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Studies on nitrite utilization by soil microorganisms.

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STUDIES ON NITRITE UTILIZATION BY SOIL MICROORGANISMS

A Thesis Presented

By

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September 1969

To my parents

for their understanding

and encouragement

which they have always given

me in whatever I choose to do.

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I also wish to thank Dr. William S. Mueller and Dr. John H. Baker for serving as members of my committee and for the time they spent helping me with the preparation of my thesis.

"Speak to the earth,

and it shall teach thee"

Job XII:8

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INTRODUCTION

Nitrogen transformations in the soil have been a major area of study since the beginning of soil microbiology. The importance of nitrogen to the growth of higher plants perhaps best explains the reason for investigations in this area by many of the early soil microbiologists. Due to biological and chemical processes, inorganic nitrogen occurs as ammonium, nitrite, or nitrate. The biological pathways and ecological interactions involved in the formation and degradation of these compounds are not well understood. The oxidation of ammonium to nitrate changes the oxidation state of nitrogen from -3 to +5, therefore involving the removal of a total of eight electrons from the nitrogen atom. With the exception of nitrite, which is present in relatively small quantities, the intermediate compounds involved have not been isolated in natural systems. It is thought that this is due to the fact that these compounds are either unstable or are immediately utilized by biological systems and therefore not detectable.

Although most of the credit for the microbial oxidation of ammonium to nitrite is given to the autotrophic genus Nitrosomonas, the large heterotrophic population of the soil microflora has also been shown to be responsible for this process. Varying ecological factors may, under certain

conditions, create an environment that would increase the importance of the oxidative processes of the heterotrophs. The genus of autotroph considered most important in the oxidation of nitrite to nitrate is Nitrobacter, but the transformation of nitrite is not solely due to the activities of this organism. Heterotrophic nitrite utilizers are also present in the soil which reduce nitrite to amines, to form amino acids and incorporate them into cellular proteins. Also, a small number of heterotrophs has been found to produce nitrate as a by-product of their metabolism. In the nitrogen cycle of oxidation and reduction, the nitrates formed are also subject to reduction to nitrogen gas or to utilization by microorganisms.

The biological activities involved in the utilization of nitrite are of particular interest since nitrite is known to be toxic to many organisms though generally never found in large amounts in the soil. Hypotheses have been made as to the physiological effects of nitrite and why certain microorganisms maintain the ability to utilize it while others cannot. The purpose of this research was to assess the extent to which the broad range of soil microorganisms is capable of utilizing nitrite and to define the physiological factors involved in nitrite utilization by such microorganisms.

LITERATURE REVIEW

Although nitrite is known to be inhibitory to microbial growth (32), there are several reports that certain microorganisms are capable of utilizing this form of nitrogen. Among the soil microflora, the chemoautotroph, Nitrobacter is recognized as utilizing nitrite as its energy source, producing nitrate in this process (2). In pure culture, nitrite was found toxic to growing cultures of Nitrobacter only when the initial $NaNO_2$ concentration exceeded 1 g/l at pH 7.0. Even higher quantities were tolerated once growth was underway (32). Heterotrophic microorganisms, with differing degrees of efficiency will also utilize nitrite as a source of nitrogen (28). It has been reported that Neurospora will grow equally as well with nitrite as with nitrate and or ammonium (28). Arthrobacter globiformis, a member of a genus that is a ubiquitous soil inhabitant, has been shown to give the same cell yield when it is grown on nitrite, ammonium, or nitrate. It tolerated a maximum nitrite level of 525 μg/ml at a pH of 7.0 and with 1% glucose as a carbon source (17). In studies on heterotrophic nitrification, nitrite is sometimes used by microorganisms as the nitrogen source. Aspergillus flavus was grown on nitrite with a resulting loss of nitrite from the media (4). Hora, et al., also studied fungal isolates, the majority of which were Penicillium and Aspergillus, on 0.5 g/l $NaNO_2$ (20).

Since soil heterotrophs utilise nitrite, it follows then that they may compete with Nitrobacter for this compound. Arthrobacter globiformis has been shown to be a significant competitor for available nitrite in the soil (18). When cell suspensions of Arthrobacter and Nitrobacter were mixed and incubated in the presence of a carbon source, no nitrate was detected indicating no growth of Nitrobacter, but there was added growth of Arthrobacter as measured by cell weight. Nitrobacter, whether alone or in the presence of non-nitrite utilizers, converted nitrite to nitrate regardless of the presence of carbon (18). This shows that a change in the ecological conditions of the environment can affect the growth of the various nitrite utilisers and strengthens the assumption that all the nitrite is not utilized by the chemosautotroph, Nitrobacter. This might be expected since the relatively small populations of Nitrobacter could not account for the maintenance of the low nitrite concentration in the soil (26).

The nature of the inhibitory effect that nitrite has on the growth and respiration of many microorganisms is obscure (32). Two hypotheses have been made concerning this phenomenon. The first one suggests that inhibition depends on the undissociated HNO_2 penetrating the cell (6). Castellani and Niven studied the effects of various factors on the inhibition of Staphylococci by $NaNO_2$. When glucose was added either before or after sterilizing, there was no change in the bacteriostatic effects of nitrite with aerobic cultures,

but under anaerobic conditions the sterilization of glucose in the medium enhanced the bacteriostatic effect of nitrite 30 fold. Calculations showed nitrous acid was the substance associated with bacteriostasis anaerobically as it was aerobically. When sucrose was used in place of glucose, autoclaving the medium did not increase the bacteriostatic effect during anaerobic growth as had been shown with glucose as the carbon source (6). In studies that compared the effects of nitrite on the growth and respiration of Nitrobacter and certain heterotrophs, washed cells of Nitrobacter oxidized nitrite at a reduced rate when the pH was as low as 5.6 (32). In contrast to this, the respiration of E. coli, Pseudomonas aeruginosa, Hansenula anomala, and Saccharomyces cerevisiae was instantly and irreversibly inhibited at comparable nitrite concentrations (1 g/l $NaNO_2$) over a pH range of 4.5-5.6. Nitrite inhibition was correlated with the concentration of undissociated HNO_2 which would increase at a lower pH. It was thought possible that inhibition was caused by nitrous oxide formed chemically from nitrite in an acid medium but this seemed unlikely since nitrite oxidation at pH 5.6 by Nitrobacter showed the oxygen uptake corresponded to: $NO_2^- + 1/2\ O_2 \rightarrow NO_3^-$ (32).

The second hypothesis is derived from the evidence pointing to the interaction of nitrite with the cellular heme pigments as the site of inhibition (21). It was reported that at pH 6.0 the oxygen uptake by Bacillus cereus was

strongly inhibited by nitrite thus implying an interference with the cytochrome system (21). Spectrophotometric observavations supported the hypothesis that one site of nitrite inhibition is the cytochrome system (32). Silver (31) found that: (1) Pseudomonas cells rich in cytochrome c were more resistant to nitrite inhibition than deficient cells, (2) Nitrite or its degradation products have a great affinity for hemes in general, (3) the instantaneous nature of nitrite inhibition at low pH also is indicative that some respiratory enzyme is involved. It has also been found that the nitrite oxidase of Nitrobacter is closely related to cytochromes (1). Another report adds that cytochrome linked enzyme systems are involved in nitrite reduction in certain microorganisms (39).

Bacterial cytochromes may be classified as cytochromes a, b, and c on the basis of their absorption spectra. This does not necessarily mean that the electron transport relationships are similar to those in animal mitochondria. The most widely distributed cytochromes are of the c type and in aerobes they generally resemble those of mammals. Several bacteria appear to possess more than one autoxidizable cytochrome of the a type and another widely distributed one among bacteria is a heme-protein that has been designated cytochrome O (41). In a review by Smith, bacterial cytochromes were compared and contrasted with those of mammalian systems. Some Bacillus spp. showed spectra similar to mammalian or

yeast tissues, but the absorption peaks of the cytochrome components of most bacteria are different from those of mammalian tissues (9,34). Even if the bacterial cytochromes have absorption peaks similar to mammalian cytochromes, they may have entirely different physicochemical and enzymic properties (9,35).

The great diversity of metabolic types among bacteria makes for greater diversity in composition of the electron transport chain. Even for a given organism, change in environmental conditions may alter the nature and concentration of the electron transport catalysts (9). Different oxidizable substrates in the growth media have produced changes in the relative content of cytochromes (3). The pH of the medium may also affect the yield of the respiratory chain pigments (23). As would be expected, a deficiency of iron in the medium results in a decrease of cytochrome content (38). The relative amounts of the different cytochromes may also change with oxygen tension and the age of the culture (36).

In a further demonstration of the toxic effects of nitrite on cytochrome, Arthrobacter globiformis grown on nitrate showed well developed cytochrome c peaks at 550 and 521 mμ and clearly defined cytochrome shoulders at 564 and 530 mμ. In ammonium grown cells these peaks and shoulders diminished appreciably and in nitrite grown cells little or no cytochrome appeared to be present. Since this microorganism will grow on nitrite, it would appear that there is an alternate

pathway for electron transport and energy yield that is operative in cells grown with either nitrite or ammonium (17).

With heterotrophs, the reduction of nitrite may be carried out by cytochrome-independent electron transport enzymes. Flavoproteins have been found concerned with reduction of nitrate, nitrite hydroxylamine, and organo nitro compounds (10). Nitrite reductase from Azotobacter vinelandii and Neurospora have DPNH and TPNH for an electron donor and the prosthetic group is FAD (10). Fedorov and Ilina (14) reported the ready reduction of nitrate and nitrite by soil actinomycetes to ammonium derivatives with hydroxylamine as a probable intermediate. The nitrate reductase of nitrite assimilation is a molybdoflavoprotein whereas the nitrate reductase of nitrate respiration appears to be a cytochrome system (30). Pyridine nucleotide-nitrite reductase was detected in extracts of Neurospora (29) which subsequently led to the purification and characterization of pyridine nucleotide-hydroxylamine and nitrite reductases (28,42). Both enzymes in Neurospora were shown to have a flavine requirement and unidentified metal components (28,29). With the conversion of nitrite and hydroxylamine to ammonium in the presence of Bacillus pumilus it was reported that methylene blue or $FADH_2$ but not DPNH served as an electron donor (37). Based on metal deficiency and inhibitor studies, Medina and Nicholas found that nitrite and hyponitrite reductases required iron and copper, and hydroxylamine reductase required manganese (25).

A sequence of intermediates was proposed for nitrate reduction in Neurospora in which nitrate was first reduced to nitrite, then to hyponitrite, followed by hydroxylamine and finally forming ammonium (25). In the case of nitrite reduction there is some question as to the nature of the first reduction products, although most assume that ammonium is the final compound in the reduction reactions (24). In contrast to nitrate to nitrite reduction, there are undoubtedly several steps in the reduction of nitrite to ammonium (24).

Along with the evidence shown that nitrite inhibits heterotrophic growth due to its oxidizing effects on cytochromes, it is known that cytochromes participate in nitrite oxidation by the chemoautotroph Nitrobacter. The results of spectroscopic examination of Nitrobacter cells under various conditions, suggest that a cytochrome with an absorption maximum at 551 mμ in the reduced state is intimately concerned with nitrite oxidation by Nitrobacter (22,33).

Anaerobic nitrate reduction is known to occur by cytochromes. The influence of growth conditions on the synthesis of cytochrome c_{552} and the activities of NADH-NO_2 oxidoreductase and NADH cytochrome c oxidoreductase have been studied in E. coli K_{12}. This cytochrome is synthesized only during anaerobic growth. Nitrite added to anaerobic cultures stimulated cytochrome c_{552} synthesis as well as nitrite and cytochrome c reductase activities. These were decreased by high concentrations of glucose or amino acids and absent or

low in aerobically grown cells (7). Soluble cytochromes in E. coli were found when grown anaerobically in a semi-synthetic medium containing $NaNO_3$. The ability to synthesize cytochrome c_{552} during anaerobic growth seems widely distributed in E. coli and related facultative anaerobes (16). Nitrate reduction by Pseudomonas aeruginosa grown under anaerobic conditions produces a pink coloration. In a thick suspension of cells, absorption bands at 552 mμ and 521 mμ were observed and suggest the presence of a type of cytochrome c (40). Therefore it appears that cytochromes are involved in the reduction of nitrate to nitrite, nitrogen gas or ammonium where nitrate is the terminal electron acceptor and also in the oxidation of nitrite to nitrate which occurs with Nitrobacter. But, when heterotrophs are grown aerobically with nitrite as the sole nitrogen source, nitrite may inhibit growth through exhibiting its toxic effects on the cytochromes so that they become inoperative. The presence of alternate pathways for nitrite utilization or a different form of bacterial cytochrome would then explain why some heterotrophs can grow aerobically with nitrite.

There is also a possibility that the oxidizing effects that nitrite has on cytochrome is reversible by the addition of reducing agents. For example it is known that mammalian cytochrome c is reduced by ascorbic acid (19). The precise mechanism of ascorbic acid function is not known with certainty. It acts as a hydrogen carrier for redox systems within the cell

and hence is important in cell metabolism. There is evidence to suggest that this vitamin influences cellular respiration and stimulates the oxidation of amino acids (11). The ease of oxidation and reduction of ascorbic acid is the property of the vitamin which has been of paramount importance in the search for its functions at the cellular level. The redox potential is between that of the pyridine nucleotides and cytochrome c (5). It is known that nonenzymatic reduction of nitrite by ascorbate occurs if the ratio of ascorbate to nitrite is greater than 5:1 or if it is under highly acid conditions (12). Therefore, taking into consideration the conditions where ascorbate can directly reduce nitrite, it is possible that ascorbate could act as the reducing agent of cytochromes and thus reverse the hypothesized oxidizing effects produced by nitrite on many microorganisms.

MATERIALS AND METHODS

Isolation of microorganisms

Nitrite utilizing isolates were taken from a fertile field soil which had not been recently fertilized. The soil was obtained locally through the greenhouse facilities of the Department of Plant and Soil Science, University of Massachusetts. Platings were made in triplicate at soil dilutions ranging from 10^{-6}-10^{-8}. To make the dilutions a 10 g sample of soil was added to a dilution bottle containing 95 ml sterilized .01 M phosphate solution and shaken by hand vigorously for two minutes. Ten ml of this dilution was then transferred to a 90 ml sterilized blank and shaken. This process was repeated until a 10^{-8} dilution was reached. From each of the 10^{-6}-10^{-8} dilutions, 1.0 ml was added to each of three petri plates. Fifteen to 20 ml of sterile growth medium was added to each plate and lightly rotated to achieve an even distribution of the organisms throughout the medium. Control plates were made at 10^{-7} and 10^{-8} dilutions on nutrient agar medium.

The isolation medium used was Morris medium (27) modified so that 50 μg/ml nitrite was the sole nitrogen source (see Appendix). Plate counts were made after four days incubation at room temperature (26-30°C). The differentiation of bacteria, actinomycetes, and fungi was based on cultural characteristics

and microscopic examinations. The isolates taken from the plates were maintained on nutrient agar slants.

Growth of isolates on various concentrations of nitrite-nitrogen

The isolates were grown in liquid modified Morris medium (see Appendix) with $NaNO_2$ in concentrations ranging from 50-4000 μg/ml. N as NO_2^-. The bacterial isolates were incubated in duplicate or triplicate on a rotary platform shaker at 26°C for seven days in 50 ml. quantities of media in 300 ml Nephalo flasks. Growth was measured turbidimetrically with a Klett-Summerson Colorimeter. The actinomycete and fungal isolates were incubated in 50 ml quantities in 250 ml Erlenmeyer flasks and growth was measured by dry weight of the mycelial pad.

Ascorbic acid, when added to the media, was in a concentration ranging from .01M-.06M. It was sterilized separately by the millipore filter technique.

Analysis for Nitrite

To test for nitrite utilization by the isolates, aliquots of the culture medium were analyzed by Csaky's method for nitrite determination (8) (see Appendix).

The growing of cells for cytochrome isolation

The two isolates used to obtain cytochromes were grown on Morris medium modified so that 300 μg/ml NO_3^--N was the nitrogen source (see Appendix). They were incubated in 2800 ml

flasks on a rotary platform shaker at 26°C for three days. The resultant cells were then harvested and used to obtain cell free extracts.

Isolation of cytochrome

After the cells were harvested by centrifugation in a refrigerated Sorvall centrifuge, the cell paste was frozen in a mortar and ground for 8-10 min. in the cold (5°C) with an equal weight of alumina. It was washed out of the mortar with two volumes of cold 0.1 M phosphate buffer, pH 7.0. The extract was separated from the alumina and cellular debris by centrifugation at 7000 rpm for 15 minutes in a refrigerated centrifuge. The extract was then dialyzed in the cold for 24 hrs. against cold doubly distilled water.

Spectral analysis of cytochromes

The absorption spectra of the cytochrome preparations were obtained with a Beckman DBG spectrophotometer equipped with a 10 inch recorder. Spectra were obtained for the visible region, from 700 to 320 mμ.

The procedure followed for the spectral analysis of the standard mammalian cytochrome c was taken from Umbreit et al. (39). The powdered cytochrome c (Nutritional Biochemicals Corporation, Cleveland, Ohio) was dissolved in distilled water giving a final concentration of approximately 1×10^{-4} M cytochrome solution. To .2 ml of this solution was added 1.7 ml H_2O, and 1.0 ml phosphate buffer, pH 7.4. To

oxidize this preparation 0.1 ml $K_3Fe(CN)_6$ was added followed by the addition of 0.1-1.0 mg $Na_2S_2O_4$ to produce the reduced cytochrome.

The cytochrome extract was oxidized by bubbling O_2 into the cuvette for 2-3 minutes by means of a Pasteur pipette and was reduced by the addition of 0.1 to 1.0 mg of $Na_2S_2O_4$. When the cytochromes were treated with nitrite, 0.1 ml of 4 M $NaNO_2$ was added per 2.0 ml of extract in the cuvette to give a final concentration of .02 M nitrite. The addition of ascorbate was made by adding .02 ml of 1.0 M ascorbate per 2.0 ml of extract to give a final concentration of .01 M ascorbate. The ascorbate solution was prepared in a phosphate buffer so that the final pH was 7.0. These concentrations of nitrite and ascorbic acid were the same as those used in the growth studies.

RESULTS

Ecological Studies

Populations of nitrite utilizers in the soil

The condition of the soil was an important factor in the isolation of nitrite utilizing microorganisms. When the soil had been dried or subject to greenhouse pasteurization (180°F, 20 min.), the bacterial and fungal counts were greatly reduced and the actinomycete population was enhanced whereas this did not occur on the control plates. Particularly prevalent was an actinomycete which had a pink pigmentation. This was almost the exclusive microorganism on some plates. This pink coloration appeared after 3-4 days incubation with the nitrite containing media but it did not appear until 6-7 days with the control nutrient agar plates.

