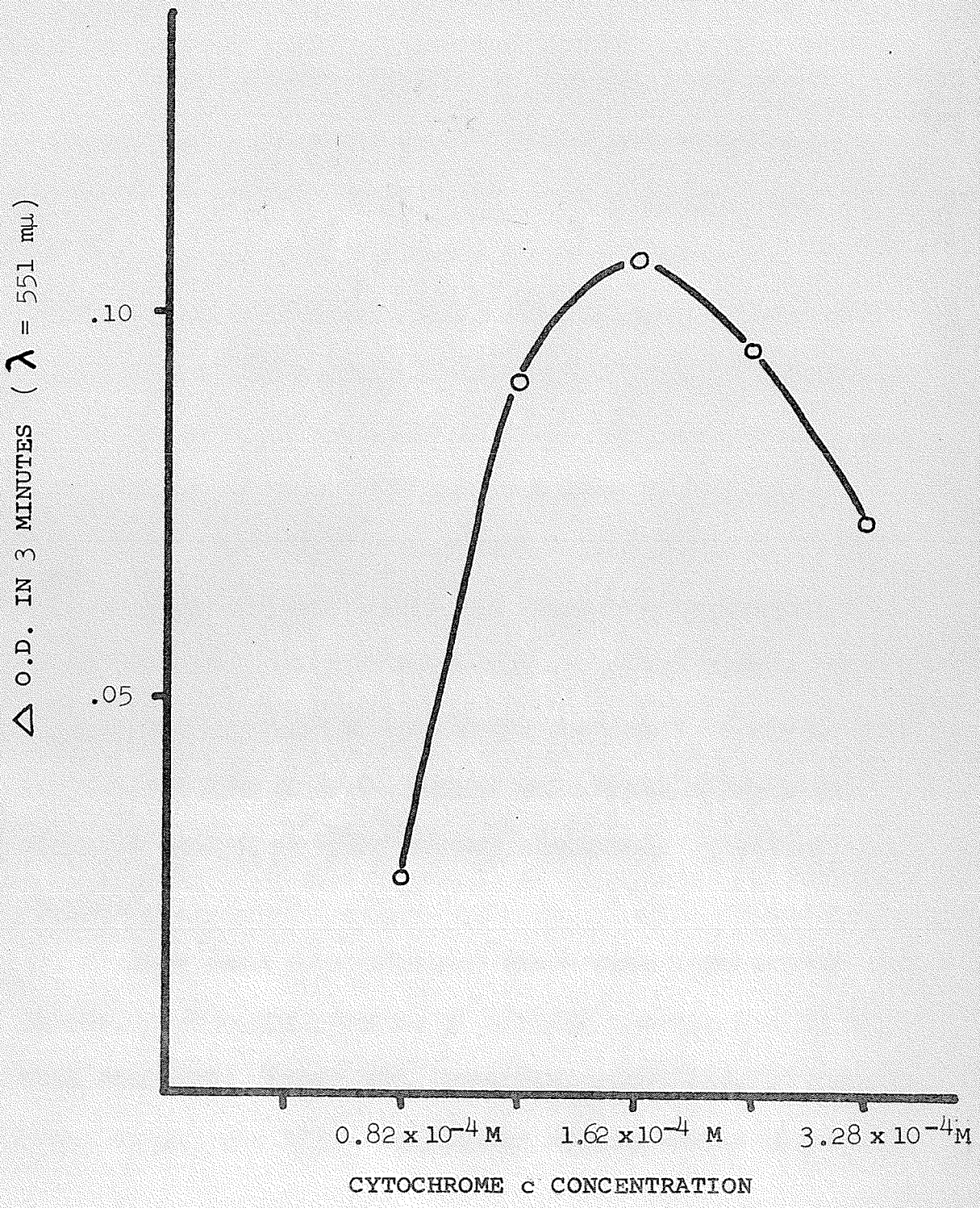


Figure 11. Effect of cytochrome c concentration on nitrite-cytochrome c reductase.

