PURIFICATION AND CHARACTERIZATION OF THE ADAPTIVE NITRATE REDUCTASE IN *AGROBACTERIUM RUBI* (CONN.)

by

Chester S. Stachow

A thesis

submitted to

The Faculty of Graduate Studies and Research

University of Manitoba

In partial fulfilment

of the requirements for the degree

Master of Science

1962

ACKNOWLEDGMENTS

The writer expresses his sincere gratitude to Dr. N.E.R. Campbell, Department of Microbiology, University of Manitoba, for his assistance and advice throughout the course of this investigation. Sincere gratitude is expressed, as well, to Dr. M.I.H. Aleem and to Dr. B.D. Sanwal for their most helpful consideration and advice.

TABLE OF CONTENTS

INDEX OF TABLES AND FIGURES

ABSTRACT

ABSTRACT

The induced nitrate reductase of Agrobacterium rubi was purified 130 fold by repeated precipitation, adsorption, and chromotography. The pH optimum was 7.5 with a substrate concentration of 10^{-3} M. Of various electron donors tested, only pyridine nucleotides were active. Dialysis against cyanide caused loss of activity which could be partially restored by the addition of iron and molybdenum. Various sequestrating agents inhibited enzyme activity, FAD markedly stimulated it. The role of the enzyme in electron transport in the organism will be discussed in the light of these results.

INTRODUCTION

INTRODUCTION

It is accepted that nitrate constitutes the main source of nitrogen for higher plants with the possible exception of the Leguminosae. There exists a symbiotic relationship among the Leguminosae and the genus Rhizobium enabling them to utilize atmospheric nitrogen.

In contrast, most soil microorganisms prefer ammonium salts as their nitrogen source. However, in soil the concentration of ammonia is relatively low in comparison to nitrate. Under partially anaerobic soil conditions, nitrate normally available for plant nutrient, may be depleted through utilization in respiration by soil microorganisms. In this respect the nitrate would act as the terminal electron acceptor in place of oxygen. Utilization of nitrate by microorganisms has been reported by Pollock (1946) and Silver (1954) to be initiated through the activity of an adaptive enzyme, nitrate reductase. An exception to this finding was reported by Bastarrachea (1961) who found the nitrate reductase in Mycobacterium

tuberculosis to be a constitutive enzyme.

Members of the genus Agrobacterium (Conn.) are indigenous to the soil and include both saprophytic and pathogenic species. Within the group, Agrobacterium rubi was selected for adaptation studies with respect to nitrate reduction. In addition, the purification and characterization of nitrate reductase in this species was undertaken to provide a comparison with nitrate reductase isolated from other organisms and characterized as molybdo-flavoproteins. It is important to emphasize that such comparisons are important, though not conclusive evidence as to the nature of the enzyme nitrate reductase in other biological systems.

HISTORICAL

HISTORICAL

Many of the original and fundamental investigations concerning the nature of nitrogen metabolism were conducted on microorganic cell preparations. In this respect many microorganisms have demonstrated the ability to reduce nitrate to ammonia. Subsequently, this may be incorporated to form amino acids, proteins, and other nitrogenous cell components. This process is known as 'nitrate assimilation'. On the other hand, 'nitrate respiration' or 'dissimilatory nitrate reduction' represents the utilization of nitrate or some of its reduction products as terminal electron acceptors in place of oxygen. 'Nitrate assimilation' and 'nitrate respiration' are usually considered to be two major processes of nitrogen metabolism in microorganisms and high plants.

Although the reduction of nitrate was observed as early as 1868 by Schönbein and somewhat later by Quastel (1925), the enzyme which catalyzes the reaction was not isolated in cell-free preparations until 1934 by Green,

Stickland and Tarr working with Bacterium coli. Yamagata (1938, 1939) obtained a cell-free extract of nitrate reductase from Escherichia coli which was inhibited by cyanide but unable to oxidize reduced coenzyme I. Some workers claimed that the enzyme was a sulfhydryl protein identical with cytochrome b, inhibited by carbon monoxide, and having a flavin requirement. On the other hand, Joklik (1950) was unable to demonstrate any effect with -SH reagents or carbon monoxide. He reported that certain dyes and flavoprotein were active as electron carriers between hydrogenase and nitrate reductase. However, the nature of the immediate natural electron donor still remained obscure.

The nitrate reductase of Neurospora, which catalyzes the reduction of nitrate to nitrite by reduced triphosphopyridine nucleotide (TPNH), has been purified and characterized as a metolloflavoprotein with flavin-adenine dinucleotide (FAD) as the prosthetic group (Evans and Nason, 1952 and Nason and Evans, 1953). Nason and Evans (1953) found that inhibition of this enzyme by p-chloromercuribenzoate (p-CMB) was reversed by cysteine or

glutathione. This strongly suggests that -SH groups are present in the nitrate reductase of Neurospora. Subsequently, Nicholas and Nason (1954b) demonstrated the metal component of nitrate reductase to be molybdenum.

Nitrate reductase of the type characterized in Neurospora (a molybdo-flavoprotein) seems to be widely distributed as evidenced by its isolation in; Bacillus pumilus (Taniguchi, 1953), Rhizobium japonicum (Evans, 1954, Escherichia coli (Nicholas, 1955), Pseudomonas aeruginosa (Fewson, 1961) and others (Nicholas, 1954; Silver, 1957; and Sadana, 1957).

Many schemes have been postulated for nitrate reduction in Escherichia coli. It is probable that the various pathways reported by different workers using this organism can be accounted for by the variety of hydrogen donors used in the assay and by the varied conditions under which the organism was grown. However, Fewson (1961) suggests that the preparations probably all contained the same terminal nitrate reductase, that is, the molybdo-protein.

Several reviews have been published on the subject

of nitrogen metabolism (Virtanen, 1952; McElroy and Glass, 1956; Nicholas, 1958; and Fewson, 1961a).

MATERIALS AND METHODS

Culture

A wild strain of Agrobacterium rubi (P-60) obtained through the courtesy of the Institute of Microbiology, Science Service, Department of Agriculture, Ottawa, was used in the investigation. This culture displays a characteristic inability to produce nitrite from nitrate as reported in Bergey's Manual of Determinative Bacteriology, seventh edition.

For stock culture and propagation purposes this species was cultured in a Mannitol-casamino-acid medium of the following composition: 0.5 gm K_2HPO_4, 0.3 gm $MgSO_4 \cdot 7H_2O$, 0.2 gm NaCl, 5.0 gm casamino-acids (vitamin-free), 1.0 gm Mannitol in 1000 ml distilled water.

Adaptation of the Culture

A twenty-four hour old broth culture, incubated at 25° C, was centrifuged and washed three times in sterile physiological saline (0.85% NaCl). The cells were

resuspended in a nitrogen-free Mannitol medium and allowed to respire for 24 hours without aeration. This constituted the inoculum for adaptation studies.

In general, two methods were attempted to stimulate nitrate reduction in the wild strain. First, a method was followed in which the cells were streaked on plates of Mannitol casamino-acid medium to which varying concentrations of nitrate (KNO_3) were added. In addition, a second method was used in which respired cells were streaked on plates of casamino-acid-free Mannitol medium to which varying concentrations of potassium nitrate were added as the sole source of nitrogen. In both cases the concentrations of nitrate varied from 0.05 to 2.0 percent.

Streak cultures were incubated at 25° C for 48 hours. Colonies appearing after this time were sub-cultured to media of the same composition. Sub-culturing was continued until growth for a 24 hour incubation period was judged to be at the maximum for the medium employed.

The Mannitol-casamino-acid-NO_3 broth medium was dispensed in one liter flasks at the rate of 500 ml per flask. After inoculation, cultures were incubated at 25° C

for 24 hours. At two hour intervals, for the first 12 hours, then at 20 and 24 hours, duplicate 0.5 ml aliquots were withdrawn for determination of nitrite according to the methods of Snell (1949).