The plate counts of an unamended fertile field soil which had not been dried, pasteurized, or had any recent applications of fertilizer, contained a variety of nitrite utilizing microorganisms. A wide range of soil bacteria, actinomycetes, and fungi appear to be able to utilize nitrite as a nitrogen source. The microbial populations in the soil dilution platings (Table 1) show that 50 µg/ml nitrite-nitrogen causes only a tenfold decrease in numbers of bacteria and fungi. The actinomycete population shows a smaller decrease in numbers. Approximately 24 hrs. additional incubation time was required

TABLE 1.--Populations of microorganisms from soil dilution platings utilizing nitrite as a sole source of nitrogen/g soil

Media	Bacteria	Actinomycetes	Fungi
Nitrite medium (Morris)	$3x10^8$	$4x10^7$	$7x10^5$
Nutrient Agar	$1x10^9$	$8x10^7$	$9x10^6$

for colonies to appear on the nitrite containing plates. The colonies were smaller in size than those appearing on the control plates.

Isolation of nitrite utilizing microorganisms

Individual isolates were selected from the nitrite containing medium. The criteria used for differentiation were their cultural and morphological characteristics. Microscopic examination showed ten of the bacterial isolates to be rods; and one a slow growing coccus which displayed an orange pigmentation. There were both Gram negative and positive rods. Some also showed motility. These results are summarized in Table 2. Two species of actinomycetes were isolated, each one having different coloration. There were five fungal isolates; among them Trichoderma, Penicillium, and Aspergillus. Trichoderma was present in the soil dilution platings in much greater numbers than any of the other fungal isolates.

Growth Studies

Growth of isolates on various nitrite concentrations

The isolates were grown on a range of concentrations of nitrite to determine the tolerance levels of the test organisms to these concentrations. The bacterial isolates were grown on modified Morris medium (see Appendix) containing 0, 50, 100, 150, and 300 μg/ml NO_2^--N respectively as the sole

TABLE 2.--Nitrite utilizing bacteria from the soil

Isolate No.	Classification	Morphology	Gram Stain	Motility	Pigmentation
1		short coccoidal rods	G-	-	0
2	Arthrobacter	rods becoming coccoidal in stationary phase	G var.	-	brown
3		long rods	G+	+	0
4		medium coccoidal rods	G var.	-	brown (H_2O soluble)
5		small rods	G var.	+	0
6	Pseudomonas	medium rods	G-	+	0
7		medium coccoidal rods often occurring in pairs	G-	+	0
8	Nocardia	mycelial	G-	-	0
9		long rods	G-	-	0
10		medium rods	G-	+	0
11		coccus	G+	-	orange

nitrogen source. After 24 hrs. incubation, ten of the isolates growing in 50 μg/ml NO_2^--N had higher growth rates than those in media containing no nitrogen. With concentrations of 150 μg/ml NO_2^--N there was a lag period of approximately 24 hrs. before the logarithmic phase of growth began. After seven days incubation, the stationary phase having been reached, growth in media containing 150 μg/ml NO_2^- -N was in general equal to or greater than the cultures that had contained 50 μg/ml NO_2^--N. At a concentration of 300 μg/ml NO_2^--N, definite inhibition of growth was found with six of the isolates, five of them did not grow at all. The one coccoidal isolate was found to be inhibited at all concentrations of nitrite. Figures 1-10 show the growth rates obtained with the ten bacterial isolates on 0, 50, 150, and 300 μg/ml NO_2^--N.

To confirm that the nitrite was in fact utilized by these isolates, the residual nitrite was analyzed by Csaky's method at the beginning of the incubation period and again at the termination of the experiment (8). In all cases the utilization of nitrite was concurrent with the growth of the organism. Figure 11 presents the results of the analyses made after seven days incubation and compares them to the corresponding growth of the organism which had been measured in Klett units. Virtually all the nitrite was utilized at concentrations of 50 and 150 μg/ml but at 300 μg/ml NO_2^--N, the nitrite was not completely utilized and the decreased growth is reflected in the residual nitrite in the medium.

Fig. 1-10 Growth of Bacterial Isolates on 0-300 $\mu g/ml$. NO_3^--N

Fig. 1 Isolate No. 1

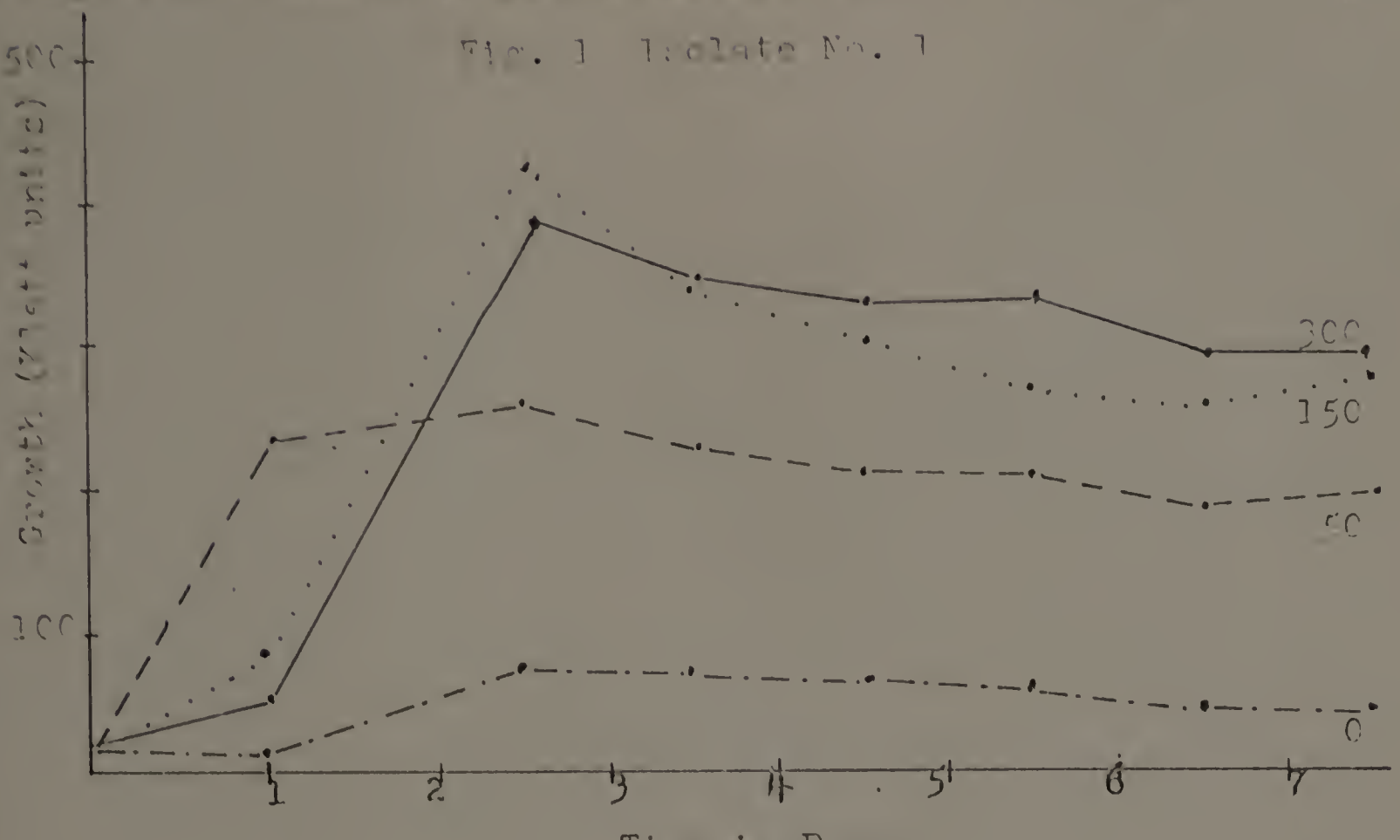
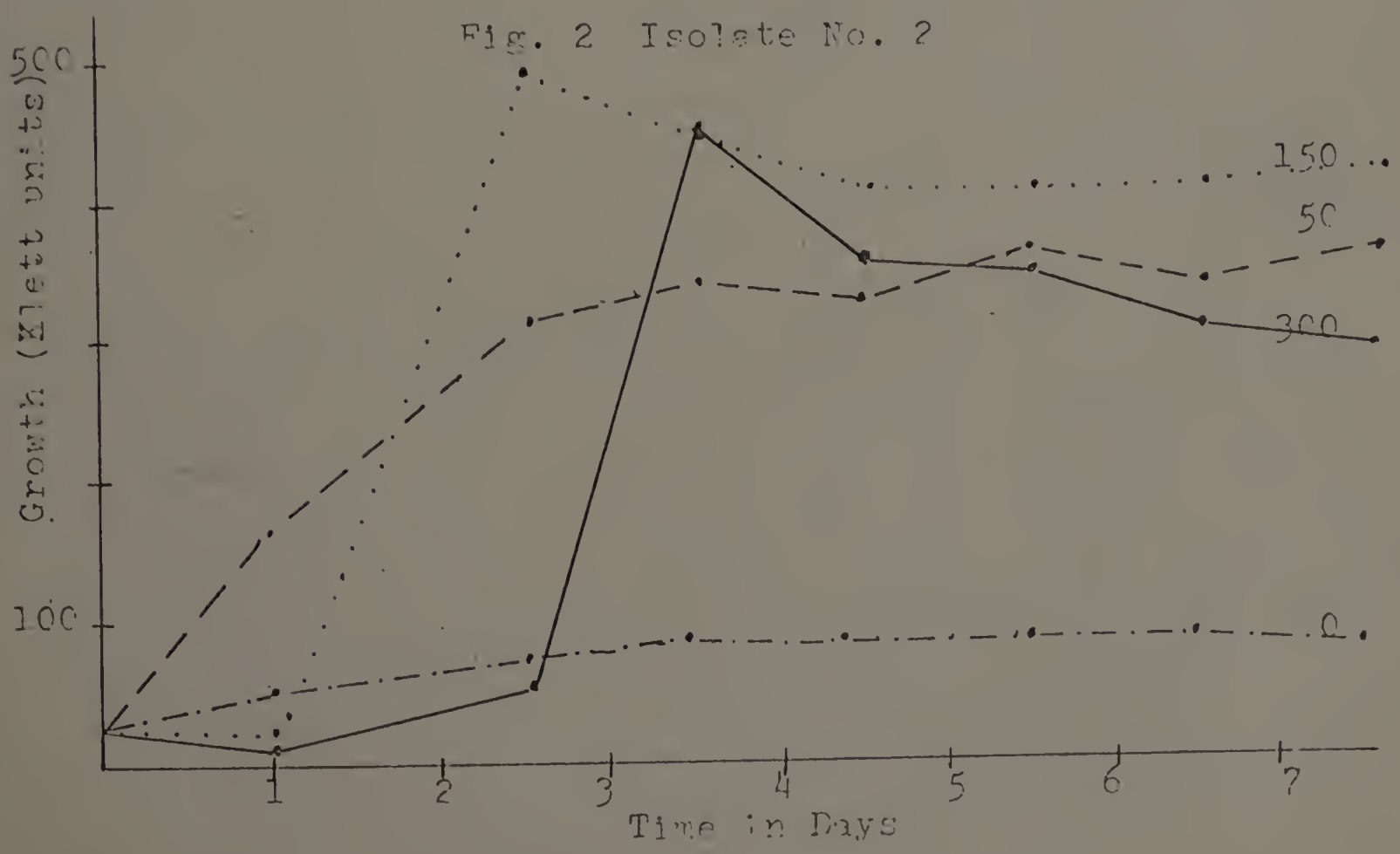


Fig. 2 Isolate No. 2



Growth Rate (cont.)

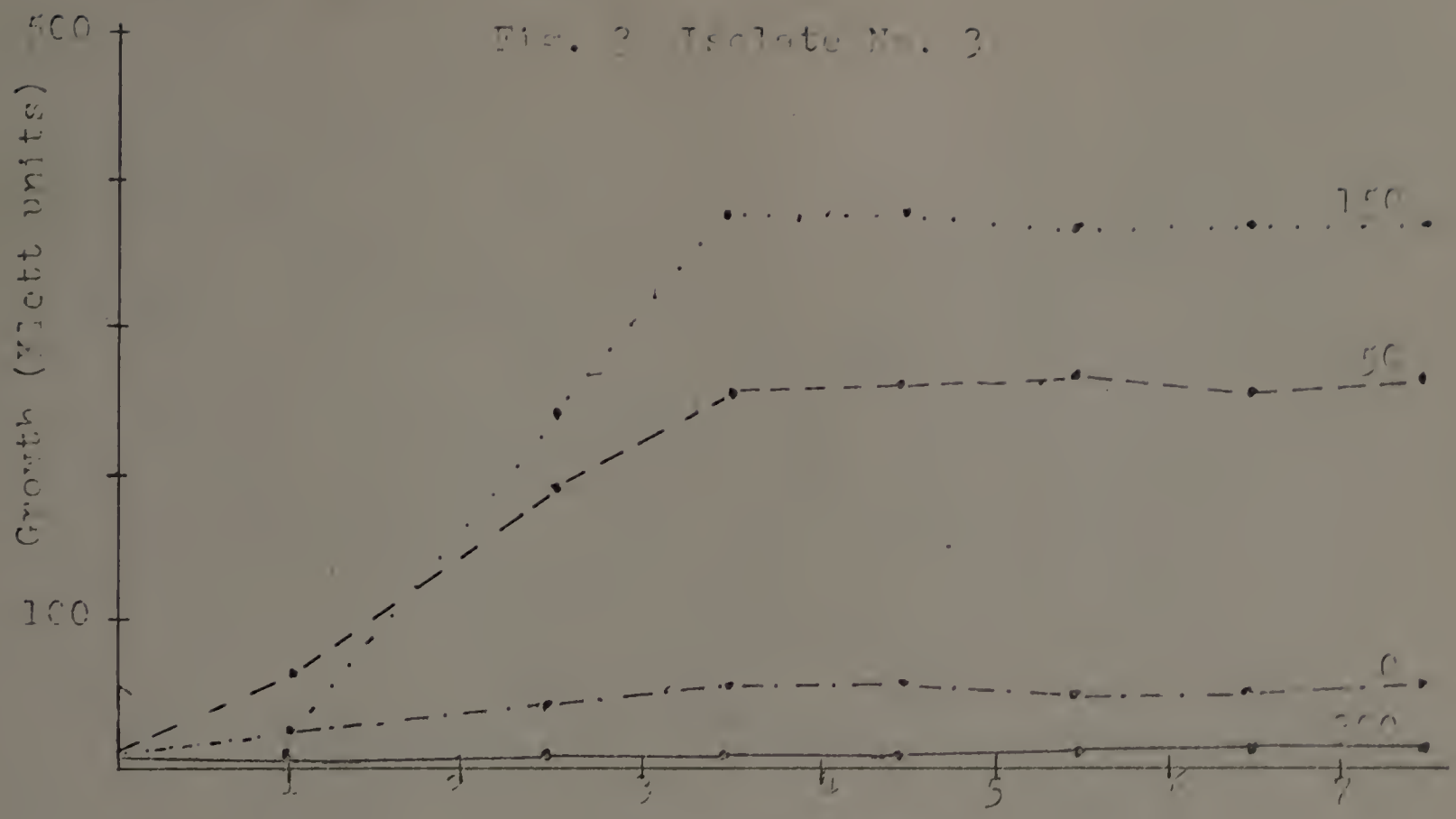


Fig. 3 Isolate No. 3

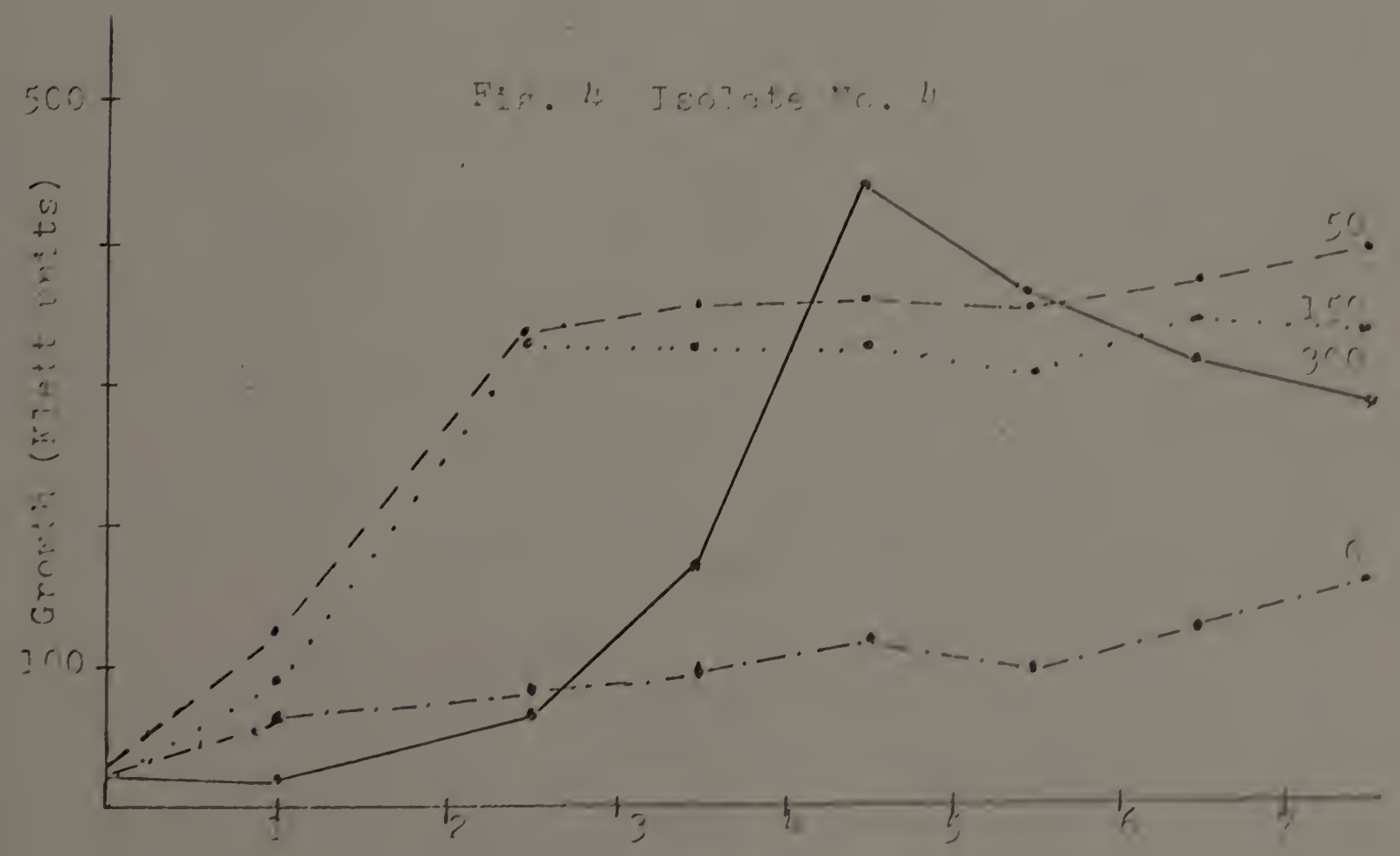


Fig. 4 Isolate No. 4

Growth Rates (cont.)