DISCUSSION

From the data obtained in the course of this investigation, it would seem that the oxidative reaction sequence of nitrification found in Asp. flavus, Asp. wentii and Pen. atrovenetum follows a pathway comparable to that found in the chemoautotrophic bacteria Nitrosomonas and Nitrobacter. Cell free mycelial extracts were capable of oxidizing ammonium to hydroxylamine, nitrite and nitrate in the presence of added TPN and mammalian cytochrome c. Artificial electron carriers such as methylene blue, 2,6-dichlorophenol indol phenol and phenazine methosulfate could partially replace cytochrome c in the system, and under anaerobic conditions, these dyes were reduced. Both ferrous and ferric iron, copper and cobalt stimulated ammonium oxidation while nickel completely inhibited the reaction.

Data were also obtained which demonstrated the oxidation of hydroxylamine to nitrite and nitrate by cell free extracts. Added TPN, mammalian cytochrome c, ferrous iron, copper and $KClO_3$ stimulated hydroxylamine oxidation.

Manometric measurements of oxygen uptake yielded a poor correlation between oxygen uptake and nitrite production. This result may have been obtained because of the toxic nature of the substrate and its possible inhibition of the electron transport system. Another possible explanation is the action of a fungal peroxidase which could facilitate the utilization of available oxygen by the system.

The oxidation of hydroxylamine was shown to be coupled to cytochrome c reduction, the reaction being catalyzed by the enzyme hydroxylamine-cytochrome c reductase. The fungal enzyme had its pH optima at 6.5 while the Nitrosomonas enzyme exhibited maximum activity at a pH of 8.5. The purified Nitrosomonas enzyme was stimulated by flavins but this stimulation was not effected on crude enzyme preparations (Aleem and Lees, 1963). No flavin stimulation was observed in crude cell free fungal enzyme preparations, but possibly a stimulation would be detected on the purified enzyme. Both the bacterial and fungal enzymes were markedly stable and both showed initial increases in activity during prolonged storage at 4° C. The similarities, with the exception of pH optima, would seem

to indicate that enzymes from both systems are quite similar in nature.

Nitrite oxidation was stimulated by added mammalian cytochrome c but not by added flavins or pyridine nucleotides. It was possible to couple nitrite oxidation to dye reduction with both methylene blue and 2,6-dichlorophenol indol phenol being reduced by the system. The maximum oxygen uptake as measured by manometric techniques occurred at a substrate concentration of 5×10^{-4} M. Higher concentrations were found to be inhibitory. In contrast to this, the nitrite oxidizing system of Nitrobacter is not inhibited by concentrations as high as 7×10^{-2} M (Aleem and Alexander, 1958) thus indicating a major difference in the two systems. As in the Nitrobacter system, nitrite oxidation is coupled to cytochrome c reduction by the enzyme nitrite-cytochrome c reductase. The fungal enzyme is very unstable so no attempts were made to purify the enzyme.

Nitrate reductase could be detected in cultures growing with ammonium or nitrate as the sole nitrogen source. In cultures using ammonium, the enzyme could not be detected until after the logarithmic phase of growth. As ammonium

represses the formation of the adaptive nitrate reductase (Kinsky, 1960) one would suspect that the concentration of ammonium must decrease before the enzyme would be formed. The enzyme was stimulated by DPNH and FAD with added iron, copper and molybdenum having no effect. In the fungal system, the reduction of nitrate may serve two purposes: (1) the assimilation of nitrate to form protein and (2) it may act as a terminal electron acceptor under conditions of reduced oxygen tension.

Although the bacterial and fungal nitrifying systems do appear to be quite similar, there is one notable difference. This is the failure of allyl thiourea to inhibit ammonium oxidation by fungal cell free extracts. Either the mechanism of ammonium oxidation is different in the fungal system or possibly allyl thiourea inhibits the transport of ammonium through the Nitrosomonas cell wall. The problem could be resolved by either determining the effect of allyl thiourea on ammonium oxidation by intact mycelia or better still, by obtaining cell free extracts of Nitrosomonas which are capable of oxidizing ammonium.