Since Nicholas (1957) had reported that trace amounts of Mo and Fe stimulated nitrate reductase activity, an experimental series was conducted to determine if a similar effect could be demonstrated in the species under investigation. The following trace elements; iron, molybdenum, copper and manganese were added individually to the basal medium (5, 10, 2 and 10 µg/l, respectively). As well, a mixture of trace elements (Larsen, 1953) was used at the rate of 0.1 ml per liter of basal medium. Nitrite determinations were made during incubation at the intervals previously described. The final composition of the medium used subsequently in the investigation is as follows:

0.5 gm K_2HPO_4

0.3 gm $MgSO_4 \cdot 7H_2O$

0.2 gm NaCl

5.0 gm Casamino acids

5.0 gm KNO_3

1.0 gm Mannitol

0.1 ml Trace element complex

1000.0 ml Distilled water

Cell Preparation

Cells cultured at 25° C for 72 hours in the final medium were harvested by centrifugation at 27,000 x g using a KSB continuous flow attachment on a Servall SS-3 centrifuge at 4° C. To ensure the removal of residual nitrite, the collected cells were washed repeatedly with 1.0% (w/v) NaCl until the final wash-supernatant gave a negative test for nitrite. Cells thus prepared were frozen and stored at -17° C until required.

Extract Preparation

The frozen cells were thawed slowly at 4° C, then resuspended in an equal volume of 0.1 M Tris (hydroxymethyl) aminomethane-HCl (pH 7.5) buffer containing reduced glutathione (10^{-3} M final concentration).

Cells were disintegrated in a Raytheon ultrasonic

generator (10 kc/sec.) at 7° C. To establish the optimum sonication time, a series of exposures of 5, 10, 15, 20, 25, 30, 40 and 60 minutes were carried out. The whole sonicate was centrifuged at 2000 x g for 10 minutes at 4° C.

Assay for the Determination of Nitrate Reductase

Nitrate reductase activity in the whole sonicate and in subsequent purified fractions was determined aerobically using the following assay mixture:

0.15 ml KNO_3 (10^{-3} M)

0.10 ml DPNH (10^{-3} M)

0.10 ml FAD (10^{-4} M)

0.10 ml Glucose (0.1 M)

0.10 ml Glucose-oxidase (0.1 gm/10 ml)

0.02 - 0.10 ml Enzyme

0.1 M Tris-HCl buffer (pH 7.5) was added to the above reaction mixture to yield a final volume of 1.0 ml. Two control assays were used; in one, the electron donor DPNH was omitted and, in the other, the enzyme fraction was inactivated by boiling for 15 minutes. The reaction

mixtures were then incubated at 25° C for 30 minutes. Following incubation 5.0 ml deionized water was added to the reaction mixture. The nitrite formed was determined by adding 0.5 ml of a 1% (w/v) sulphanilamide to stop the reaction followed by 0.5 ml of the 0.02% (w/v) n-naphthylethylenediamine-dihydrochloride. After allowing 20 minutes for maximum color development the color intensity was determined in a Klett-Summerson colorimeter using a green filter (540 mμ).

To prevent the adsorption of nitrite to the surface of protein present in the reaction mixture, frequently it became necessary to add 0.4 ml Sodium lauryl-sulfate (0° sat.) to the reaction mixture immediately before the addition of the sulphanilamide. This precaution was necessary only when the protein content of the enzyme preparation contained 10 to 20 mg of protein per ml.

Immediately after color development the insoluble protein of the reaction mixture was removed by centrifugation at 10,000 x g for 15 minutes.

Definition of Unit and Specific Activity

One unit of enzyme activity was defined as that amount of nitrite (mμ Moles) formed in 30 minutes. Specific activity is expressed as units per milligram of protein.

Enzyme Purification Procedure

The whole sonicate was diluted with twice its volume in 0.1 M Tris-HCl buffer (pH 7.5) and centrifuged at 2000 x g for 10 minutes at 4° C. The resulting supernatant and pellet were tested for the presence of the enzyme and the supernatant constituted the starting material for further purification. To ten ml of the supernatant 2.43 gms of solid ammonium sulphate ($(NH_4)_2SO_4$) was added slowly to yield a 40% saturated ammonium sulphate solution. The mixture was equilibriated at 4° C for 15 minutes with continuous stirring. After equilibriation, the solution was centrifuged at 27000 x g for 15 minutes and the supernatant and precipitate fractions were tested for nitrate reductase activity. The supernatant was

discarded, and the precipitate suspended in twice its volume of 0.1 M Tris-HCl buffer (pH 7.5). This constituted fraction one.

The next step of purification was the fractional adsorption of the enzyme to calcium phosphate gel (20 mg per ml dry weight). Twice the volume of calcium phosphate gel was added to fraction one. The $CaPO_4$ gel-enzyme suspension was kept at 4° C for 15 minutes with intermittent stirring, then centrifuged at 5,000 x g for 5 minutes. Since enzyme activity was found to be in the supernatant, an equal volume of calcium phosphate gel was added to it. Equilibration treatment was the same as previously cited for fraction one. Again the enzyme activity was found in the superantant after centrifugation. Similar calcium phosphate gel treatment was repeated twice more.

Subsequently, two ml of alumina C γ (30 mg per ml dry weight) were added to 10 ml of the supernatant fraction thus prepared and allowed to stand at 4° C with intermittent stirring. After 15 minutes the fraction was

centrifuged at 5,000 x g for 5 minutes.

The supernatant was then adsorbed to a DEAE-cellulose column. A continuous gradient method was used to elute the enzyme from the cellulose column. A phosphate (0.2 M, pH 7.4) - NaCl (4 M) mixture was used for this purpose.

A spot-plate method was used for rapid determination of nitrate reductase activity in each 5 ml fraction eluted from the cellulose column. This method consisted of adding 4 drops of a "standard assay mixture" and same amount of a "standard control assay mixture" to separate wells of a spot plate. The term "standard assay mixture" refers to a mixture containing all the components of the assay system described previously in method of assay. The "standard control assay mixture" is a mixture corresponding to the control assay mixture in which DPNH was omitted. To start the reaction, 2 drops of each enzyme fraction was added to the "standard and control assay mixtures" and incubated at 25° C for 10 minutes. Then 3 drops of sulphanilamide and n-naphthylethylenediamine

hydrochloride were added to the assay mixtures to develop the color. To confirm the spot plate findings, those fractions yielding the characteristic red color by the spot tests were assayed as described previously in "Methods".

Nitrate reductase activity appeared in three separate 5 ml fractions. In order to increase the enzyme activity, the fractions were pooled, the protein precipitated by ammonium sulphate (60% sat.) and the resulting precipitate was dissolved in five ml of a 0.1 M Tris-HCl (pH 7.5) buffer after centrifugation. The fractions containing ammonium sulfate were dialyzed overnight in the cold against running tap water prior to protein determination (Lowry, 1951).

Metal Components

The purified enzyme was dialyzed against potassium cyanide and 0.1 M Tris-HCl-glutatione (pH 7.0) for three hours using glutatione soaked membranes as described by Nicholas and Nason (1954a). The potassium cyanide was

removed from the enzyme extract by dialysis against cold deionized water for another three hours. To test for the possible reactivation of nitrate reductase, the metals, iron, molybdenum, manganese, magnesium, zinc and copper were added to the assay system at a final concentration of 10^{-2} and 10^{-3} M.

Flavine Components

Varying concentrations of FAD were added to the assay mixtures to determine if stimulation of enzyme activity by the flavine component could be observed. To remove residual and bound flavines, the enzyme preparation was exposed to ultraviolet light (2600 A°) at 4° C for varying time intervals (10, 20, 40 and 60 minutes) in the dark as suggested by McElroy (1954). One ml portions of the enzyme preparation were dispensed in each of two planchettes: one was placed directly under the u/v source at a distance of 4 cm; the other was shielded from the u/v source and served as the control. Irradiated and control samples subsequently were used in reaction assay mixtures both in the presence and absence of added flavine.