Fig. 5 Isolate No. 5

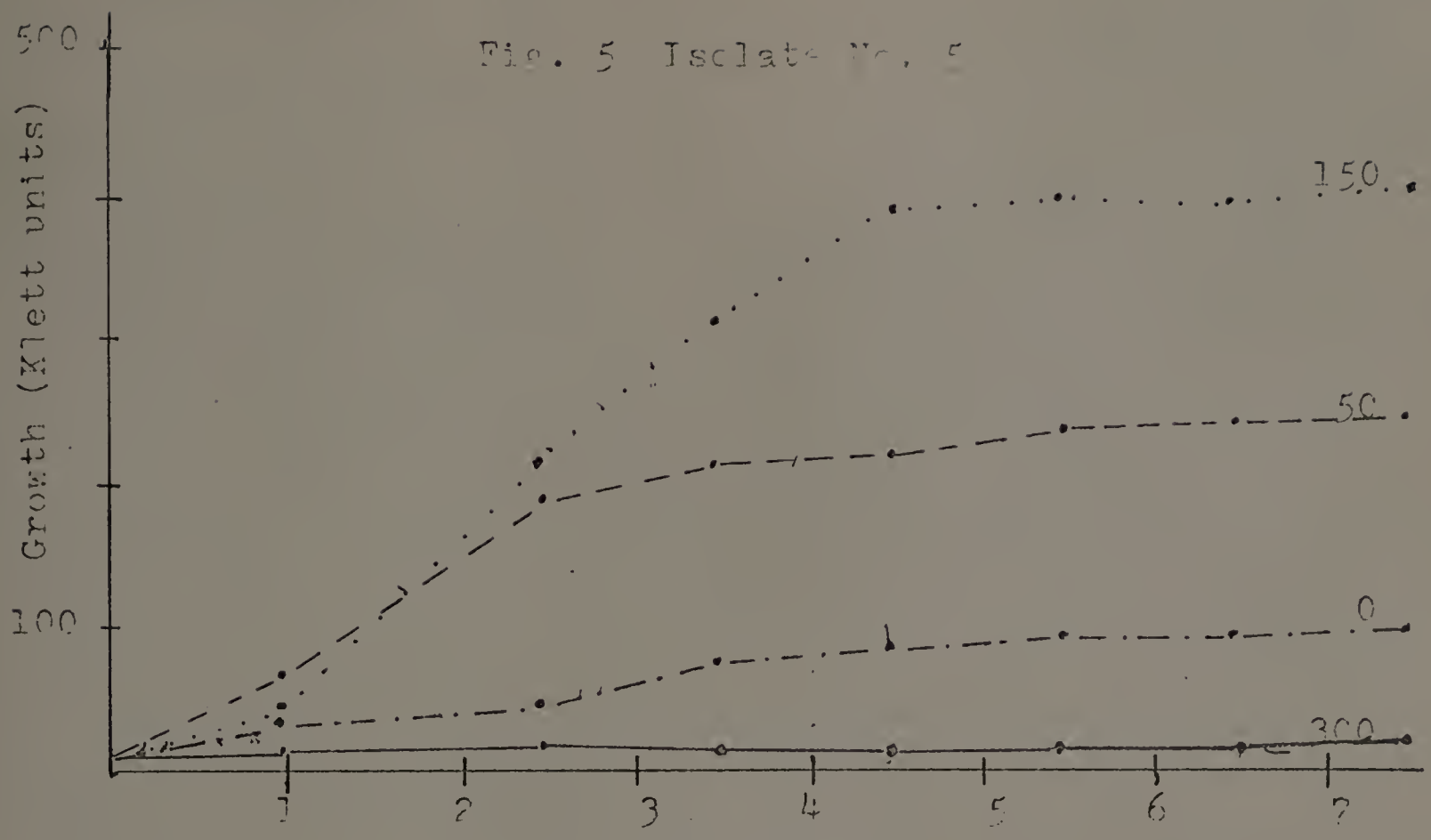
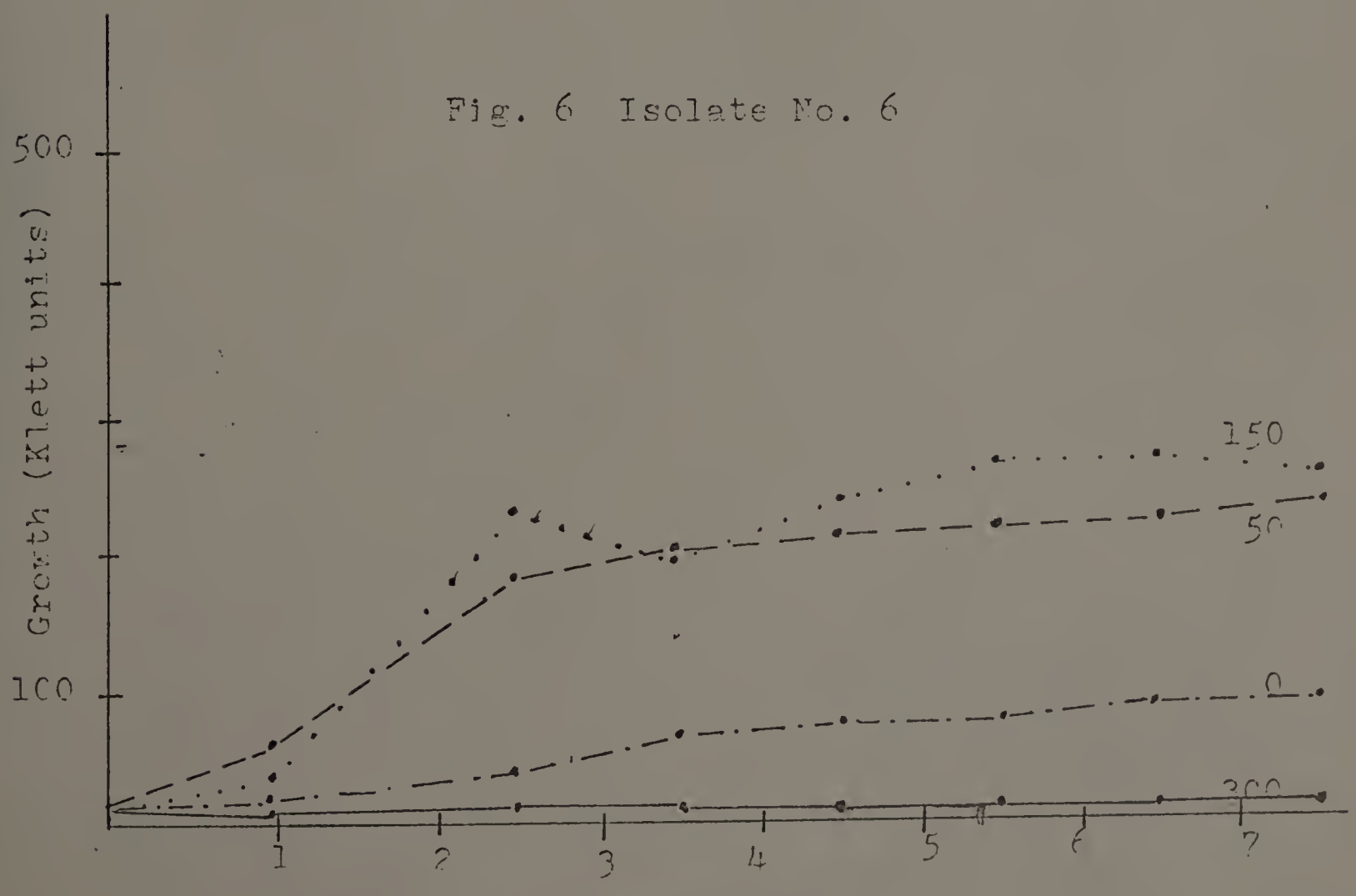


Fig. 6 Isolate No. 6



Growth Rates (cont.)

Fig. 7 Isolate No. 7

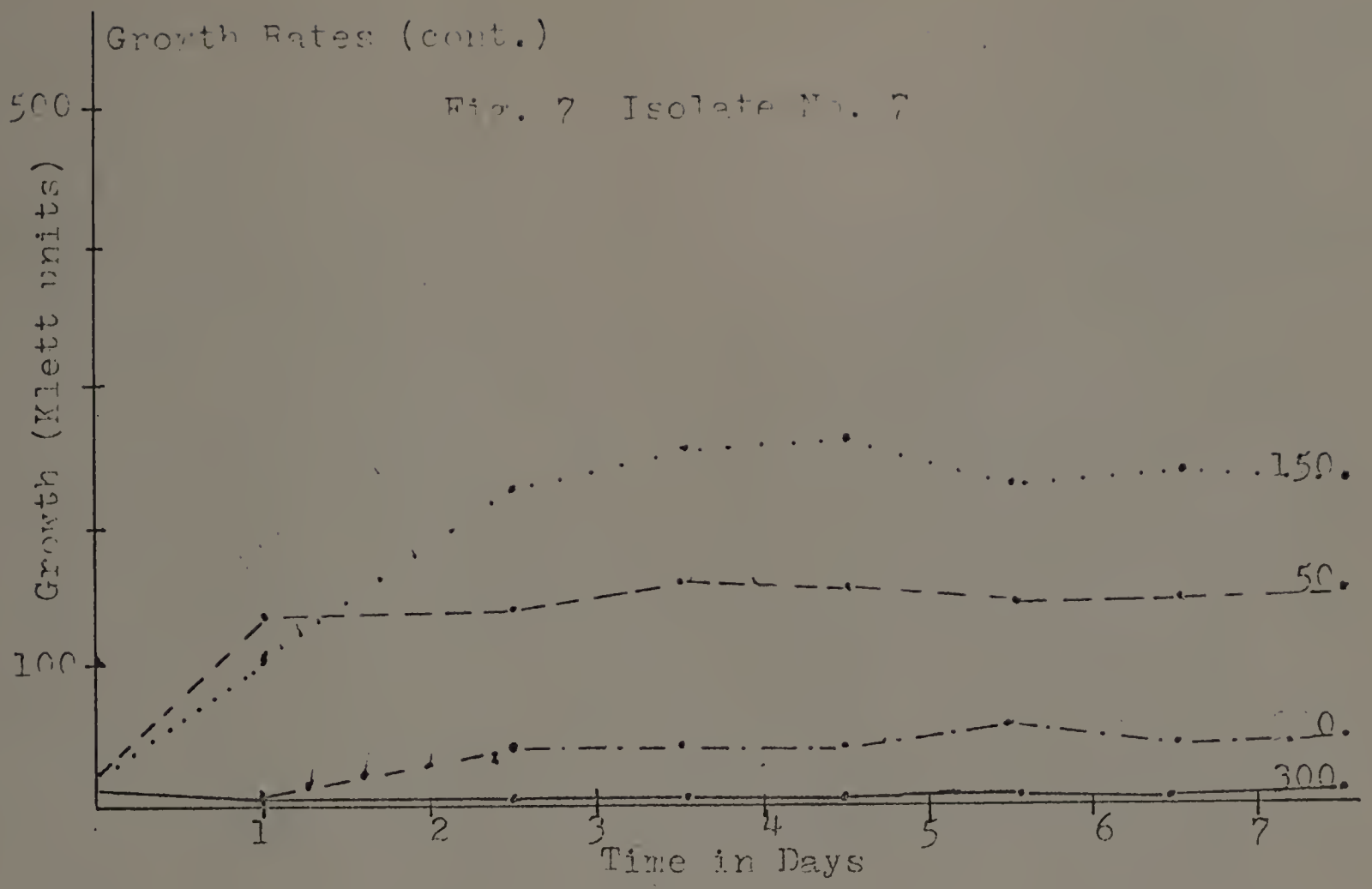
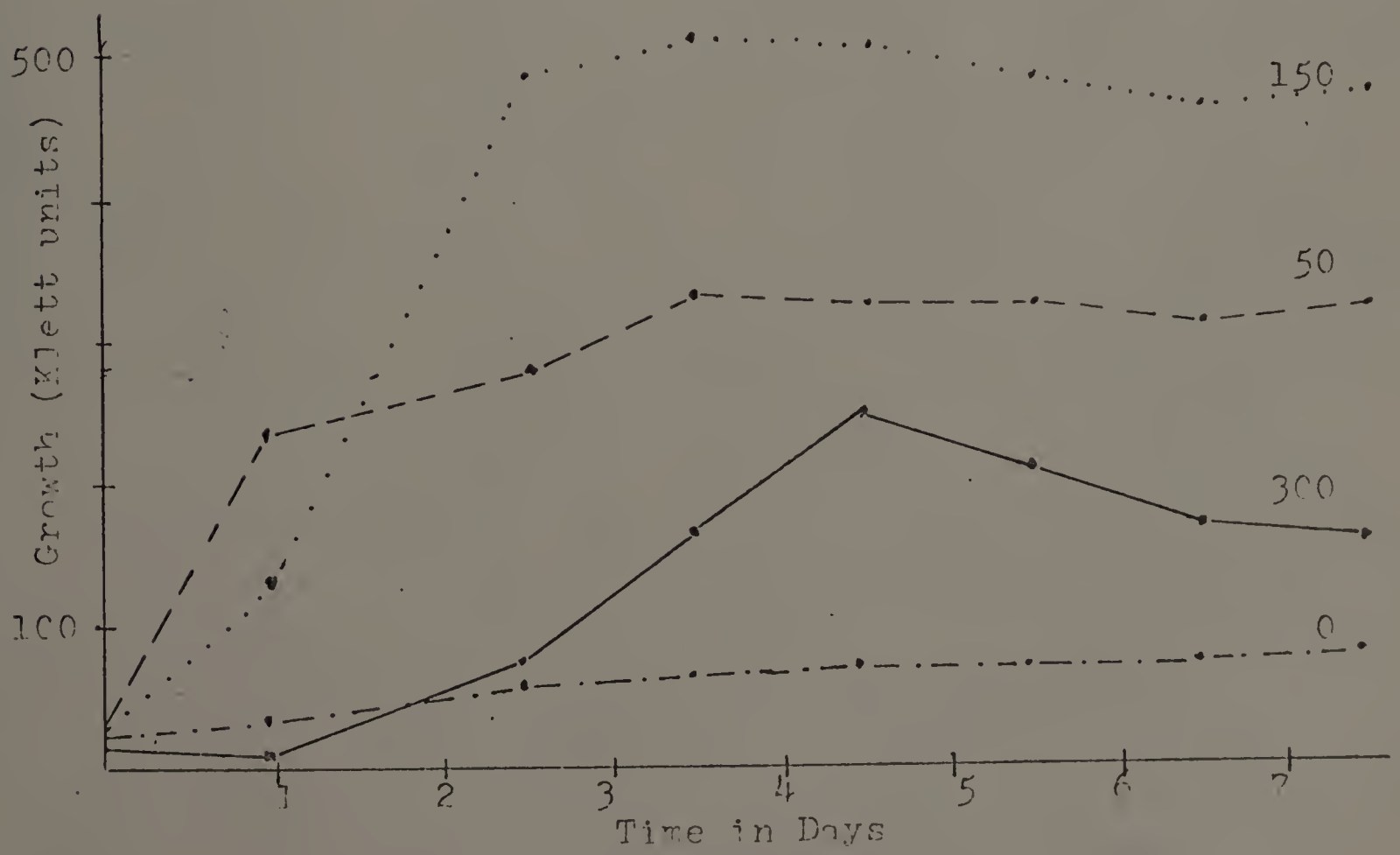


Fig. 8 Isolate No. 8



Growth Rates (cont.)