Because of the inherent complexity of the crude

fungal extracts, it was difficult to obtain data to demonstrate a good nitrogen balance in the reaction mixtures. In many extracts the relative activities of the oxidizing enzymes were different so time studies yielded somewhat variable data as to the accumulation of the oxidation products. Although the maximum growth of the fungi (in terms of weight per unit volume of medium) occurred after at least 7 days of incubation, younger cultures appeared to have more active enzyme systems. The possibility exists that a nonenzymatic interaction occurs between hydroxylamine and nitrite to form nitrous oxide or nitrogen (Iwasaki and Mori, 1958). This would result in a loss of nitrogen from the system and partially explain the difficulties involved in obtaining a good nitrogen balance. The problem is further complicated by the operation of both oxidizing and reducing systems in crude cell free extracts. These systems are responsible for causing continuous fluctuations in the nitrogen balance. The only way to overcome this is by the purification of the separate components of the system.

The above problem can be partially overcome by heating the crude extracts at 50° C for 5 minutes. This treatment

inactivates both the nitrite oxidase, i.e. the nitrite-cytochrome c reductase without harming the rest of the system. Both of the previously mentioned enzymes were inactivated by periods of storage at 4° C exceeding 7 days while the ammonium and hydroxylamine oxidases retained their activity (in part at least) for periods up to 3 months.

It would appear from the data obtained that the pathways of heterotrophic nitrification closely resemble those of autotrophic nitrification. Although Alexander and Marshall (1962) suggest that the reactions of nitrification in Aspergillus flavus involve β-nitropropionic acid as an intermediate, it seems far more likely that the pathway should be similar to that found in the autotrophic bacteria. These workers reported that cell free extracts of Asp. flavus could produce nitrite but not nitrate from β-nitropropionic acid. They suggest because of their findings that β-nitropropionic acid is a likely intermediate in heterotrophic nitrification. As they found a fungal peroxidase present in their enzyme extracts, it is possible that the reaction

they describe is a peroxidase cleavage of the compound and has nothing at all to do with nitrification (Little, 1957).

Schatz and Mohan (1955) reported that Streptomyces nitrificans was capable of growing in a medium with 0.2% urethan as both the carbon and nitrogen source. The extracts obtained from these organisms oxidized ammonium to nitrite, but had no action on hydroxylamine or nitrite. They also report that nitrite was produced from the oxidation of urea. In an earlier report, Isenberg, Schatz et al (1954) stated that Streptomyces could produce nitrite from urethan and other carbamates. No nitrate was formed. It seems probable that prior to the nitrification of the carbamates, a cleavage is effected which yields free ammonium. Thus, the pathway of nitrification would involve not organic nitrogen compounds but only inorganic nitrogen compounds.

In the heterotrophic fungi, it is doubtful whether the reactions of nitrification supply any appreciable amount of energy to the organisms. As the medium is supplemented with sucrose, the fungi obtain more than enough energy from the oxidation of the carbohydrate.

Attempts to grow the fungi in an inorganic medium were not successful. This may however be due to an inability to fix carbon dioxide. It is possible that the oxidation of ammonium serves another purpose, that of detoxification. Perhaps the fungi are not able to tolerate high concentrations of ammonium and find it advantageous to store their supply of nitrogen in a form less toxic. After the fungi have oxidized most of the excess ammonium to nitrate a nitrate reductase is induced and the fungi may now assimilate the nitrate into cell protein, thus avoiding possible inhibition of growth.

Although it appears as if heterotrophic and autotrophic nitrification are essentially the same, there is still much work to be done to obtain more information on the nature of the heterotrophic nitrifying enzyme systems.

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