Reduced Cofactors

Methylene blue (10^{-4} M), FAD (10^{-5} M), cytochrome c (3%), FMN (10^{-5} M) and menadione (10^{-3} M) were reduced by hydrogen gas in the presence of a palladium-asbestos catalyst according to the method of Smith (1954). Five ml portions of the various cofactors in the oxidized form were dispensed in evacuation tubes (simple test tube with side-arm). Hydrogen gas was passed through the cofactor-catalyst mixture by means of a fine-tipped glass tube inserted secured in a tightly-fitting one-hole stopper. Exhaust gas was removed through a collection manifold attached to each side-arm by means of rubber tubing. Tubes containing flavine cofactors were tightly wrapped in aluminum foil to prevent photoinactivation of the flavines during the reduction treatment. After three hours of gassing, the rubber connectors on the side-arms and inlet connections were securely clamped to maintain a slightly positive internal hydrogen pressure on the evacuation tubes. The tubes were tilted to permit the reduced cofactors to collect in the rubber extensions of

the side-arms. Samples for assay mixtures were removed as required by means of a graduated 1.0 ml hypodermic syringe which had been flushed repeatedly with hydrogen gas before insertion in the rubber side-arm connector above the clamp.

Nitrate reductase activity in the presence of these reduced cofactors was determined anaerobically in Thunberg tubes. With the exception of the cofactors employed in place of reduced dipyridine nucleotide, the assay mixture was the same as for aerobic determinations. Precautions were taken to prevent autooxidation when the reduced cofactors were being added to the reaction mixtures. Immediately after the addition of cofactors (the last component added to the reaction mixture), thunberg tubes were evacuated and incubated at 25° C for 30 minutes.

RESULTS

RESULTS

Adaptation

Despite repeated trials it was evident that the wild strain of A. rubi could not be cultured in a medium containing nitrate, nitrate or ammonium sulphate as the sole nitrogen source. However, the addition of a casamino acid supplement to the mannitol nitrate medium permitted good growth by the culture within 48 hours. Evidence of the accumulation of nitrite under these cultural conditions is presented as the control curve in Fig. 1a.

Sonication

The optimum sonication time was found to be 15 minutes as illustrated in Fig. 2. The crude sonicate contained a strong DPNH oxidase but no TPNH oxidase. To reduce interference by DPNH oxidase, glucose (0.1 M) and glucose-oxidase (0.1 g/10 ml) were added to the reaction mixture.

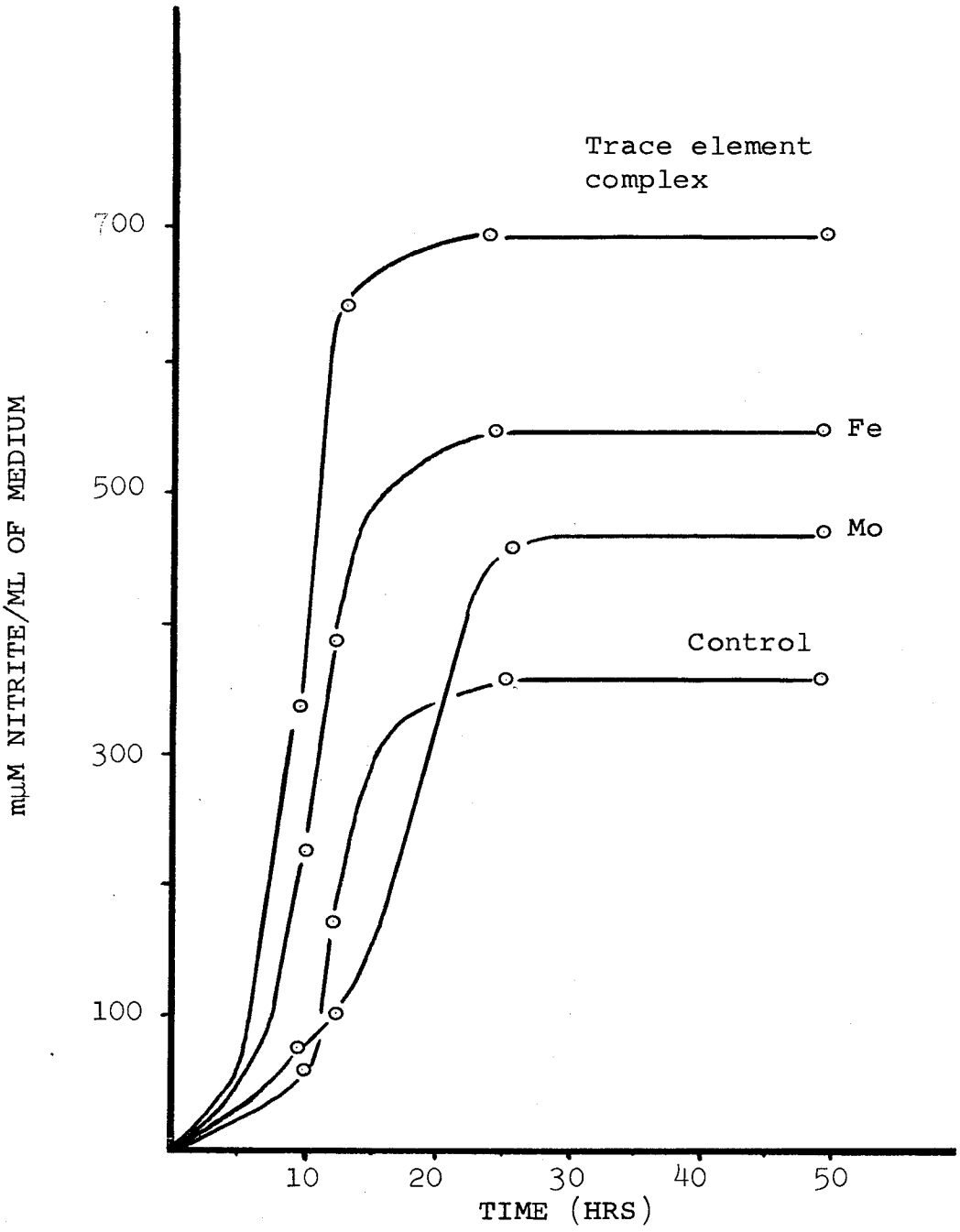

Figure 1a. Effect of added metals on nitrite accumulation in the growth medium.