Fig. 9 Isolate No. 9

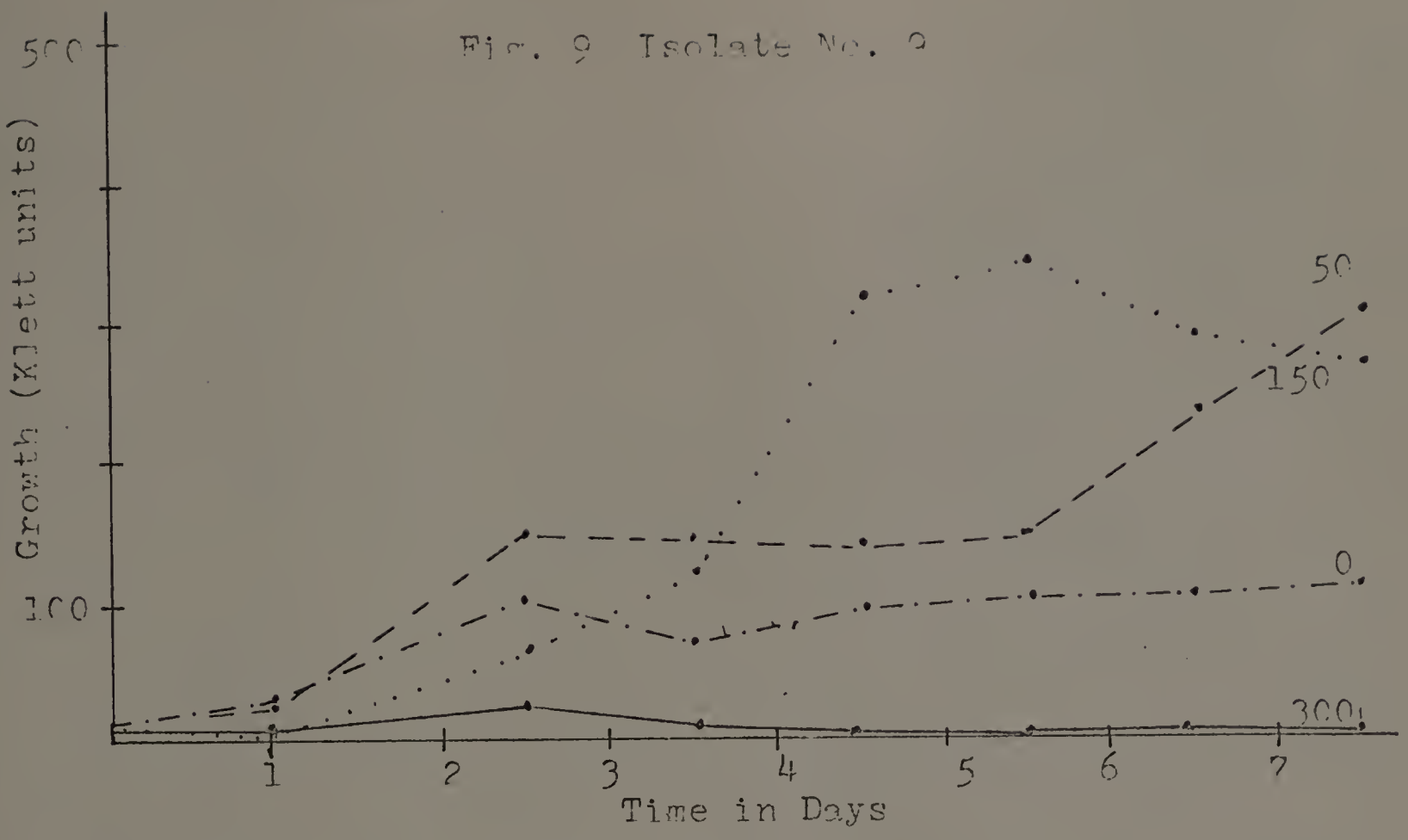
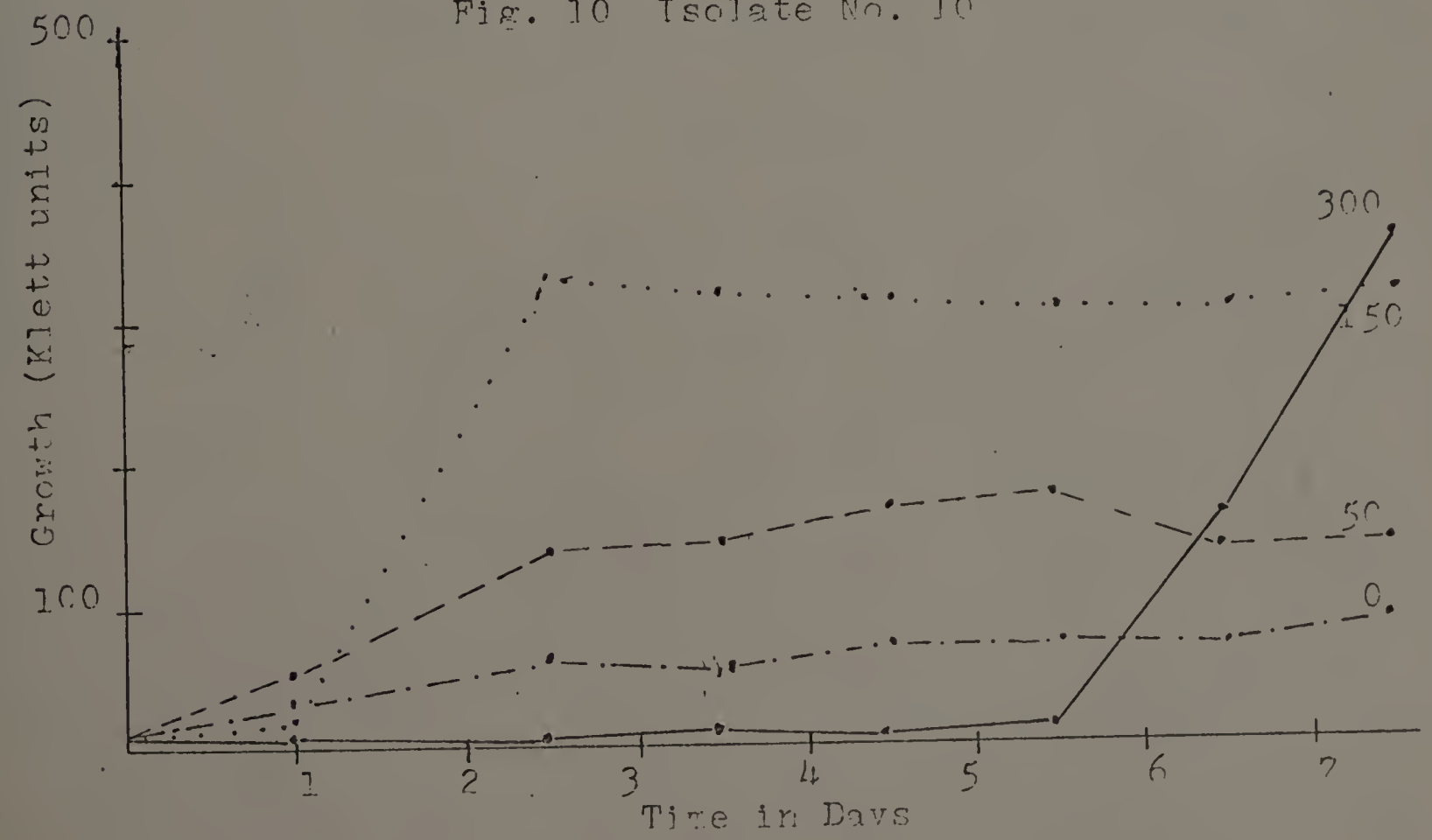


Fig. 10 Isolate No. 10



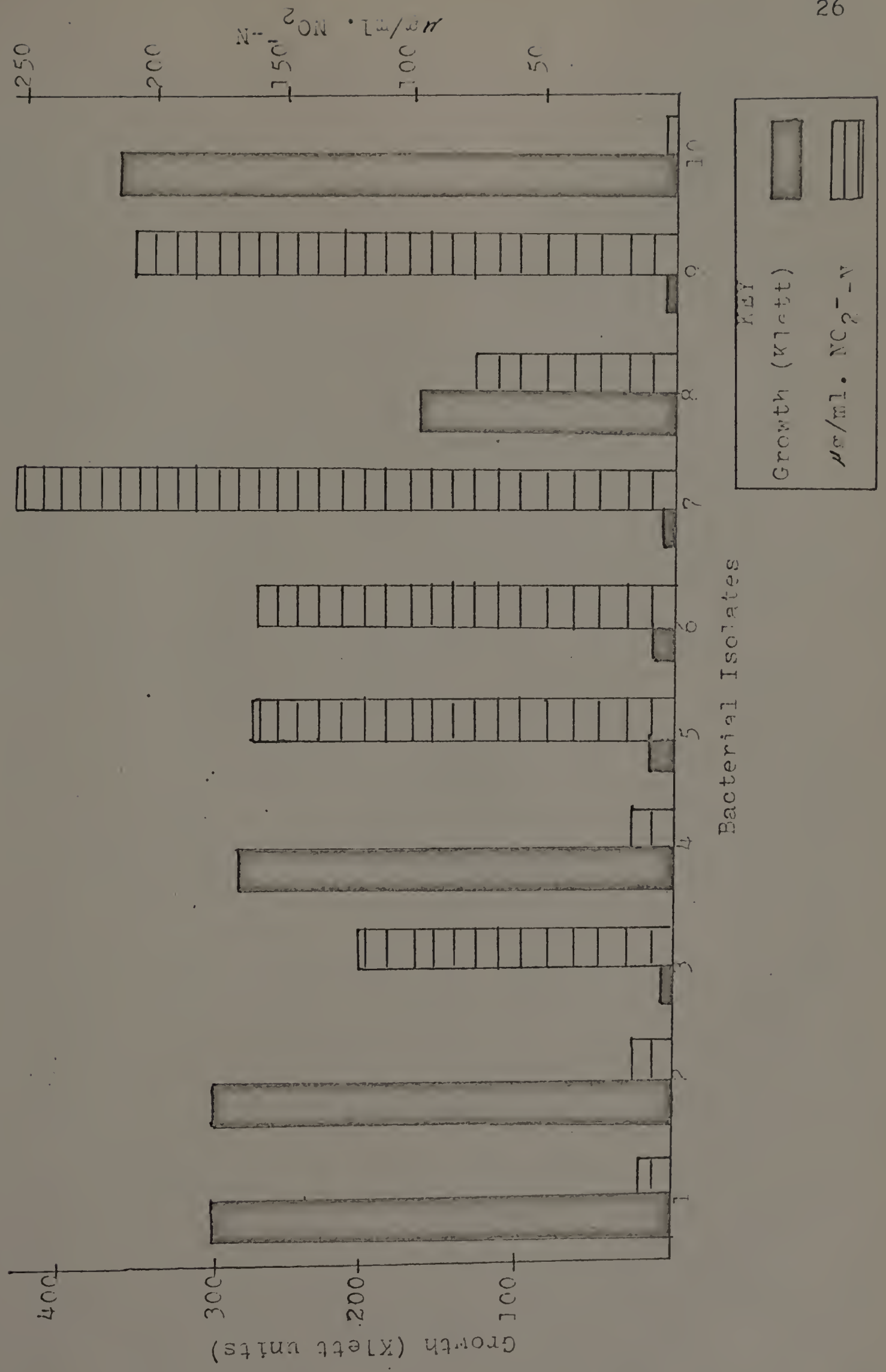


Fig. 11 Residual Nitrite vs. Growth of Isolates 1-10 on 300 μg/ml • NO_2^--N

Studies of the actinomycete and fungal isolates were made with parallel nitrite concentrations and growth was measured by dry weight of the mycelial pad. Contrary to the results of the bacterial nitrite utilizers, three of the fungi were not at all inhibited by the highest concentration of nitrite-nitrogen. This was indicated by a continued increase in dry weight in the cultures with the highest nitrite content. A graphic representation of the dry weights of these mycelial isolates and the corresponding nitrite concentration of the media is presented in Figures 12-18. The residual nitrite of the media was also measured and again there was a correlation between nitrite utilization and growth as is shown in Table 3.

Further studies were made on the pink pigmented actinomycete (P-1) and the Trichoderma sp. since they were found on the soil plates in such large populations. They were grown at higher concentrations of nitrite to see what level inhibited or reduced growth. In the case of actinomycete P-1 it was already known that optimum growth on nitrite was at 150 μg/ml NO_2^--N, but it was found to grow on nitrite-nitrogen concentrations up to 1500 μg/ml. The Trichoderma sp. tested grew in extremely high concentrations of nitrite reaching its maximum growth at 2500 μg/ml NO_2^--N. At concentrations of 3000, 3500, and 4000 μg/ml there was a visual decrease in growth with each increment of nitrite. However it was not inhibited completely even at 4000 μg/ml NO_2^--N.

Fig. 12-16 Growth of Fungal Isolates on 0-300 $\mu g/ml.$ NO_2^--N

Fig. 12 Trichoderma sp.

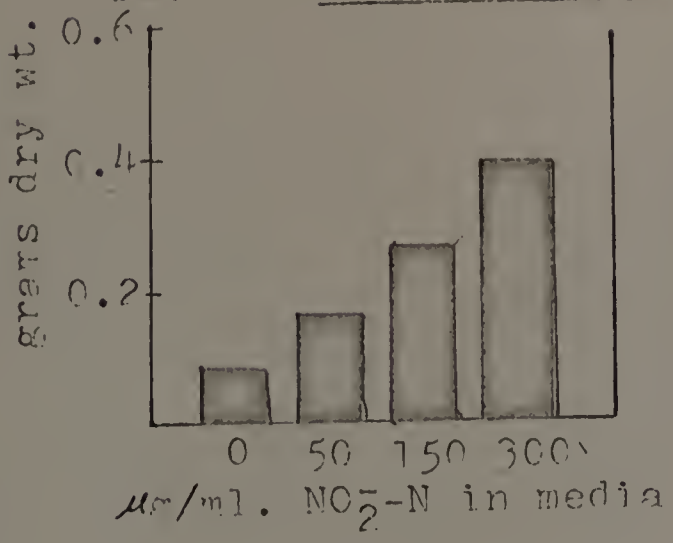


Fig. 13 Penicillium Sp.

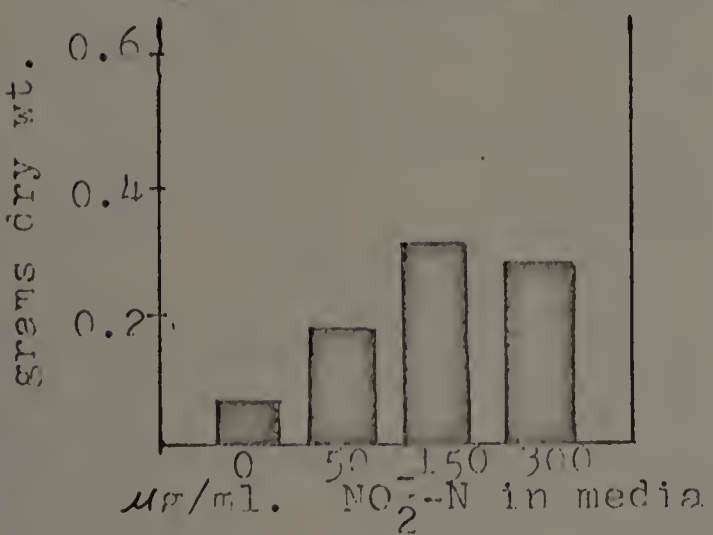


Fig. 14 Asperrillus sr.

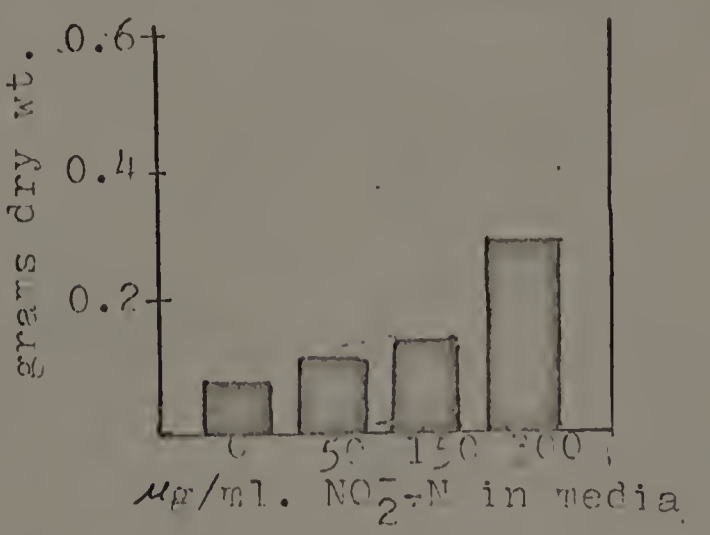


Fig. 15 Aspergillus sp.

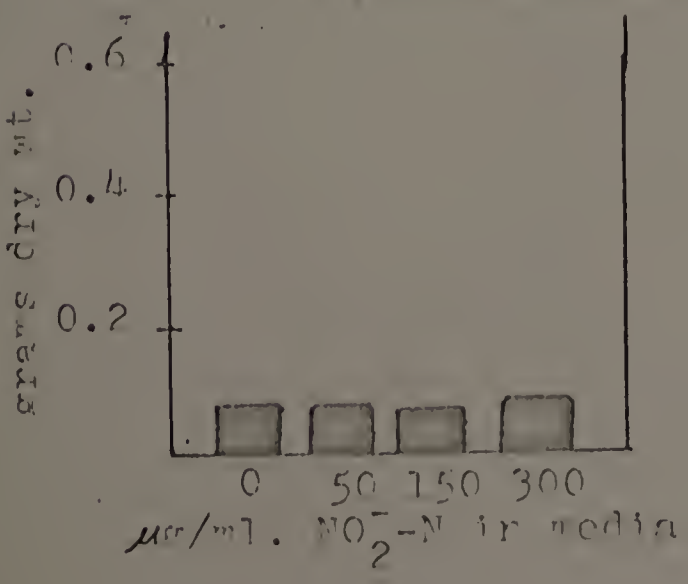


Fig. 16. Trichoderma sp.

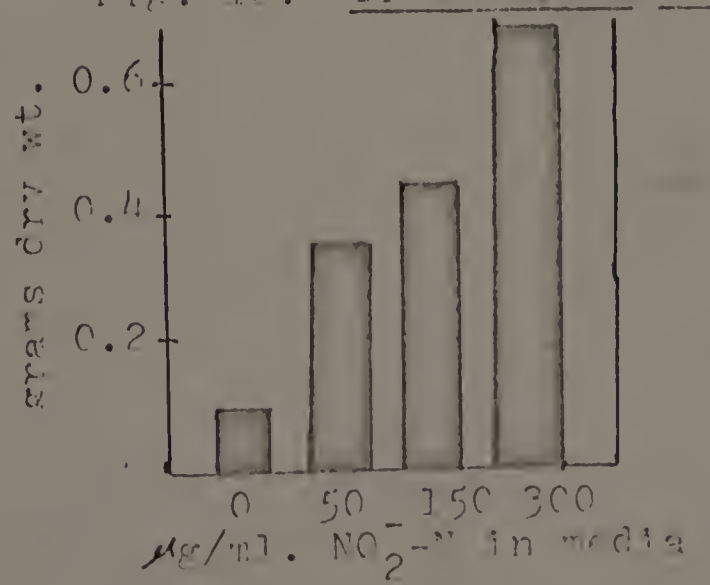


Fig. 17-18 Growth of Actinomycete Isolates on NO_2^- Containing Media

Fig. 17 Isolate P-1

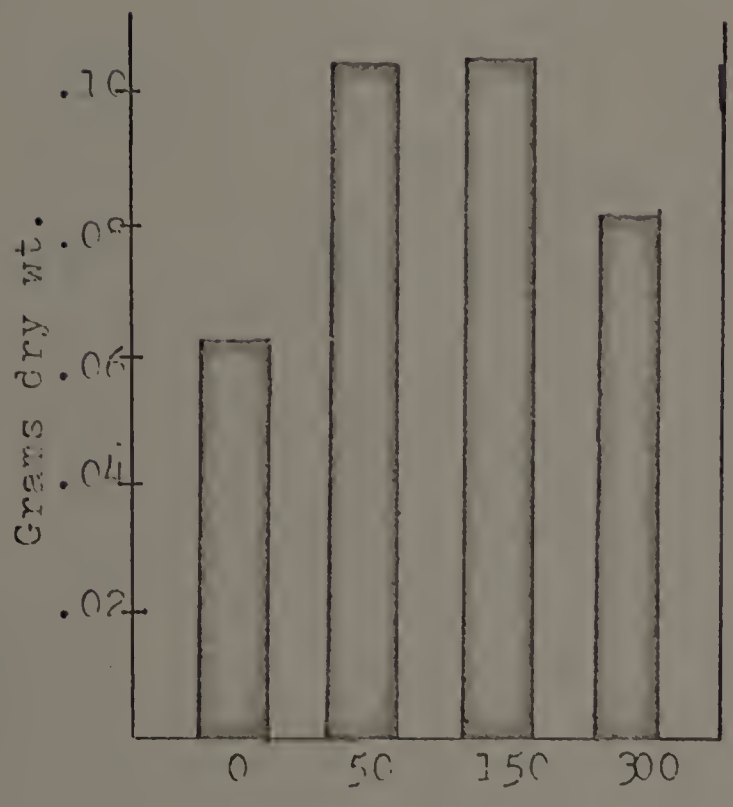


Fig. 18 Isolate B-1

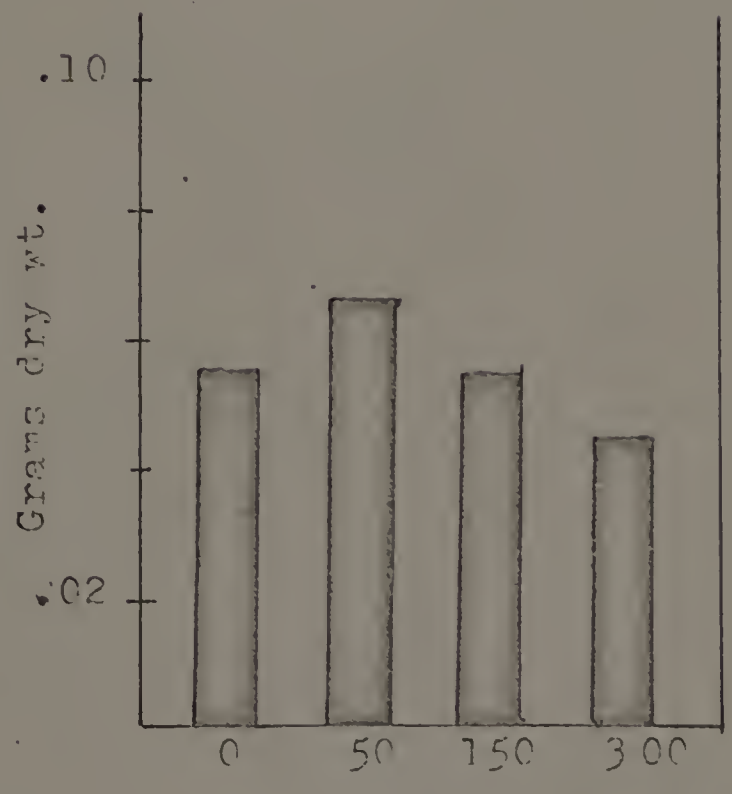


TABLE 3.--Growth of soil isolates and residual nitrite in nitrite containing medium