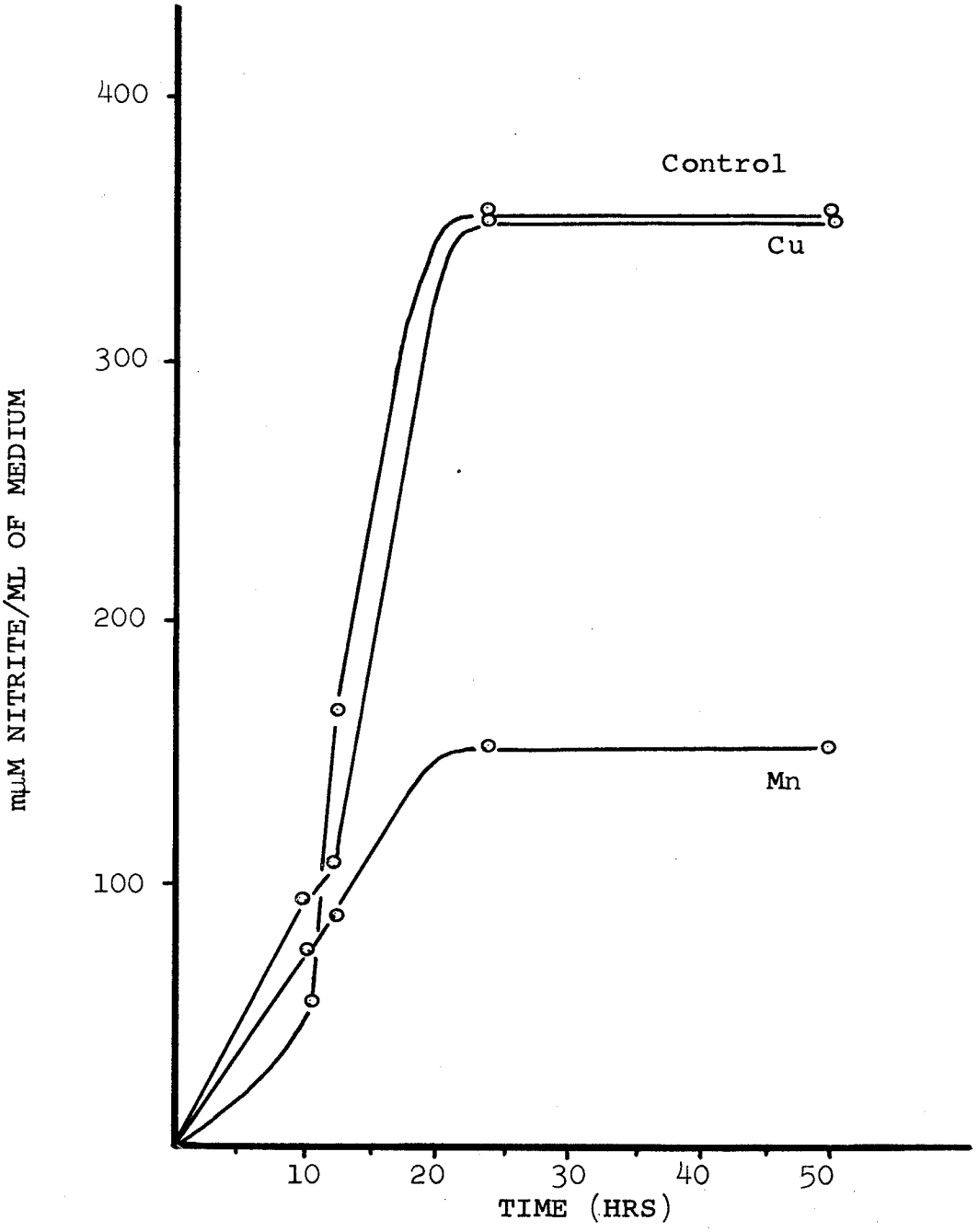

Figure 1b. Effect of added metals on nitrite accumulation in the growth medium.

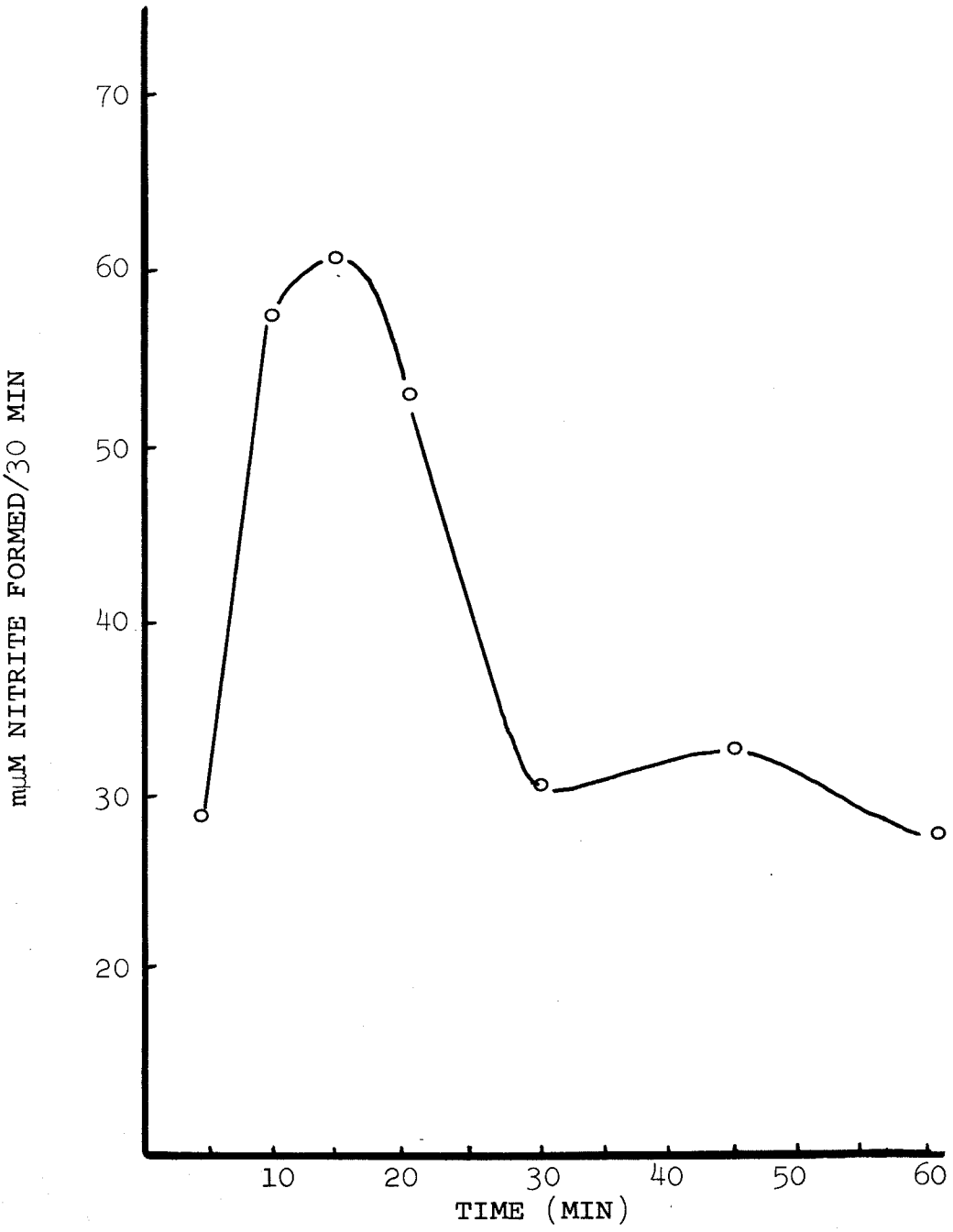

Figure 2. Effect of sonication time on nitrate reductase activity.

Enzyme Purification

A summary of the purification procedure is given in Table 1 and represents an over-all purification of 130 fold. After Ca-PO_4 gel adsorption, further purification was attempted by column chromatography using Sephadex G-50 and DEAE-cellulose. Purification afforded by the latter was markedly higher than that obtained with Sephadex G-50.

Enzyme Concentration and Incubation Time

The linearity between enzyme concentration and amount of nitrite produced is indicated in Fig. 3. The time of incubation was also proportional to the nitrite produced over the range, 0 to 35 minutes, Fig. 4.

pH Optimum

Optimum enzyme activity was obtained at pH 7.5 in 0.1 M Tris-HCl buffer, Fig. 5. There was a marked decrease in enzyme activity when assayed in 0.1 M phosphate buffer over a pH range from 7.0 to 9.0.

TABLE 1

Purification of nitrate reductase from **A.** rubi
Cells were suspended in equal volume of 0.1 M Tris-HCl (pH 7.5) buffer and disintegrated by ultra sonication for 15 min at 7° C. The enzyme and protein assays are described in Methods

Fraction	Procedure	Volume (ml)	Total protein (mg)	Protein mg/ml	Total Units mμM NO_2/30 min	Specific Activity	Recovery %	Purification
1	Crude extract, centrifuged at 3000 x g for 15 min. Supernatant was used	10	185	18.5	21	11.3	-	-
2	Precipitate from 0 to 40% ammonium sulphate saturation of fraction 1 centrifuged at 10,000 x g for 15 min dissolved in 0.1 M Tris-HCl buffer, pH 7.5.	10	102	10.2	30	29.3	142	2.6
3	Calcium phosphate gel (20 mg/ml) added to fraction 2 at twice the volume. Centrifuged at 5000 x g for 5 min. Activity in the supernatant solution.	30	187.50	6.25	30	48.0	142	4.2
4	Calcium phosphate gel (equal vol) added to fraction 3. Centrifuged at 5000 x g for 5 min. Activity in the supernatant.	21	61.53	2.93	24	82.7	114	7.3

cont'd

Table 1 (cont'd)

5	Calcium phosphate gel (equal vol) added to fraction 4. Activity in the supernatant solution.	23	28.75	1.25	22	176.0	104	15.5
6	Calcium phosphate gel (half x vol) added to fraction 5. Activity in the supernatant solution.	22.5	13.95	0.62	14	225	66	19.9
7	Alumina Cγ (200 mg) added to fraction 6. Centrifuged at 5000 x g for 10 min at 4° C. Activity in the supernatant solution.	24	2.88	0.12	8	666	38	58.9
8	Fraction 7 was purified by DEAE cellulose column. The enzyme was eluted by continuous gradient method using 0.2 M phosphate buffer (pH 7.4) 4 M NaCl.	5	0.27	0.054	8	1481	38	131.0

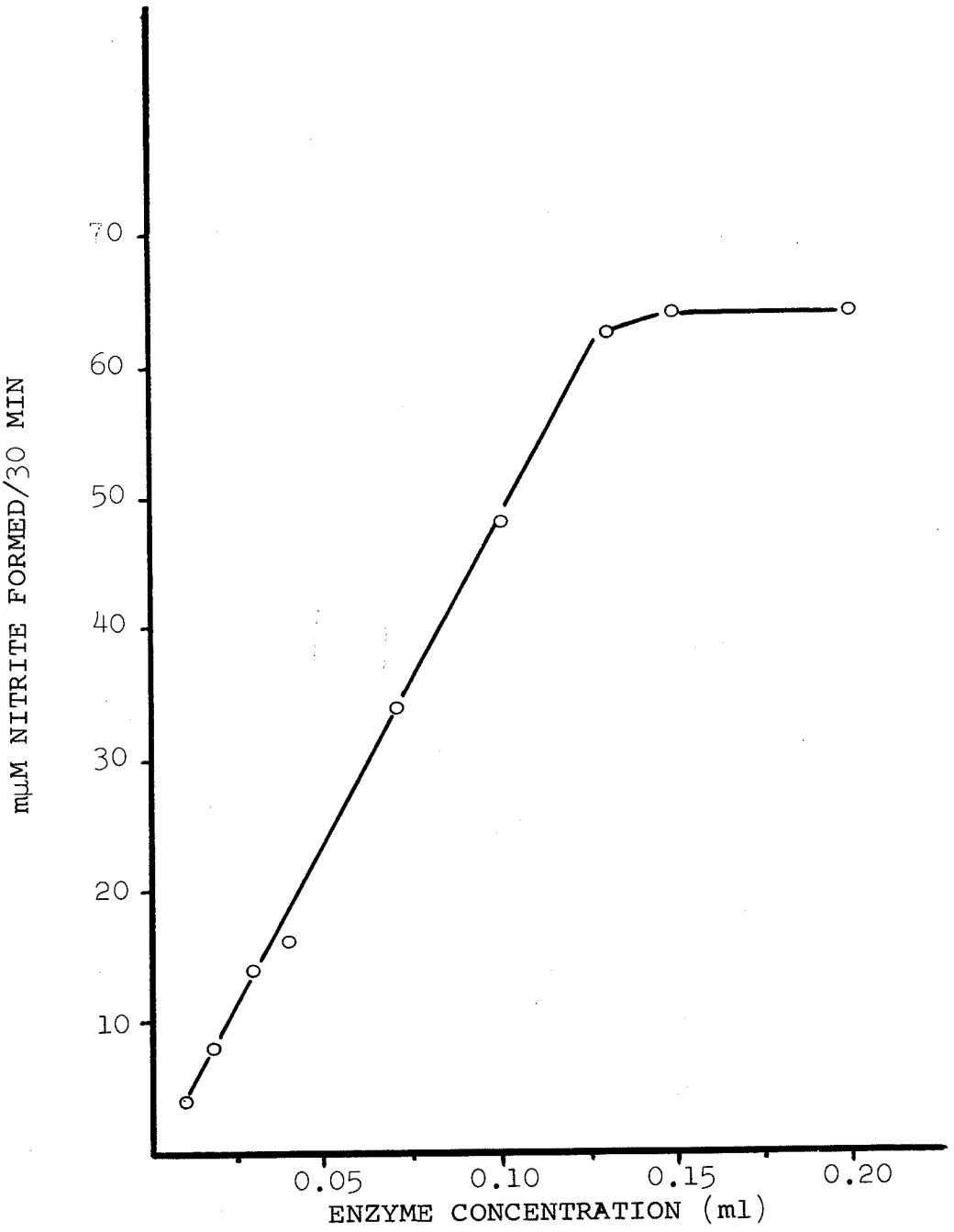

Figure 3. Effect of enzyme concentration on nitrate reductase activity.

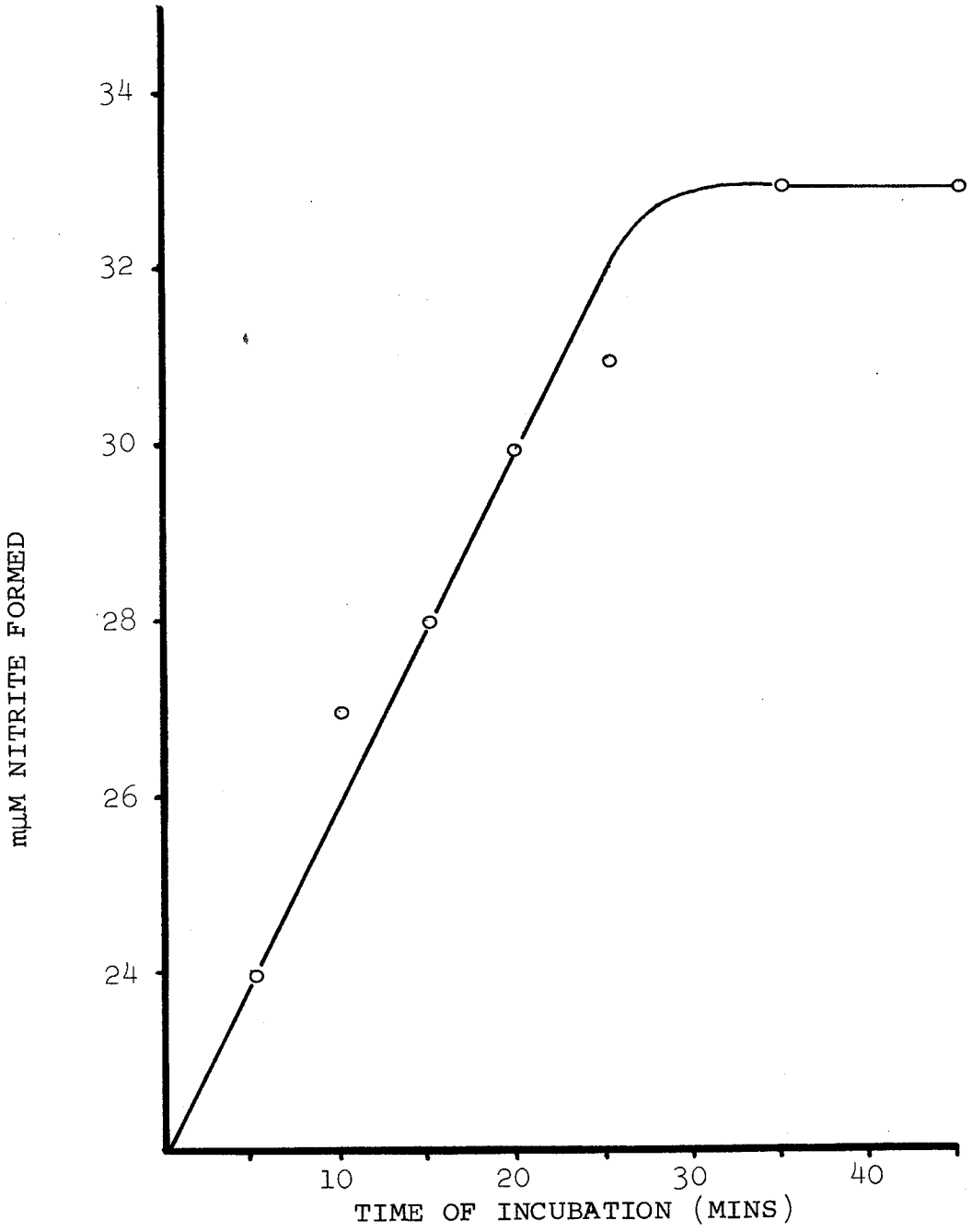

Figure 4. Effect of incubation time on nitrate reductase activity.