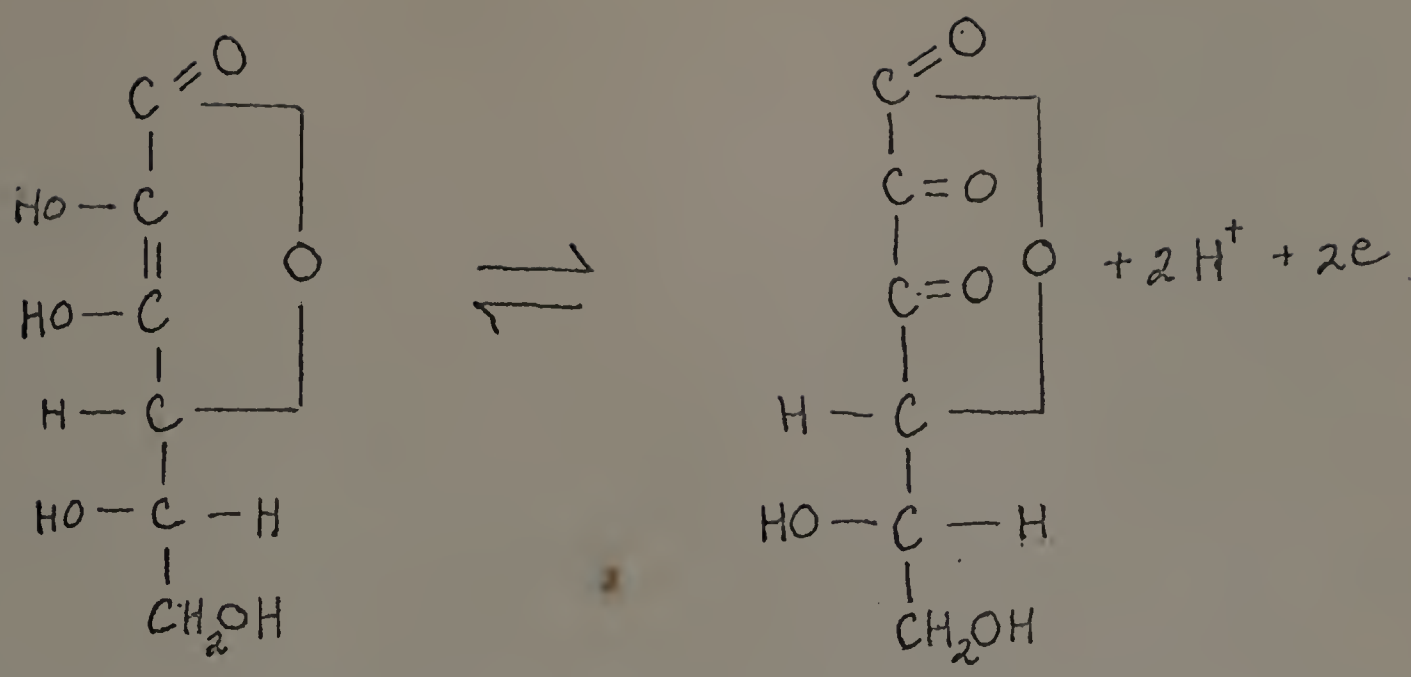
Isolate	$[NO_2^-$-N] in μg/ml in media	Dry wt. g	Residual NO_2^--N (μg/ml)
Actinomycete-P-1	0	.0601	0.01
	50	.1040	0.01
	150	.1051	0.88
	300	.0814	88.16
Brown Actinomycete	0	.0554	0.01
	50	.0654	5.17
	150	.0527	79.04
	300	.0457	119.50
Trichoderma sp.	0	.0992	0.00
	50	.1651	0.00
	150	.2312	0.49
	300	.2977	48.50
Penicillium sp.	0	.0898	0.00
	50	.1840	0.00
	150	.3005	0.65
	300	.2716	36.50
Aspergillus sp.	0	.0835	0.00
	50	.1170	0.00
	150	.1477	0.17
	300	.2744	21.90
Aspergillus sp.	0	.0893	0.00
	50	.0881	19.15
	150	.0742	80.00
	300	.0849	152.30
Trichoderma sp.	0	.0928	0.00
	50	.3472	6.69
	150	.4361	30.40
	300	.6829	92.10

Physiological Studies

Reducing agents and nitrite utilization

A hypothesis had been made that nitrite inhibition is due to its oxidizing effect on cytochrome c (31). If this were so, the addition of a reducing agent might be expected to decrease the toxicity of nitrite and allow the microorganism to grow. To test this idea, two bacterial isolates were chosen for detailed studies. One isolate, an Arthrobacter sp., was chosen for its ability to grow on nitrite at 300 μg/ml NO_2^--N while the other isolate, a Pseudomonas sp., was inhibited by this concentration of nitrite. If the addition of a reducing agent did in fact allow the Pseudomonas sp. to grow it would indicate that the toxic effect of nitrite on the cytochromes was indeed due to its oxidizing characteristics. Alternately, the fact that the Arthrobacter sp. was not inhibited by nitrite could be explained by the presence of an alternate pathway for nitrite utilization (17).

The reducing agent chosen for this study was ascorbic acid as it is known to influence cellular respiration (11) and it also reduces cytochrome c (19). Ascorbic acid is easily oxidized and reduced producing the following chemical changes:



There is considerable speculation that the vitamin functions as an electron carrier in cellular oxidation and reduction (5). Another reason for using ascorbate is the fact that it is not introducing any other form of nitrogen into the system as would occur with the addition of cysteine, an amino acid known for its reducing capacity.

Preliminary studies were carried out with a range of ascorbic acid concentrations to see if the nitrite inhibited microorganism would then grow. The two isolates were incubated for seven days in modified Morris medium (see Appendix) containing 300 μg/ml NO_2^--N and ascorbic acid ranging from 0-.06M. It is known that ascorbate can nonenzymatically reduce nitrite only if it is in a 5:1 ascorbate : nitrite ratio or higher (12). Taking this fact into consideration the ratio was kept well below this as it ranged from 1:2 to 3:1 ascorbate to nitrite. Controls containing no nitrogen were maintained with and without the addition of ascorbate.

Figures 19 and 20 present the results of this

Fig. 19 Growth of a Pseudomonas sp. with Nitrite and 0-.06 M Ascorbate

($300\ \mu g/ml.\ NO_2^-N = .02\ M\ NO_2^-$)

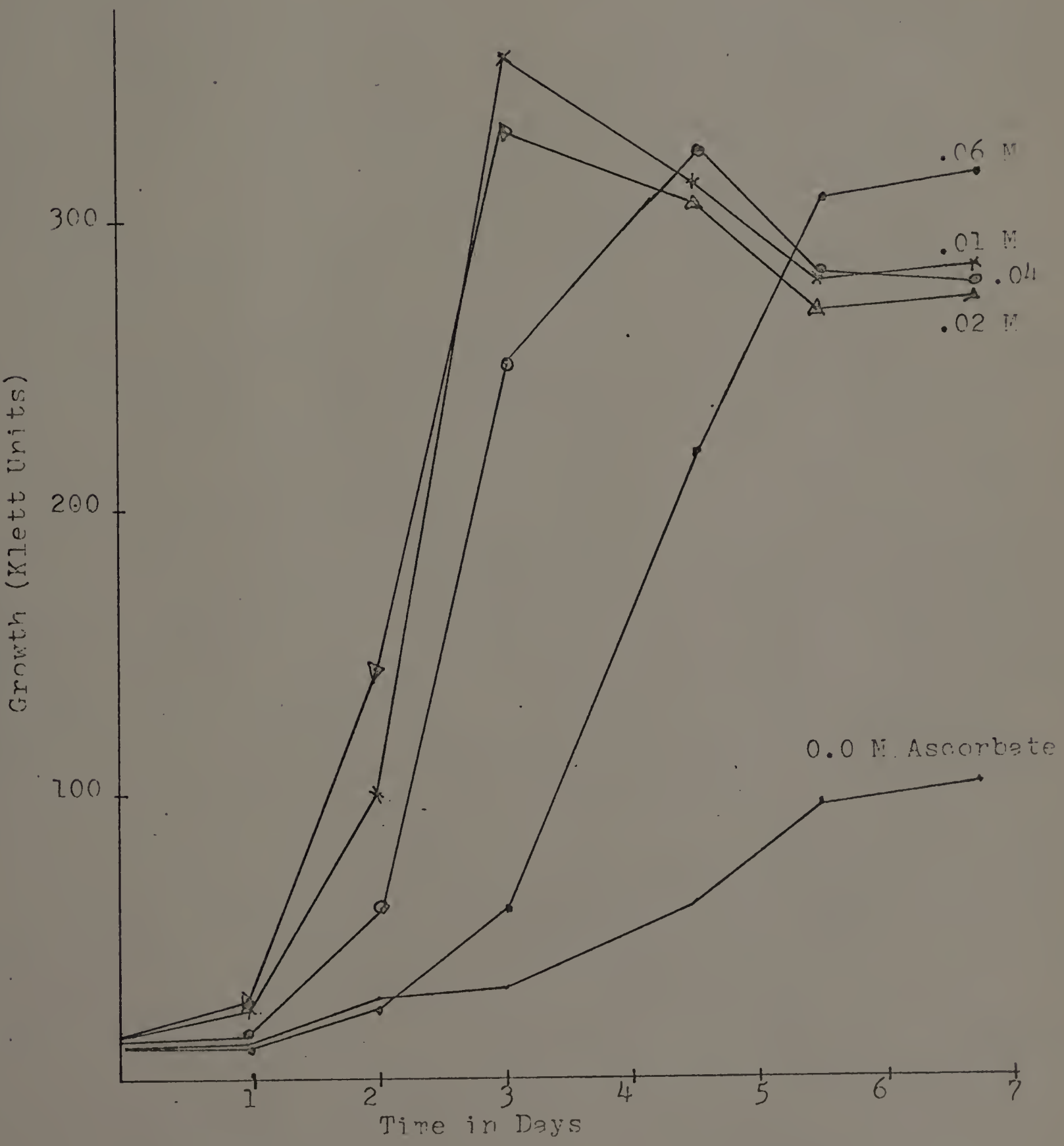
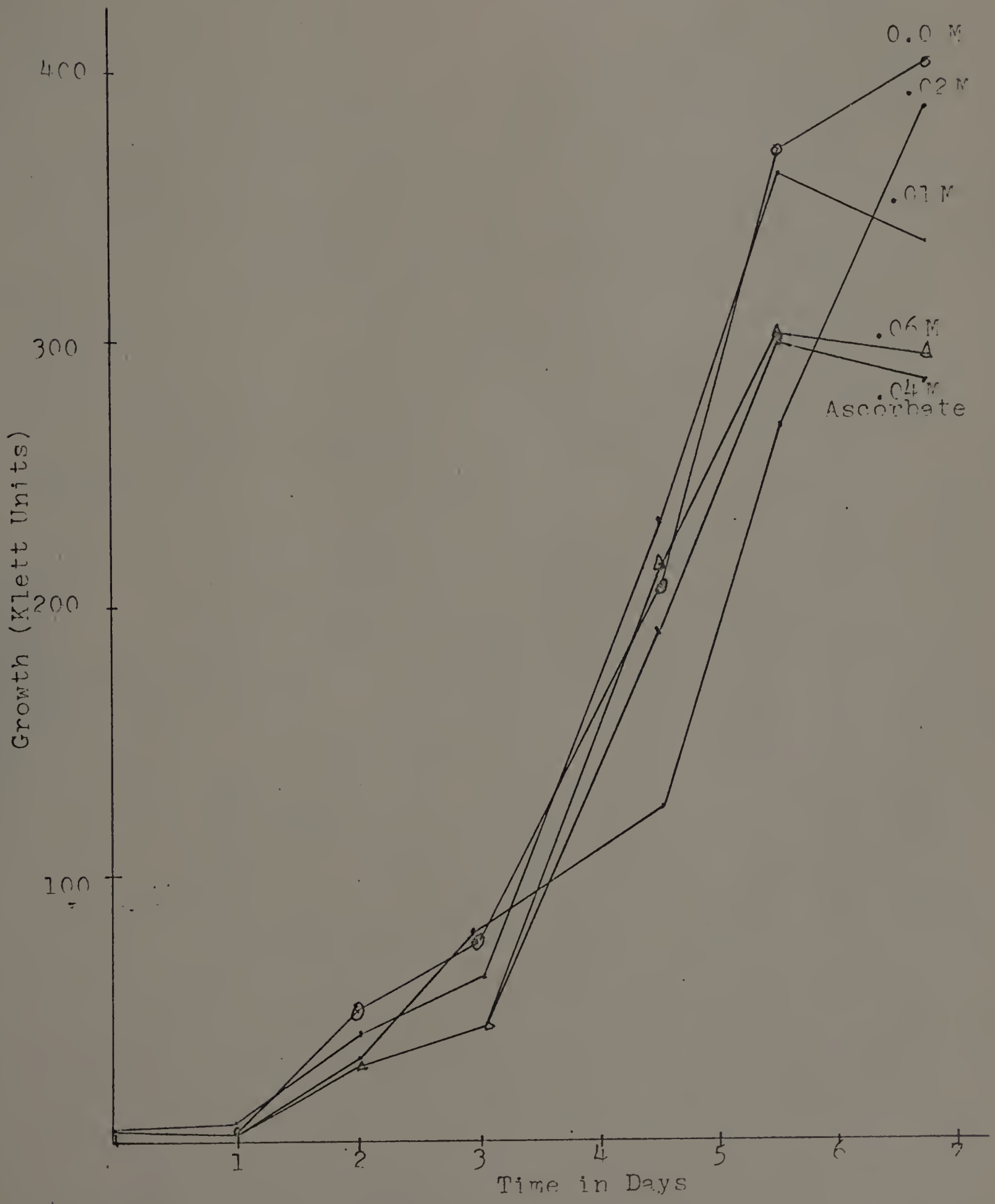


Fig. 20 Growth of an Arthrobacter sp. with Nitrite and 0-.06 M Ascorbate

(300 μg/ml. NO_2^--N = .02 M NO_2^-)



experiment. It can be seen in Figure 19 that ascorbic acid does in fact allow the Pseudomonad to again utilize nitrite. A molarity of .01-.02 of ascorbic acid allows for maximum growth with .04 and .06 M ascorbic acid temporarily inhibiting growth of this microorganism and causing a longer lag period before logarithmic growth begins. Figure 20 shows that ascorbic acid does not enhance the growth of the Arthrobacter sp., the bacterium that normally utilizes nitrite at this concentration. In fact it tends to inhibit the growth of this microorganism. The greatest growth was obtained with the cultures containing no ascorbate.

To be certain that the ascorbate did not change the nitrite nonenzymatically, uninoculated controls containing nitrite with and without ascorbate were maintained under aeration for the same incubation period previously employed and then analyzed for nitrite. Nitrite-nitrogen was found in the same amount in both controls.

The effects of ascorbic acid on growth

The two isolates were also grown on modified Norris medium containing no nitrogen source, 300 µg/ml NO_2^--N or NH_4-N with and without the addition of ascorbic acid. This was done to test for the action of ascorbic acid itself on microbial growth and also to see if it had any effect when present with other inorganic nitrogen sources. With the Pseudomonad, ascorbic acid did enhance growth when no nitrogen

source was available but did not have a significant effect when added to the ammonium or nitrate containing cultures. With the Arthrobacter sp. the addition of ascorbic acid did not enhance the growth of any of the test cultures. Figure 21 shows the growth of these two isolates with no nitrogen source and with or without .01 M ascorbic acid; it confirms that ascorbic acid affects only one of the two isolates, the Pseudomonas sp.

Ascorbic acid and nitrite utilization by other bacterial isolates

The eight remaining bacterial isolates were incubated under the same conditions with .01 M ascorbic acid and 300 µg/ml nitrite-nitrogen as the sole nitrogen source. Table 4 shows that in every case where nitrite inhibits the growth of the isolate, the addition of ascorbic acid decreased these effects and allowed the organism to grow.