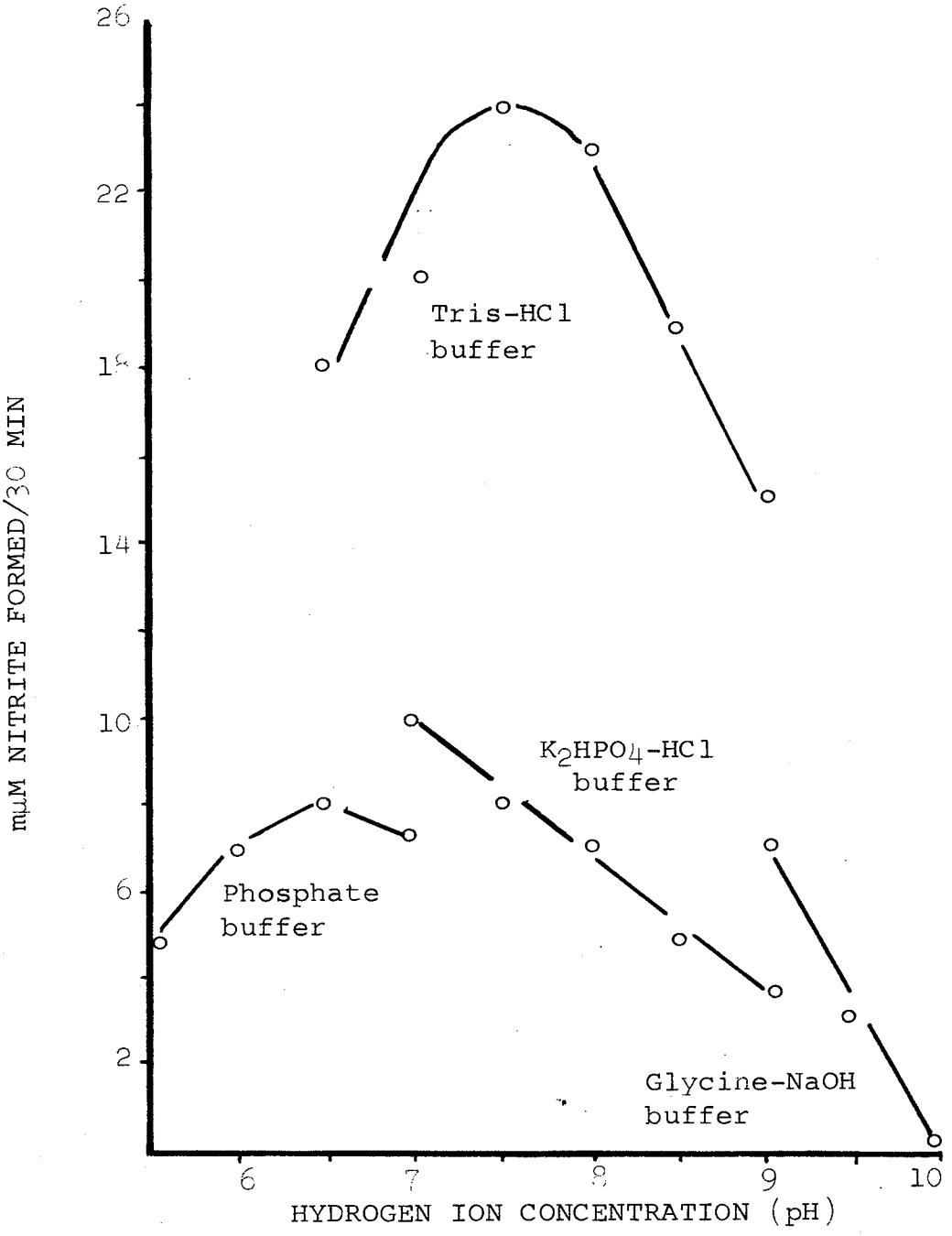

Figure 5. Effect of hydrogen ion concentration and various buffers on nitrate reductase activity.

Substrate Saturation

The dissociation constant (Km) of the enzyme-nitrate complex was calculated to be 1 x 10^{-3} moles/liter on the basis of the saturation curve, Fig. 6.

Inhibitors

Table II illustrates the percent inhibition of nitrate reductase activity by a series of compounds. Metal chelating agents, sodium azide and cyanide at 10^{-3} M final concentrations produced a substantial decrease in nitrate reductase activity. Sensitivity of the enzyme to flavine and sulfhydryl inhibitors was also noted.

Flavine Component

A marked stimulation of enzyme activity (50 percent) occurred with the addition of flavine adenine dinucleotide at a final concentration of 10^{-5} M, Fig. 7. The ultraviolet treatment (60 min) decreased enzyme activity by 72 percent. However, the addition of FAD

(10^{-5} M) to the same reaction mixture (irradiated enzyme) almost completely resorted activity. These data are presented in Table III.

Metals

The addition of iron, molybdate and a trace element complex markedly increased the nitrite accumulation in the culture medium (40 to 50 percent), Fig. 1a. Of the metals Fe^{+++}, Fe^{++}, Cu^{++}, Zn^{++}, Mg^{++}, Mn^{++}, MoO_4^- only MoO_4^- showed slight reactivation of the cyanide-dialyzed enzyme. However, this result was difficult to repeat consistently. Enzyme activity was completely reactivated when iron (ferrous sulfate) was added to a 50 percent azide-inhibited enzyme. Molybdate had no effect.

Electron Donors

Results presented in Table IV show that reduced FAD, MB, cytochrome c and menadione are not as effective as DPNH as electron donors.