Cytochrome Studies

Cytochrome c

Having found that ascorbic acid does reverse the toxic effects of nitrite, the physiology of the phenomenon was examined in closer detail. Cytochrome extracts were prepared of the two test isolates and spectral studies were made to see if nitrite did in fact affect the cytochromes as was stated in the hypothesis on the mechanism of nitrite inhibition. To have a standard to relate the results of the

Fig. 21 The Effect of Ascorbate on Growth without Added Nitrogen

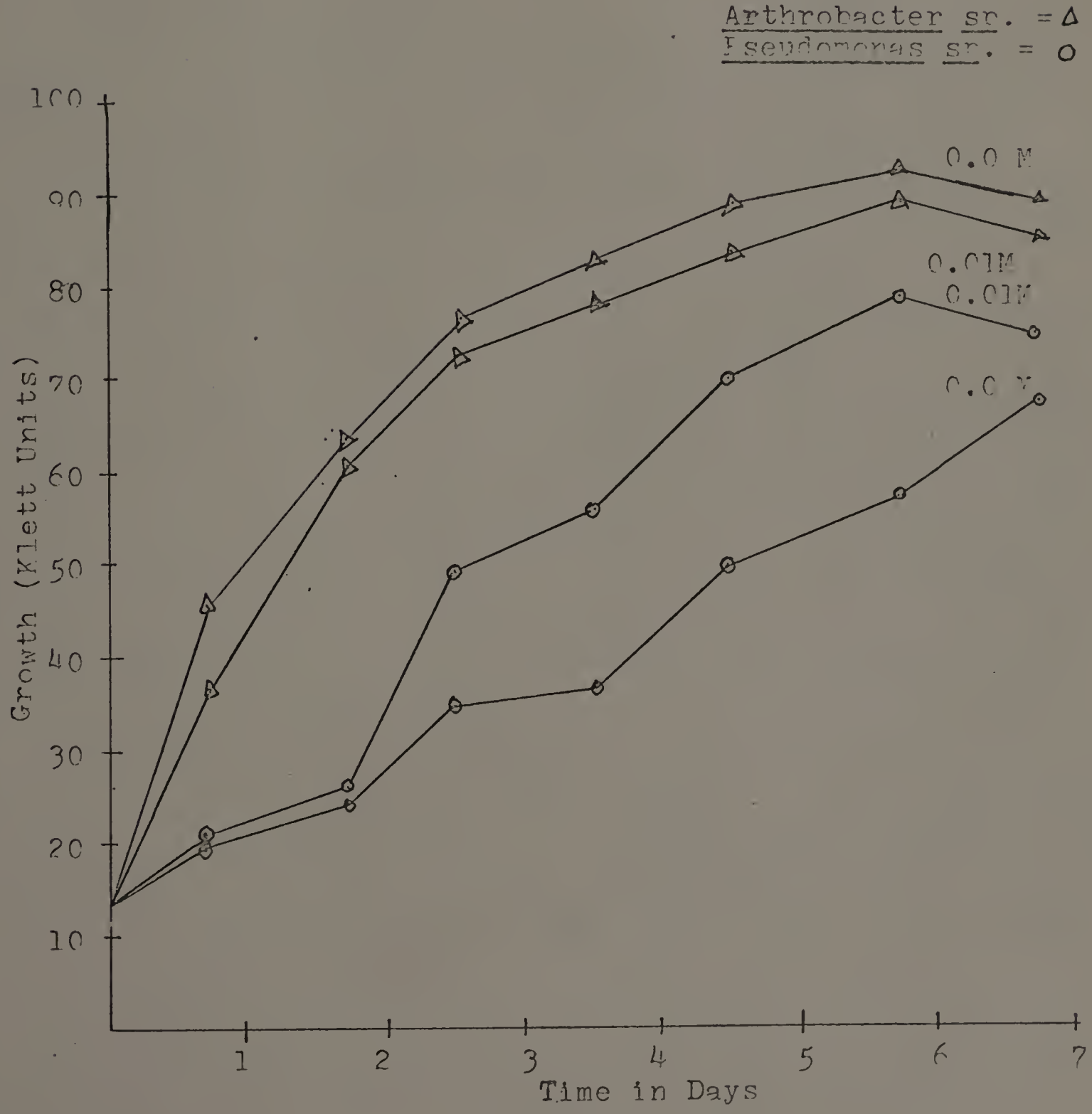


TABLE 4.--The effect of ascorbic acid on nitrite grown bacterial isolates
Growth in Klett Units

Isolate:	1		2		3		4		5		6		7		8		9		10	
Treatment:	N	NC	N	NC	N	NC	N	NC	N	NC	N	NC	N	NC	N	NC	N	NC	N	NC
hrs. incubation at 26°C																				
24	52	17	40	25	6	21	34	18	7	19	16	25	19	64	51	18	5	4	13	6
48	112	59	48	42	5	48	139	79	5	38	14	75	33	150	100	80	6	3	26	8
94	251	445	385	290	6	261	345	295	5	110	42	240	187	142	400	191	39	30	60	33
114	244	485	308	315	16	220	284	273	7	380	62	298	175	143	355	395	160	125	234	115
144	212	435	292	310	55	192	273	269	17	286	97	310	149	140	360	375	305	300	250	315

N = 300 μg/ml NO_2^--N

NC = .01 M ascorbic acid
300 μg/ml NO_2^--N

bacterial cytochromes to, the spectra of oxidized and reduced mammalian cytochrome c were recorded and these were then compared to the oxidizing effects of nitrite and the reducing ability of ascorbic acid. Figure 22 shows the oxidized and reduced peaks of cytochrome c which occur at 550, 522, and 415 mμ. Figure 23 shows the spectra resulting from treatment first with nitrite and then ascorbic acid. It can be seen that nitrite does oxidize cytochrome c: its spectra are similar to the oxidized peaks produced with $K_3Fe(CN)_6$ used in the standard method of oxidation. The addition of ascorbic acid reduces cytochrome c and the spectra produced are similar to the reduced spectra resulting from treatment with $Na_2S_2O_4$. Figure 24 shows the spectrum of cytochrome c when it has been treated only with ascorbic acid. Since it produces the same reduced spectrum there does not appear to be an interaction of nitrite with ascorbate which might have been expected to form a different spectrum.

Cytochrome extracts

Cells of the two isolates were harvested from modified Morris medium containing 300 μg/ml NO_3^--N. It was found that by the third day of incubation the Arthrobacter sp. produced a pink coloration in the medium. Upon making a spectral analysis of this solution, characteristic cytochrome peaks were shown. The cell-free extract of cytochromes also showed peaks at the same wavelengths but they were much less clearly

Fig. 22 Spectrum of
Mammalian Cytochrome c
reduced and oxidized

pH 9.4
oxidized 0.01 M $K_3Fe(CN)_6$ ——
reduced 1.1-1.0 -3. $Na_2S_2O_4$ - - - -

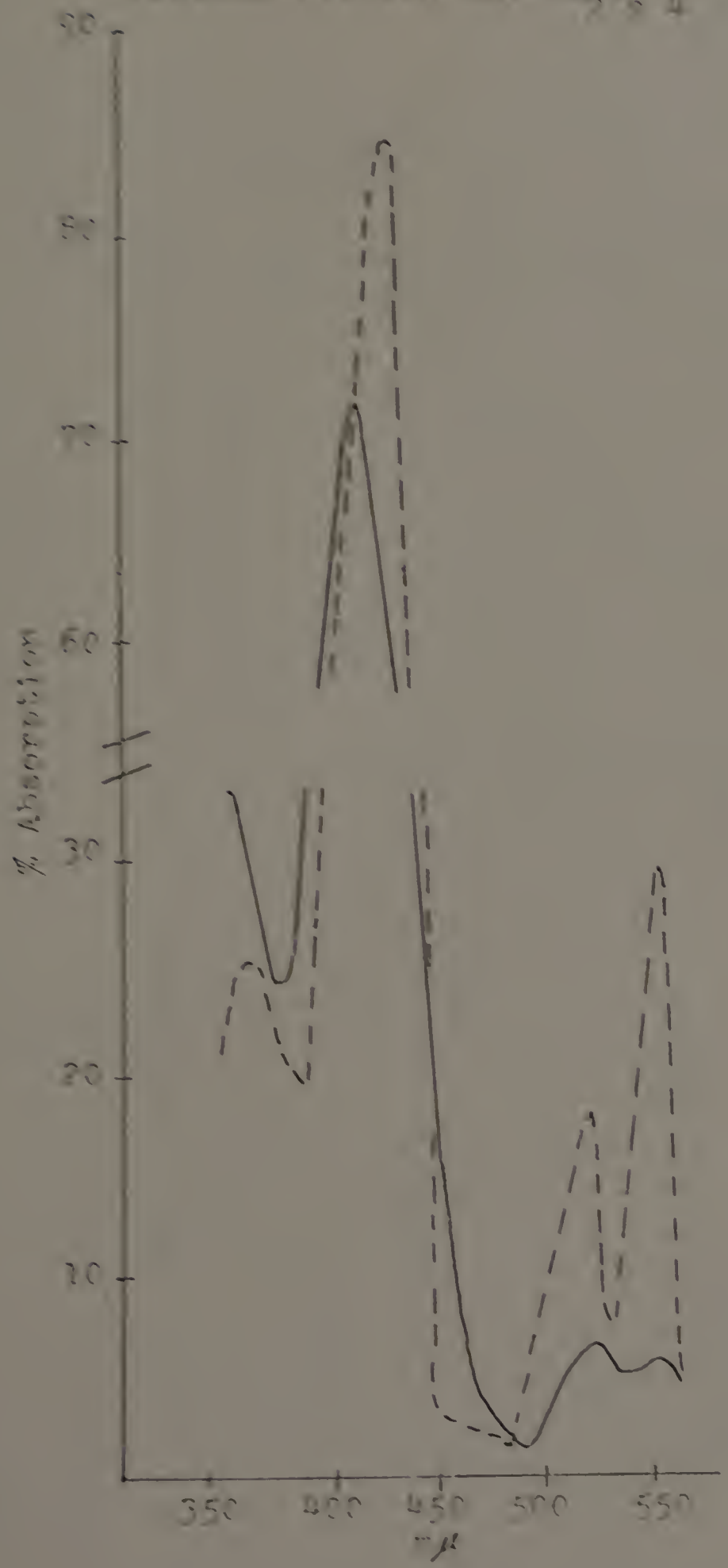


Fig. 23 Spectrum of mammalian cytochrome c reduced and oxidized

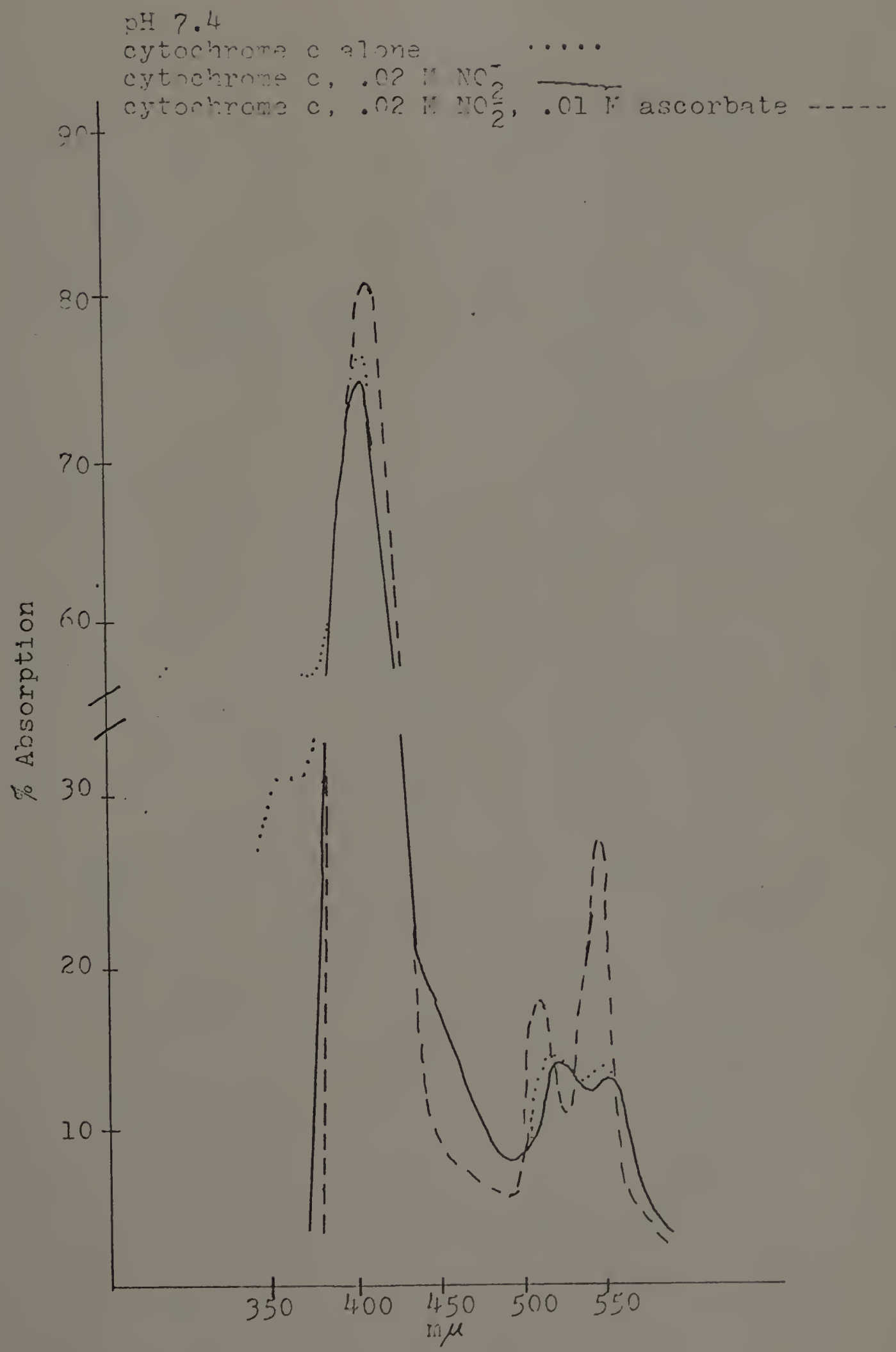
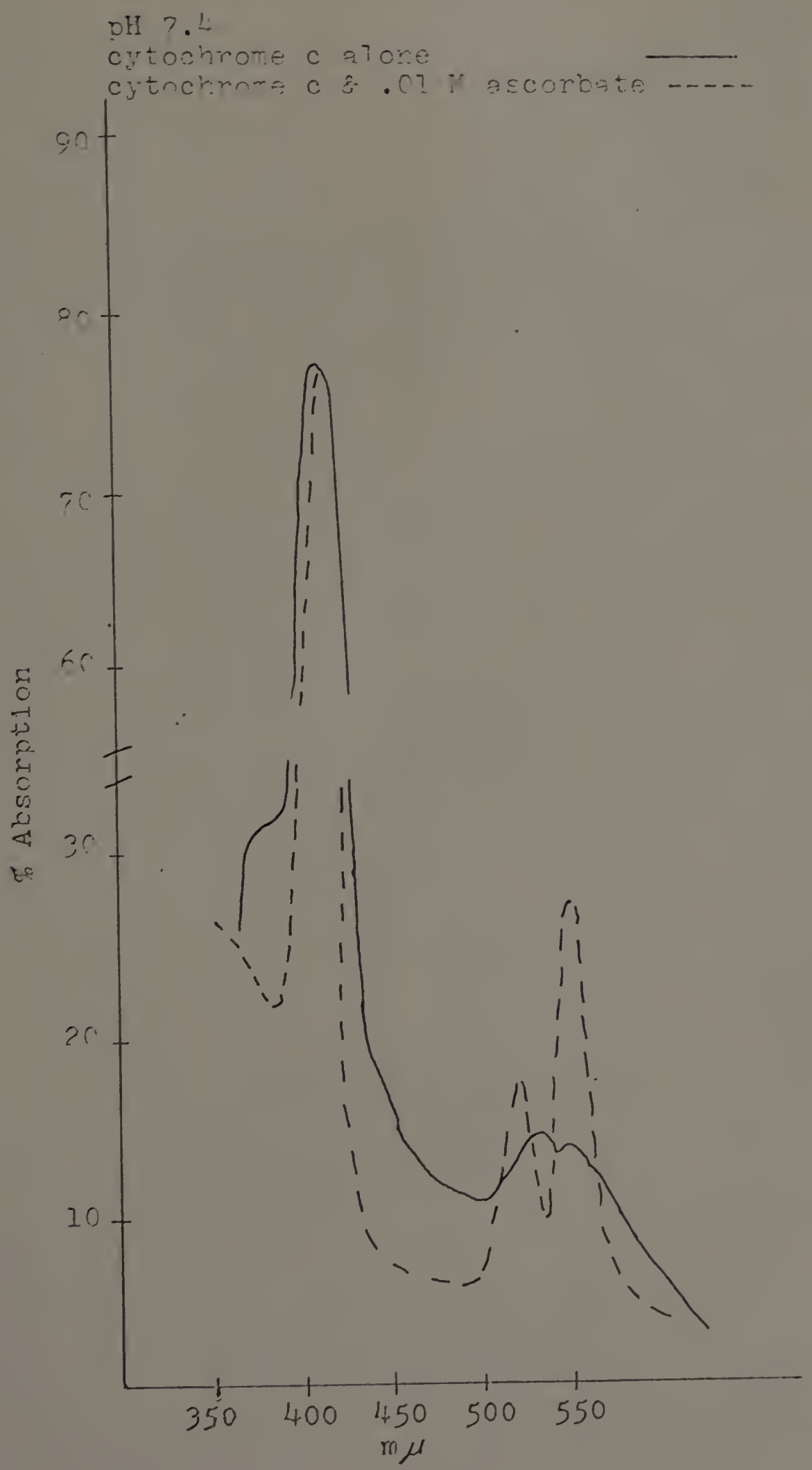


Fig. 24 Spectrum of
mammalian cytochrome c
with ascorbate added



defined. The α, β and γ peaks were at 565, 535, and 405 mμ respectively. The results of this spectral analysis could not be compared to cytochrome c as the nitrite and ascorbate did not appear to oxidize and reduce this bacterial cytochrome as it did with the mammalian cytochrome. This fact is illustrated in Figure 25 where the cytochrome is known to be oxidized and reduced and produces spectra which are not similar to those found when treated with ascorbic acid and nitrite. The reduced form showed a fourth peak occurring at 392 mμ (Fig. 25). This, and a peak also occurring at 360 mμ were the important characteristics which differentiated the oxidized from the reduced form. In comparison to these results, treatment of the cytochrome with nitrite followed by ascorbate did not produce this fourth peak and no indication of a peak at 360 mμ (Fig. 26). In fact the ascorbate addition erased a small peak which had been formed at 360 mμ by the addition of nitrite. There appears to be an interaction of nitrite and ascorbate as treatment of the cytochrome with ascorbate alone does not produce a spectrum similar to that formed when the cytochrome has been previously treated with nitrite (Fig. 27).

The results of the spectral analysis of the Pseudomonas cytochrome extract reflected the fact that nitrite can be toxic to its growth and these effects can be reduced by ascorbic acid. This fact creates a similarity with cytochrome c along with the presence of a peak at 414 mμ and shoulders at 550 and 523 mμ which are very close to those of cytochrome c

Fig. 25 Srectrum of
Arthrobacter cytochrome
reduced and oxidized

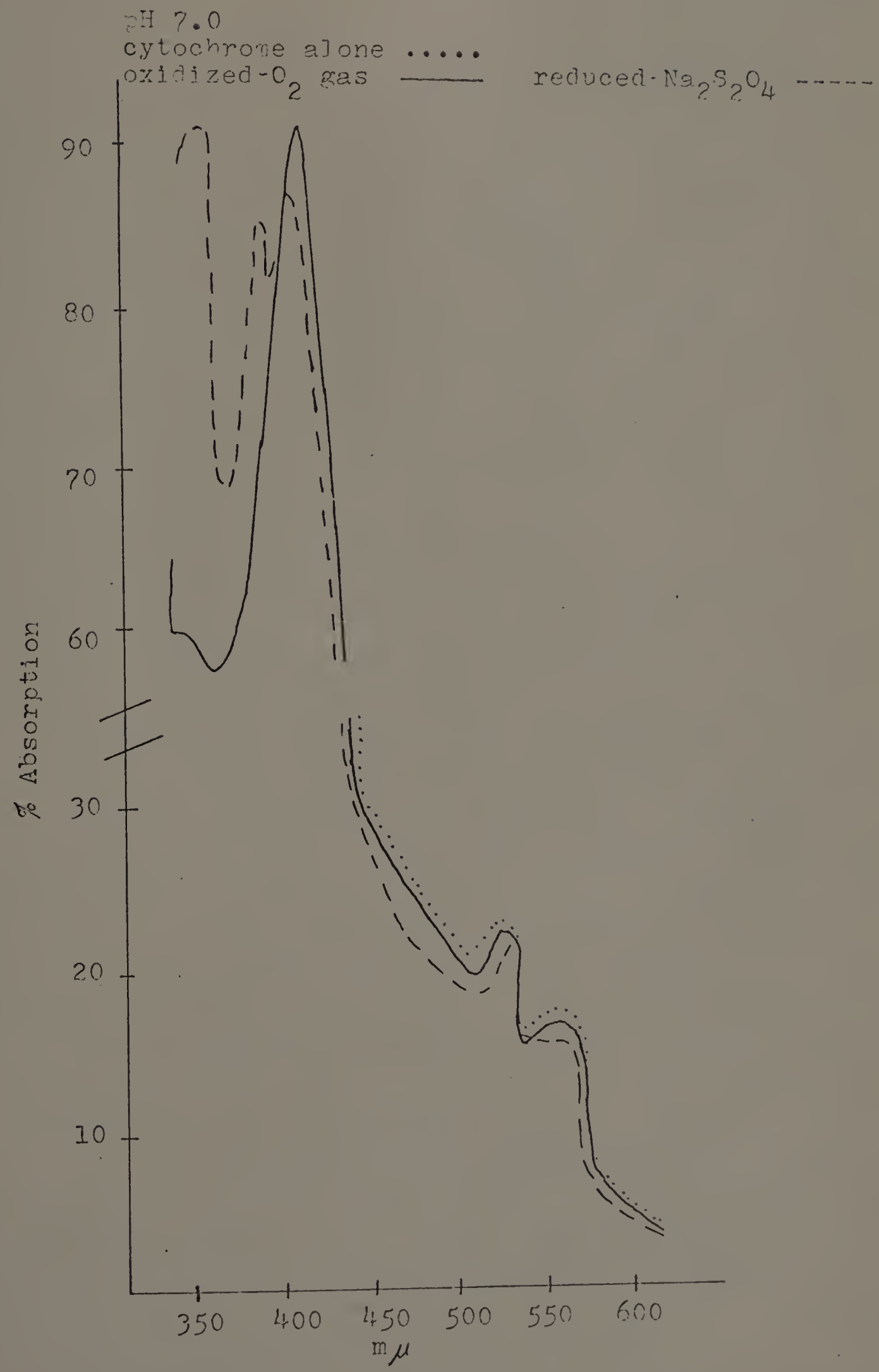


Fig. 26 Spectrum of
Arthrobacter cytochrome
reduced and oxidized

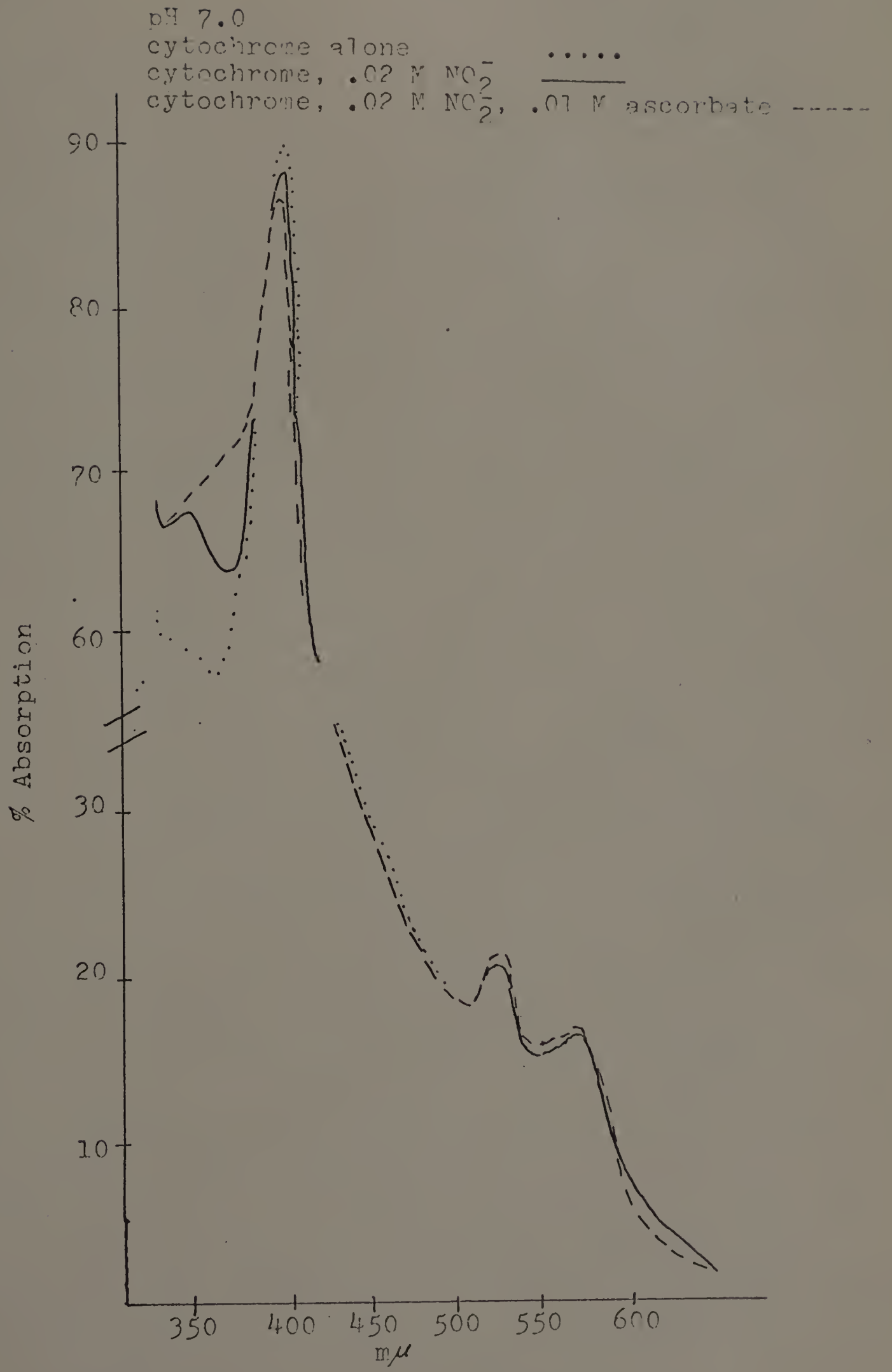
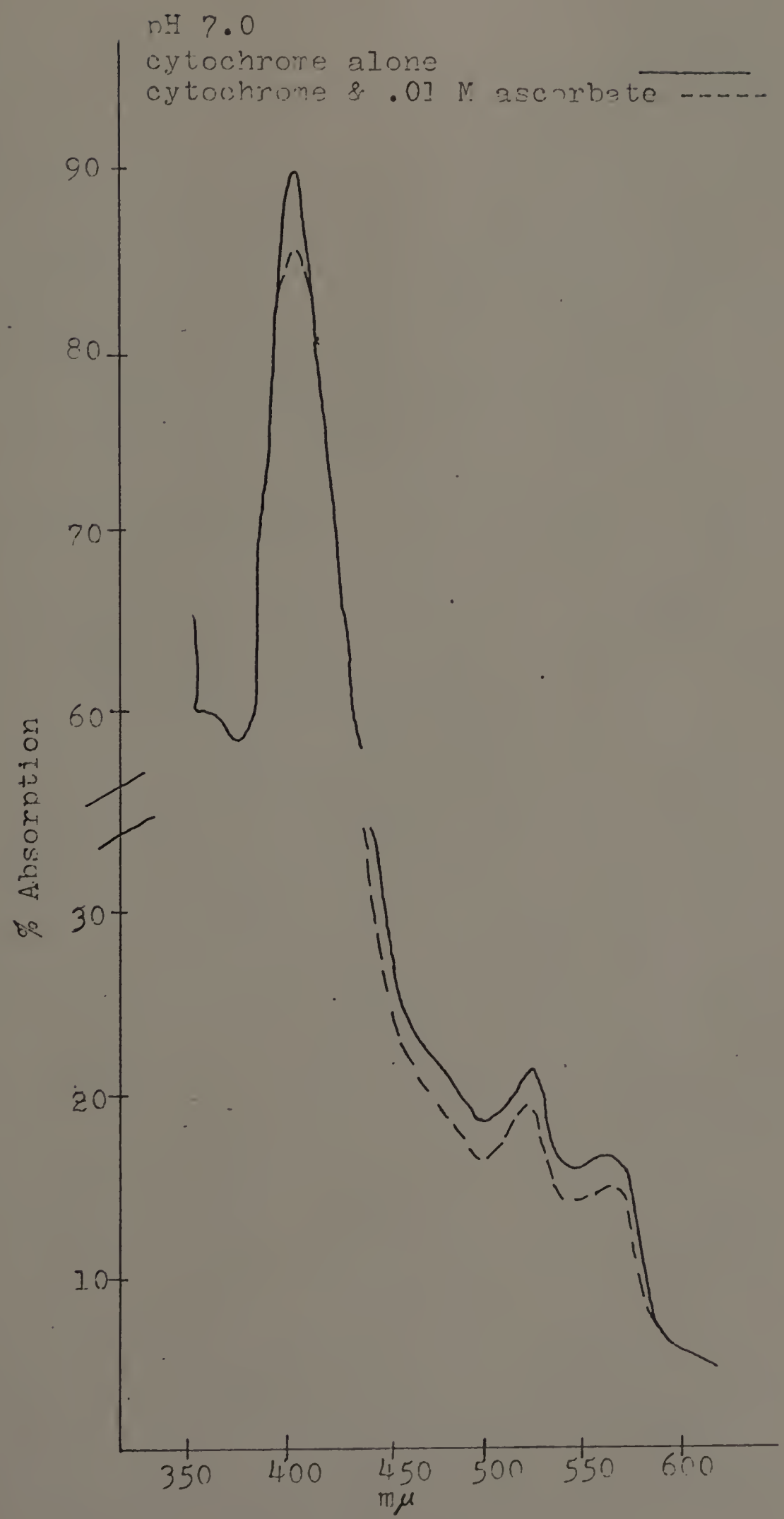


Fig. 27 Spectrum of
Arthrobacter cytochrome
with ascorbate added



(Fig. 28). The addition of nitrite also produced a peak at 360 mμ as had occurred with the Arthrobacter sp. but this is the only similarity between the cytochromes of these two bacterial isolates. This is substantiated by the following considerations: (1) The subsequent addition of ascorbate to this preparation instead of erasing the peak formed by nitrite, decreases the percentage of absorption of this peak and brings it nearer to the shoulder at 360 mμ produced by the cytochrome alone. This would indicate that ascorbate reverses the toxic effects of nitrite on the cytochrome whereas in the Arthrobacter sp. it did not tend to return the spectra to their original status. (2) The spectra resulting from the addition of ascorbate to the nitrite treated cytochrome resembles the reduced form of the cytochrome produced by the addition of the $Na_2S_2O_4$ (Fig. 29). Thus contrary to the action of ascorbate on the Arthrobacter cytochrome, it does appear to reduce the cytochrome of this Pseudomonas sp. (3) When the cytochrome is treated with ascorbate alone, a similar reduced spectrum is produced whereas it does not seem to affect the cytochrome of the Arthrobacter in any way (Figs. 27 and 30).

Thus it appears that the Pseudomonas cytochrome is similar to mammalian cytochrome c in that the α, β, and γ peaks are at similar wavelengths (Figs. 22 and 29) and ascorbate reduces both cytochromes (Figs. 23 and 28) as is shown by comparison with their respective reduced cytochrome standards (Figs. 22 and 29).

Fig. 2° Spectrum of *Pseudomonas* cytochrome oxidized and reduced

pH 7.0
cytochrome alone .....
cytochrome, .02 M NO_2^- _____
cytochrome, .02 M NO_2^- .01 M ascorbate -----