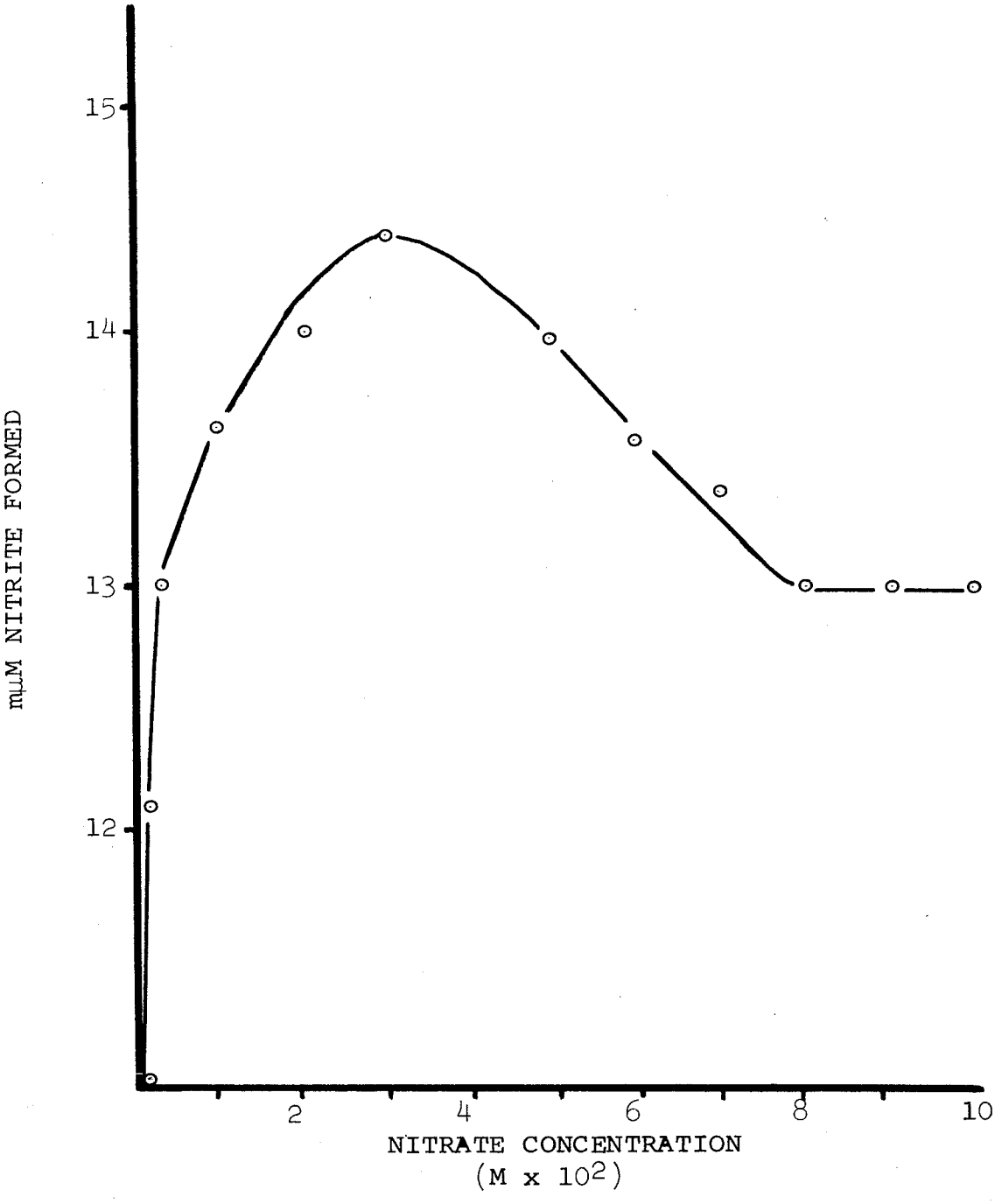

Figure 6. Effect of nitrate concentration on nitrate reductase activity.

TABLE 2

Effect of some inhibitors on nitrate reductase activity in A. rubi

Inhibitors	Final conc. of inhibitor (moles/l)	
	10^{-3}	10^{-4}
Sodium azide	100	67
2:2-dipyridyl	26	-
Disodium ethylenediamine tetraacetate	0	-
Sodium fluoride	22	-
8-hydroxyquinoline	0	11
Quinacrine dihydrochloride	100	69
Hydrazine hydrochloride	34	
Quinine sulphate	43	
Potassium chlorate	0	
Potassium cyanide	35	
Allyl urea	22	
2-4 dinitrophenol	25	
Sodium diethylenedithiocarbamate	0	
p-chloromercuribenzoate (p-CMB)	67	
p-chloromercuribenzoate + Glutathione (10^{-3} M)	48	
Copper sulphate	100	

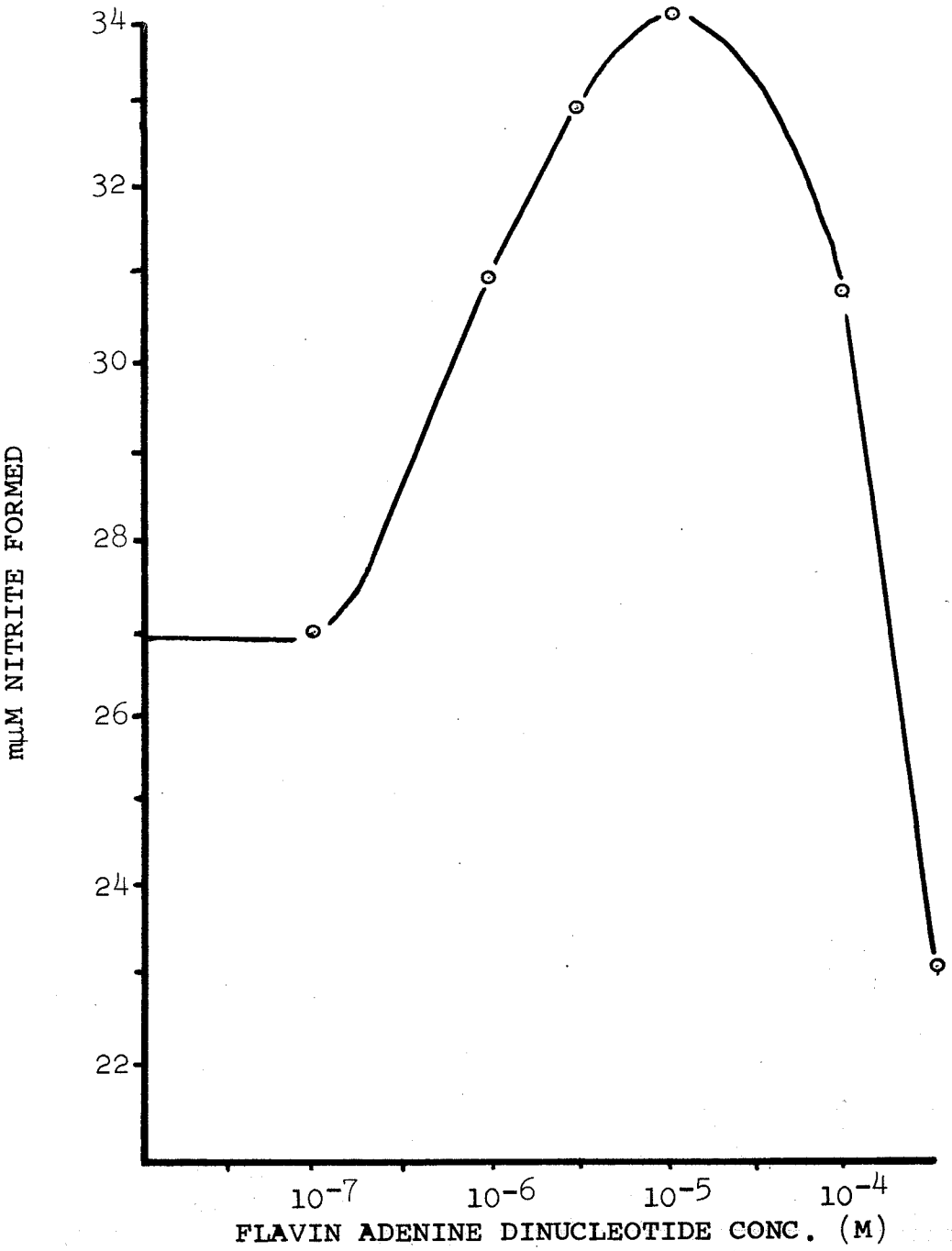

Figure 7. Stimulation of nitrate reductase activity by flavin adenine dinucleotide.

TABLE 3

Ultra-violet inactivation of flavine

Irradiation (u/v) Time (min)	Treatment	mμM NO_2 formed
0	none	59
10	minus FAD	49
	added FAD (10^{-5} M)	37
20	minus FAD	36
	added FAD (10^{-5} M)	44
40	minus FAD	22
	added FAD (10^{-5} M)	38
60	minus FAD	16
	added FAD (10^{-5} M)	37

TABLE 4

Electron donors
Enzyme activity was determined anaerobically.