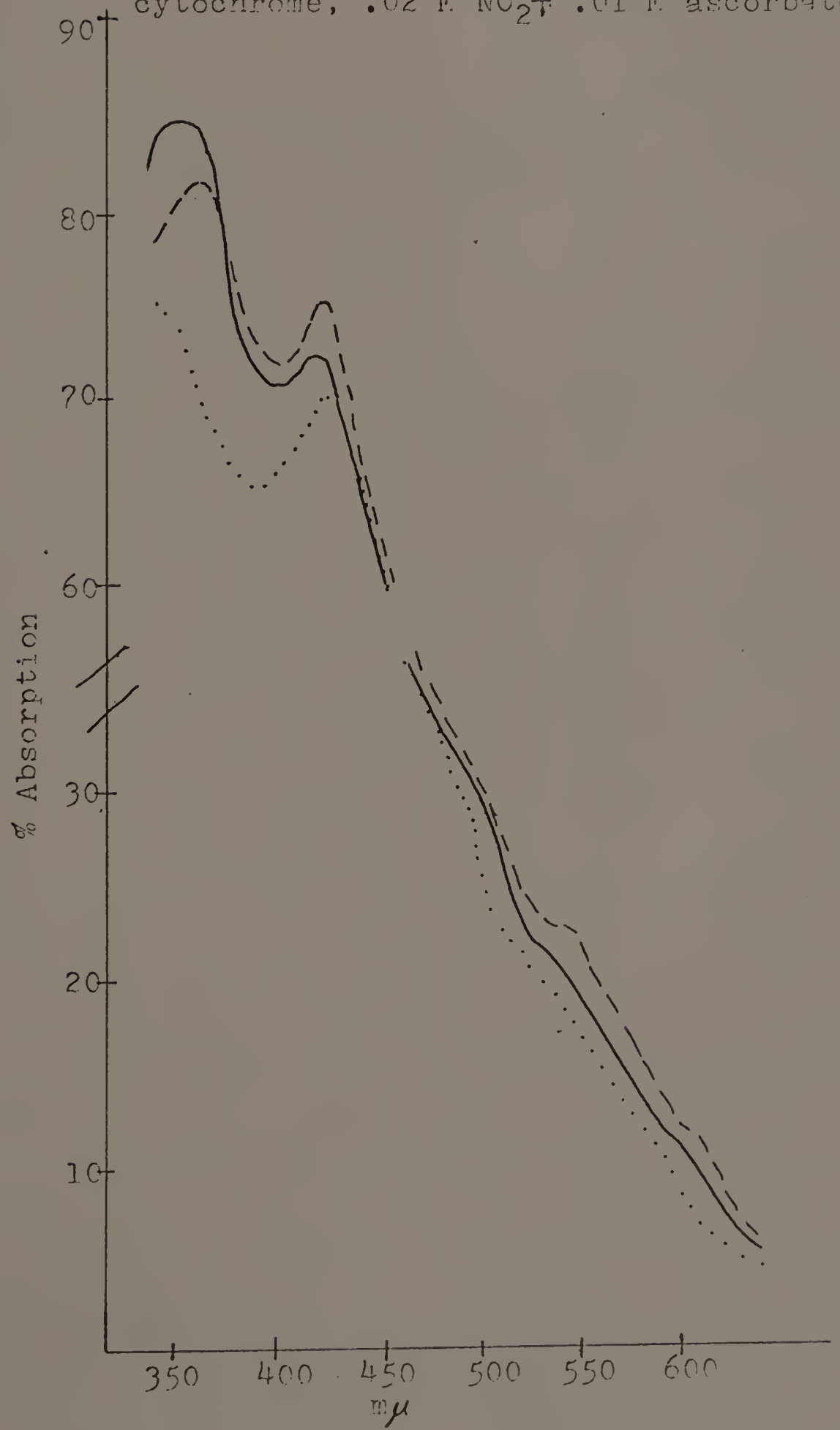


Fig. 29 Spectrum of Pseudomonas cytochrome oxidized and reduced

pH 7.0
cytochrome alone .....
oxidized - O_2 gas ——— reduced - $Na_2S_2O_4$ ----

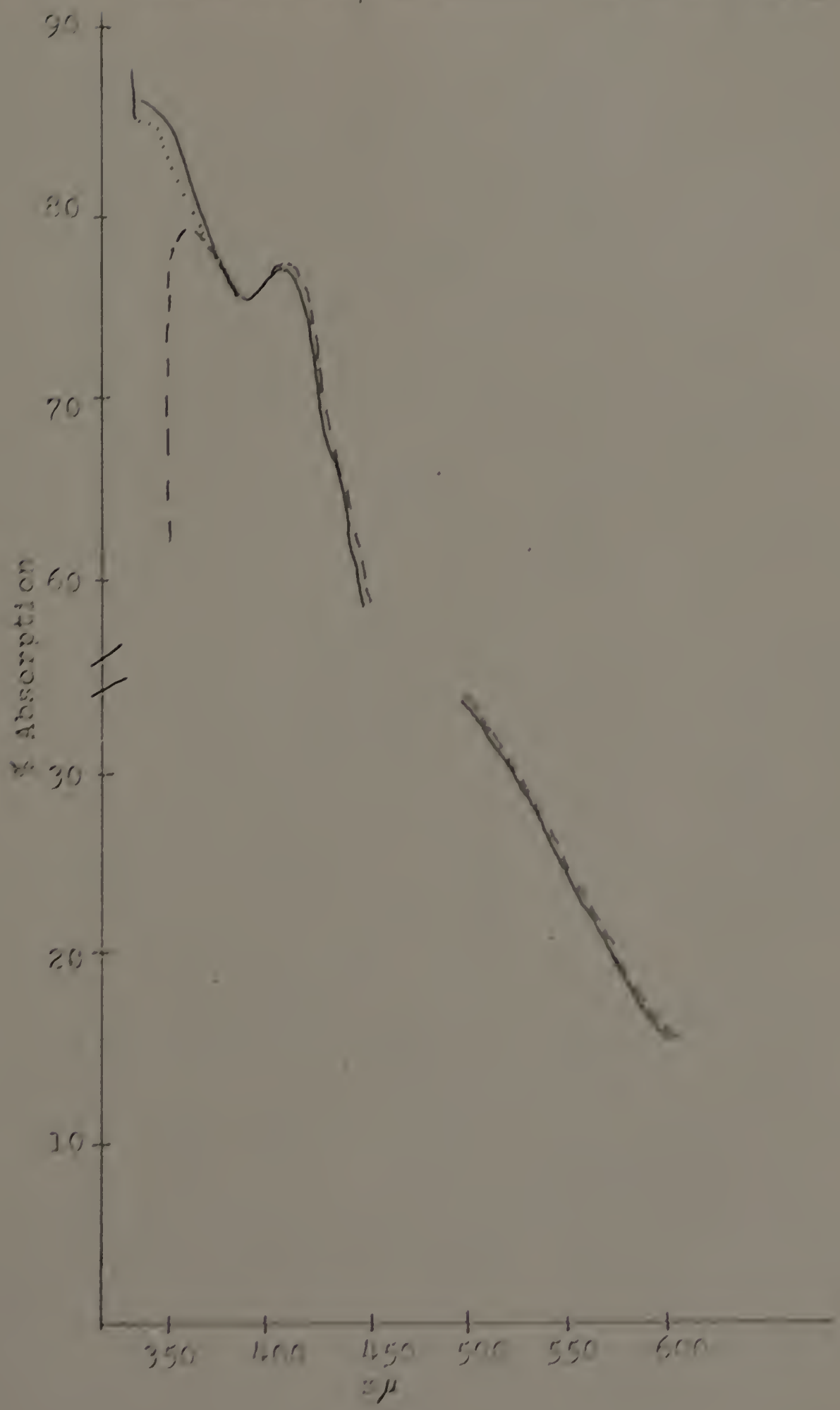
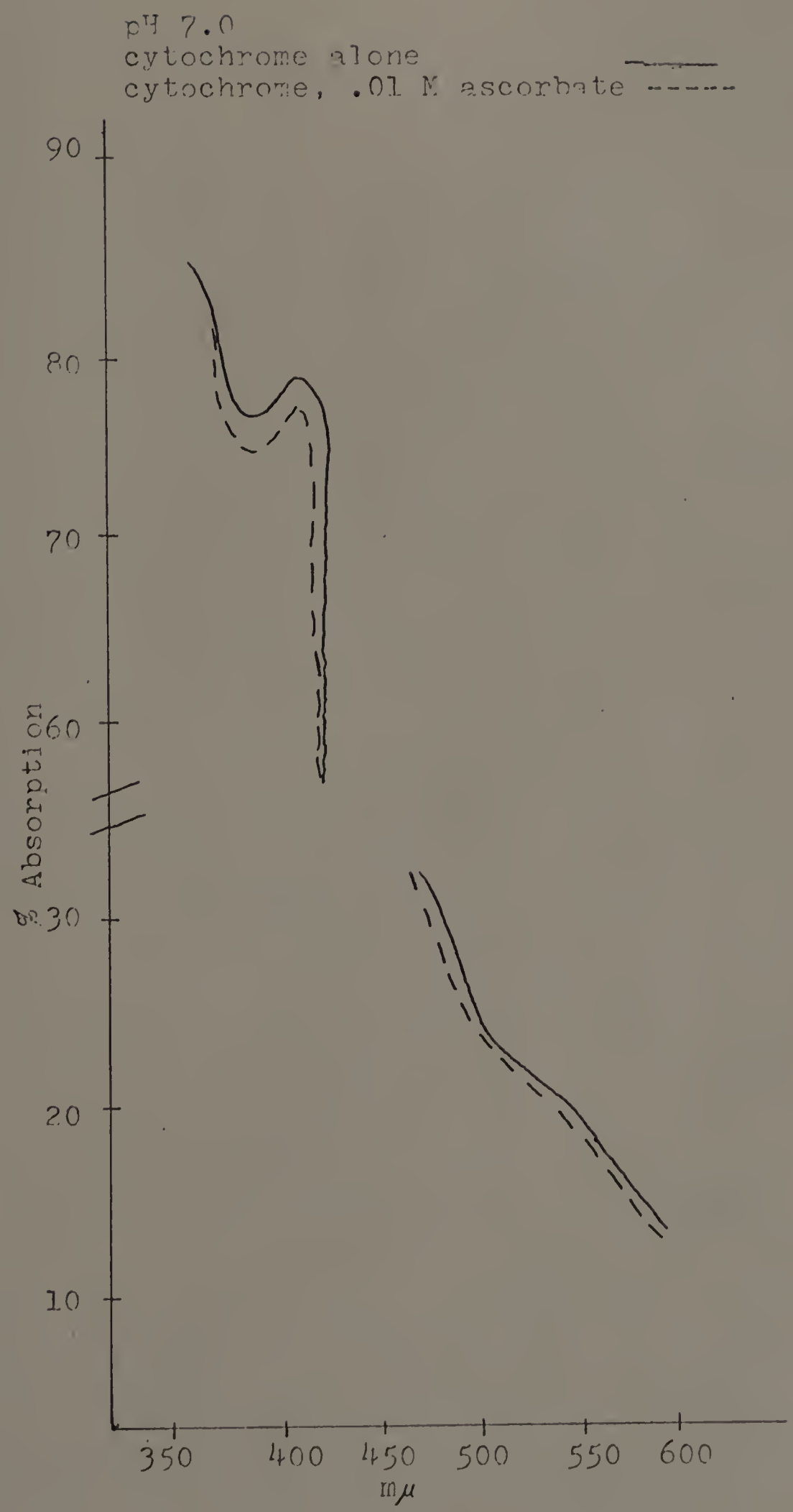


Fig. 30 Spectrum of
Pseudomonas cytochrome
with ascorbate added



The cytochrome from the Arthrobacter sp. does not show similarities with mammalian cytochrome c as the peaks occur at different wavelengths and the ascorbate does not reduce the cytochrome as is shown by comparison of Figures 25 and 26. This may then indicate the presence of an alternate pathway for nitrite reduction or the fact that this different type of cytochrome can adapt to the use of nitrite as a nitrogen source.

These results do support the hypothesis that nitrite exercises a strong oxidative effect on cytochrome c. The isolate that cannot normally utilize nitrite at the concentration used, will do so with the addition of ascorbate. This compound is known to reduce cytochrome c which is shown in the spectral analyses. The Arthrobacter spectra (which do not resemble cytochrome c) show a peak at 360 mμ with the addition of nitrite and this is the same peak produced by the ascorbate on the Pseudomonas sp. This would then indicate a relationship between this peak formation and nitrite utilization.

DISCUSSION

The results of the plate counts made on a medium containing a defined carbon and nitrite source indicate the presence of large soil populations that will utilize nitrite (Table 1). This demonstrates that nitrite is not transformed solely by autotrophs which oxidize it to nitrate. There are several reports that nitrite is reduced by soil heterotrophs to the amine level (4,18,20,28). This includes Arthrobacter, a genus present in large populations in the soil (18). Also, since heterotrophs are present in much greater populations than the autotrophs their influence on the nitrogen economy may be substantial (2). According to the counts reported here, bacterial and fungal populations showed only an approximate tenfold decrease in numbers and the actinomycetes an even smaller decrease when nitrite was the sole source of nitrogen. Nitrite utilization was not shown to be attributed to a certain class of microorganisms. There appears to be a great variety of microbial types involved since bacteria, fungi, and actinomycetes were all found to utilize nitrite. The actinomycete population was the least inhibited according to the results of the soil dilution platings. There might be a correlation between this and the known hardiness of actinomycetes to adverse environmental conditions (2).

The variable ecological factors in the soil are important to consider when discussing the effects of nitrite

utilizers on the transformations of nitrogen. Various conditions could create situations that give certain microorganisms a competitive advantage in the presence of nitrite (18). The results included in this work indicate that soil exposed to high temperatures or dehydration alter the populations of nitrite utilizers. Under these conditions, nitrite utilizing actinomycetes were still found in large numbers while very few bacteria and fungi were observed.

Another factor to take into consideration is the low level of nitrite in the soil. If it is toxic to microorganisms, what keeps it at such low levels? It appears that it must be immediately used or complexed in some manner so that it is not allowed to build up as an end product of nitrate reduction or ammonium oxidation. The results show the presence of large populations of nitrite utilizers which therefore help to explain the maintenance of this low level of nitrite in the soil.

Growth studies of these nitrite utilizing isolates indicate an ability to tolerate various concentrations of nitrite (Figs. 1-18). The isolates found to tolerate the highest nitrite concentrations (Trichoderma and a pink pigmented actinomycete) were also found to be the ones prevalent in soil which had been dried and exposed to high temperatures. Therefore, there might be a correlation between hardiness of the organism and the ability to withstand and utilize high nitrite concentrations. In the complex soil environment,

microecological niches may contain high nitrite concentrations where these microorganisms would most likely have a competitive advantage.

The pink coloration of the actinomycete appearing at different rates in the two media employed might be related to the availability of nutrients. The pink pigment appears first in the nitrite containing defined medium even though the actinomycete has a lower growth rate on this medium. This is most likely explained by the fact that it is a minimal medium, requiring the organism to synthesize many more enzymes for growth to occur whereas the nutrient agar is a richer medium providing many factors which allow for increased growth. When these available compounds in the nutrient agar were depleted the organism also produced its pink pigment on this medium.

The lag period found in nitrite utilization in both the plate counts and in the pure culture studies might in part be explained by the fact that the microorganism has to synthesize many more enzymes to reduce the inorganic nitrogen to ammonium and to incorporate it into organic nitrogen compounds. This cannot be the complete answer though, since nitrate, a more highly oxidized form of nitrogen does not produce such a long lag period in the growth of the microorganism. Nitrite itself must have some effect on organisms which makes it a toxic nitrogenous source. Although the mechanism by which it may be toxic is not known, it has been

postulated that in the case of microorganisms it may be due to the damaging effects it has on the cytochrome system (31). Nitrite, with its high oxidative capacity may completely and irreversibly oxidize the cytochromes. That the addition of ascorbic acid to the test cultures did allow growth of the nitrite inhibited isolate (Pseudomonas sp.) pointed out the reversibility of nitrite toxicity by the re-reduction of the cytochrome. The slight lag period caused by ascorbate with the Arthrobacter sp., the nitrite utilizing isolate, indicated that it may interfere with its alternate pathway for nitrite reduction. The fact that ascorbic acid in all cases enhanced growth of the nitrite inhibited bacterial isolates indicates a correlation between ascorbic acid and nitrite utilization. A certain molarity of ascorbic acid was found to allow for the best growth. Concentrations of ascorbate could be too high and growth again become inhibited which might be explained by the irreversible reduction of cytochrome c (15,19). Previous work on the reversibility of nitrite toxicity in anaerobic systems had revealed that sulfhydryl compounds such as sodium thioglycollate, cysteine, or glutathione, would reverse nitrite inhibition. Ascorbic acid and other reducing substances that do not contain a sulfhydryl compound were ineffective (6). With the results obtained here, ascorbic acid evidently has an effect on reversing the nitrite toxicity in aerobic systems.

The fact that ascorbic acid did not alter the growth

rate of the Arthrobacter sp. other than to cause a slightly greater lag period in nitrite utilization, indicated both the adaptability of Arthrobacter to nitrite utilization and the repressing effect produced by ascorbic acid. Since bacterial cytochromes vary greatly in their characteristics, the Arthrobacter cytochrome may not be affected by nitrite (9). Many reports also indicate alternate pathways that do not involve cytochromes as the method of nitrite reduction (10,13,28,29,30,37,42).

Cytochromes were isolated with the intentions of studying further the physiological effects of nitrite. The Arthrobacter and Pseudomonas isolates were cultured in a modified form of Morris medium containing 300 μg/ml NO_3^--N. Nitrate was chosen as the sole nitrogen source as this would require the organisms to synthesize the enzymes needed to reduce nitrate to ammonia. Most likely this occurs by way of the intermediate nitrite. If the cytochrome system is in fact involved, greater quantities of cytochrome should be present in these cells.

Upon spectral analyses there was a correlation found between the effects of nitrite and ascorbic acid on the cytochromes and nitrite utilization. The Pseudomonas cytochrome spectra had a similarity to cytochrome c as the changes brought about by nitrite and ascorbate could be compared with the oxidizing and reducing capacity it has on cytochrome c. Figures 28 and 29 show this similarity in that

the standard oxidized and reduced spectra of the Pseudomonas cytochrome are quite similar to the cytochrome which has been treated with nitrite and ascorbate, both producing a peak at 360 mμ. The ascorbate acts to return the spectra to that obtained with the pure cytochrome extract. The mammalian cytochrome c standard oxidized and reduced spectra are similar to that produced by nitrite and ascorbate respectively (Figs. 22 and 23). This supports the hypothesis that nitrite exhibits a toxic effect on cytochrome c through its oxidizing capacity (31). Results obtained in this work indicate the ability of a reducing agent to decrease the toxic effects of nitrite by reducing the cytochrome. The extent to which it is reduced would depend on the concentration of reducing agent used. Figure 19 supports this statement as an increased concentration of ascorbic acid is shown to inhibit growth by irreversible reduction of the cytochromes.

A study of the Arthrobacter sp. shows that nitrite does not produce any adverse effects on its cytochrome. The peaks were at 565, 535, and 405 mμ in the oxidized spectrum with a fourth one appearing at 392 mμ in the reduced spectrum. This immediately shows the dissimilarity between it and the cytochrome c peaks which are at 415, 522, and 550 mμ with no fourth peak occurring in the reduced form. A comparison of the Arthrobacter cytochrome treated with nitrite and ascorbate (Fig. 26) and the standard oxidized and reduced spectra (Fig. 25) shows no similarities in that ascorbate

does not produce a spectrum comparable to the reduced spectrum resulting from treatment with $Na_2S_2O_4$. The oxidized spectrum is not similar to the nitrite treated spectrum and nitrite appears to produce a new peak at 360 mμ (Fig. 26) which is erased as a result of an addition of ascorbic acid. These results can be related to the fact that growth studies showed no inhibition from the presence of nitrite and in fact a slight repression of growth with the addition of ascorbate. The peak occurring at 360 mμ might be principally operative in nitrite utilization since the Arthrobacter cytochrome extract of the nitrite utilizing isolate has a peak at this wavelength when treated with nitrite. In contrast to this, the Pseudomonas sp. (which is inhibited by nitrite) has this same peak only when ascorbate is added to the nitrite. When nitrite alone is added to this non-nitrite utilizing species, a peak of very high absorbance is obtained. The addition of ascorbate reduces the absorbance and returns the spectrum to one that more closely resembles the untreated cytochrome. Since it had been shown that ascorbic acid did in fact increase the growth of Pseudomonas in cultures which contained no nitrogen source (Fig. 21), it might be assumed that ascorbic acid is instrumental in some additional metabolic process not involved with nitrite reduction. The physiological functions of ascorbic acid have not yet been clearly defined and may be involved in several different processes (11). Therefore, when nitrite is present it may assume an additional function

in returning an electron carrier to a reduced state.

In conclusion, nitrite, although known to be toxic to life processes, can be utilized by many soil micro-organisms. The ability of these organisms to do this depends either on its adaptability to use nitrite as a nitrogen source or else on the reversibility of this toxicity by the action of other biochemical compounds.

SUMMARY

A large proportion of the soil microflora will utilize nitrite as a sole nitrogen source. The ability of many microorganisms to do this indicates possible competition for nitrite. Different nitrite concentrations are tolerated by various microorganisms. A bacteriostatic effect in aerobic systems can be reversed by the addition of ascorbic acid. This reducing agent reverses the oxidizing effect of nitrite on the cytochrome system. This reaction can be compared to the respective oxidizing and reducing capacities of nitrite and ascorbic acid on mammalian cytochrome c. An organism not inhibited by nitrite will be immune to nitrite and ascorbic acid as oxidizing and reducing agents. A comparison of the nitrite treated cytochrome spectrum from a nitrite utilizing organism with the cytochrome of a non-nitrite utilizer which has been treated with both nitrite and ascorbic acid shows similarities in their respective spectra. Thus nitrite utilization by microorganisms may be a result of reversibility of toxic effects, the presence of alternate pathways, or the adaptability of pathways of nitrate reduction to tolerate and reduce nitrite.

APPENDIX

1. Modified Morris (25) Media for Isolation of Soil Microorganisms

	per liter
K_2HPO_4	7.0 g
KH_2PO_4	3.0 g
$NaNO_2$	.246 g = 50 µg/ml NO_2^--N
20% $MgSO_4 \cdot 7\ H_2O$	1.0 ml
1% $FeCl_3$	1.0 ml
1% $CaCl_2$	1.0 ml
Trace elements	
28% H_3BO_3	0.2 ml
1.86% $MnCl_2 \cdot 4\ H_2O$	0.2
.20% $CuSO_4 \cdot 5\ H_2O$	0.2
.75% $NaMoO_4$	0.2
.37% $CoCl_2 \cdot 6\ H_2O$	0.2
.25% $ZnSO_4 \cdot 7\ H_2O$	0.2
Carbon source	
50% glucose sterilized separately	20.0 ml
Agar	18.0 g
Soil extract	100 ml

2. Preparation of soil extract

field soil	1000 g
tap water	1000 ml

$CaCO_3$ .5 g (acts as a flocculant for the colloidal material)

Autoclave for 20 minutes and filter to clarify.

3. Liquid Modified Morris Media

Same as (1) but contains no soil extract or agar and the NO_2^--N concentration varies from 50-4000 μg/ml

4. Liquid Modified Morris Media for growing and harvesting of cells

Same as (3) but contains 300 μg/ml NO_3^--N (2.172 g $KNO_{3/1}$) in place of $NaNO_2$.

5. Csaky's Method of Nitrite Determination (8)

Reagents

(a) Sulfanilic acid--Dissolve 10 g sulfanilic acid per liter of 30% acetic acid. Heat on a water bath to dissolve the sulfanilic acid

(b) Alpha-naphthylamine--Dissolve 3 g of α-naphthylamine hydrochloride per liter of 30% acetic acid.

Procedure

To a 3.0 ml aliquot of sample (or a smaller aliquot plus distilled water to make a volume of 3.0 ml) in an 18 x 150 mm test tube, add 1.0 ml of the sulfanilic acid reagent and 1.0 ml of the α-naphthylamine reagent. Mix, and read the optical density against distilled water at 525 mμ after four or five minutes. Refer the reading to

a calibration curve prepared from standard solutions of nitrite. The highest standard should contain 1.4 µg of nitrite. Make a blank determination.

Notes

The α-naphthylamine reagent decomposes quite readily. It was found advisable to prepare 200 ml batches of the reagent and to store the reagent in the refrigerator when not in use.

Blank determinations usually read around .02-.03 mµ. When the blank is reading beyond .03 mµ, it is best to prepare a new batch of α-naphthylamine reagent.

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