Treatment	mμM formed/30 min
FAD + DPNH	33
reduced FAD (-DPNH)	11
Methylene blue reduced (-DPNH) + FAD	no color
FAD + reduced cytochrome c (-DPNH)	no color
reduced FAD + (oxid) cytochrome c (-DPNH)	5
reduced FAD + (oxid) cytochrome c + DPNH	32
Menadione (reduced) + FAD - DPNH	9
Menadione (reduced) + FAD + DPNH	32

DISCUSSION

DISCUSSION

The results presented indicate that A. rubi can form an adaptive nitrate reductase enzyme in the presences of an inducer (KNO_3). The enzyme appears to possess a flavine and possibly a metal component as cofactors. In this respect it closely resembles the metallo-flavoprotein reductases previously isolated by a number of investigators from E. coli, Neurospora, and Soy bean leaves.

Failure of the organism to grow in a medium containing nitrate, nitrite or ammonia as the sole nitrogen sources, even after prolonged incubation (weeks), suggested that this species requires organic nitrogen supplement in the form of amino acids. Nutritonal studies (Campbell, 1960) indicated a requirement by this species for aspartic, isoleucine, leucine, phenylalanine, proline, tryptophane and valine. Accordingly, the basal salts nitrate medium was supplemented by the addition of a vitamin-free casamino acid preparation.

The adaptive nature of the nitrate reductase from

this species is clearly indicated by the results obtained from the induction experiments. This finding supports the contension by Pollock (1946) that nitrate reductase is adaptive rather than constitutive.

Initially, increases in nitrite accumulation that occurred when iron and molybdenum supplements were added to the medium suggested that these metals increased the nitrate reductase activity. However, this result can not be considered as conclusive evidence that such a dependency exists, rather, the added metals may merely stimulate the growth of the cells to provide a higher concentration per unit volume of the enzyme in question. Another explanation for the nitrite accumulation may be similar to that proposed by Nicholas (1953) in that molybdenum depressed nitrite reductase activity. Therefore, the addition of a molybdenum supplement might depress the activity of the concomitant nitrite reductase sufficiently to permit the accumulation of nitrite through the nitrate reductase system.

The reactivation of nitrate reductase by the addition of iron to the azide-inhibited enzyme suggests

that iron might be the metal component of the enzyme. However, the inhibitor, sodium azide, may react with sites on the protein molecule other than the metal. The addition of iron could possibly dissociate the azide from these sites and produce reactivation. The slight reactivation occasioned by the addition of molybdate after repeated dialysis against cyanide suggests molybdenum as the metal component of the enzyme. This is in agreement with the results obtained for nitrate reductase from Neurospora (Nicholas, 1954a), E. coli (Nicholas, 1955) and Pseudomonas areugenosa (Fewson, 1961).

The results obtained with flavine inhibitors and ultraviolet treatment strongly indicates that nitrate reductase of A. rubi contains a flavine as its prosthetic group. To show conclusively that the flavine portion of the enzyme is FAD, fluorometric analysis and the D-amino acid oxidase test are necessary in addition to the reactivation studies performed in this investigation. Taniguchi (1960) solubilized and purified a nitrate reductase from E. coli which contains molybdenum and iron, but no bound flavine. This result seems to confirm the

hypothesis of Fewson (1961) that nitrate reductase freed from the penultimate hydrogen or electron transfer system, contains molybdenum only and is devoid of flavine.

The inhibition of the enzyme by p-chloromericuribenzoate and partial restoration by glutathione (10^{-3} M) indicates the sulfhydryl nature of nitrate reductase. Fewson (1961) suggests that sulfhydryl groups of nitrate reductase from Ps. areuginosa are involved in binding the FAD to the protein. Yagi (1959) and Armstrong (1960) have shown a similar function for sulfhydryl groups in D- amino acid oxidase and yeast lactic dehydrogenase respectively. Yagi (1959) also suggested that the sulfhydryl group of the protein combines with the amino group of the adenine nucleus of FAD.

Attempts to involve cytochrome c in the reduction of nitrate failed. Neither the addition of oxidized nor reduced cytochrome c increased the formation of nitrite. These results are unlike those reported by Fewson (1961) in Ps. areuginosa, where the sequence of electron transfer is suggested to be:

$$\text{DPNH} \longrightarrow \text{FAD} \longrightarrow \text{cytochrome c} \longrightarrow Mo^{+5} \longrightarrow NO_3^-$$

Therefore, the active components of the nitrate reductase from A. rubi are postulated to be the same as the assimilary nitrate reductase system from E. coli where the sequence of electron transfer has been shown to be (Nicholas, 1954):

$$DPNH \longrightarrow FAD \longrightarrow Mo \longrightarrow NO_3^-$$

The assimilation of nitrate by N. crassa proceeds according to the following pathway (McElroy, 1955):

$$NO_3^- \longrightarrow NO_2^- \longrightarrow ? \longrightarrow NH_2OH \longrightarrow NH_4^+$$

While the present investigation has been concerned solely with the initial reductive step in this pathway, crude extracts from A. rubi also contained a nitrite reductase. The detection of intermediates and the end product beyond this point was not within the scope of this investigation and awaits further study. In the case of Neurospora (Kinsky (1961) has shown that the end product of this pathway, ammonia, in Neurospora, decreases the formation of nitrate reductase and presumably functions as a means of physiological control for nitrate utilization. This example of "feedback inhibition" is analogous to results

observed in various metabolic pathways in bacteria (Vogel, 1957; Yates, 1957; and Levin, 1961).

REFERENCES

Armstrong, J. Me D., Coates, J. H. and Morton, R. K. (1960). Flavin dissociation and inactivation of cytochrome b_2 by oxygen. Nature, 186:1033-34.

Bastarrachea, F. and Goldman, D. S. (1961). Diphosphopyridine nucleotide-linked nitrate reductase from Mycobacterium tuberculosis. Biochim. Biophys. Acta, 50:174-176.

Bergey's Manual of Determinative Bacteriology (1957). 7th edition. Ed. by Breed, R. S., Murray, E. G. D. and Smith, N. R., Baltimore: The Williams and Wilkins Company.

Campbell, N. E. R. (1960). Doctoral Thesis, "Some aspects of transformation in the genus Agrobacterium, Conn. p. 53.

Evans, H. J. (1954). Diphosphopyridine nucleotide-nitrate reductase from soy bean nodules. Plant Physiol.

29:298-301.

Evans, H. J. and Nason, A. (1952). The effect of triphosphopyridine nucleotide on nitrate reduction by purified nitrate reductase. Arch. Biochem. Biophys., 39:234-240.

Fewson, C. A. and Nicholas, D. J. D. (1961). Nitrate reductase from Pseudomonas aeruginosa. Biochem. Biophys. Acta, 49:335-349.

Fewson, C. A. and Nicholas, D. J. D. (1961a). Utilization of nitrate by micro-organisms. Nature, 190:2-7.

Green, D. E., Stickland, L. H. and Tarr, H. L. A. (1934). CCXL. Studies on reversible dehydrogenase systems. 111. Carrier linked reactions between isolated dehydrogenases. Biochem. J., 28:1812-1823.

Joklik, W. (1950). Studies on the nitrate reductase of Escherichia coli in the cell-free state. Australian J. Sci. Res., Ser. B, 3:28-44.

Kinsky, S. C. (1961). Induction and repression of nitrate reductase in Neurospora crassa. J. Bacteriol. 82: 898-904.

Larsen, H. (1953). On the microbiology and biochemistry of the photosynthetic green sulfur bacteria, p. 34.

Levin, A. P. and Magasanik, B. (1961). The effect of purines on the formation of two enzymes involved in purine biosynthesis. J. Biol. Chem. 236:184-188.

Lowry, O. H., Rosebrough, N.J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem., 193:265-275.

McElroy, W. D., Hastings, J. W., Sonnenfeld and Coulombre, J. (1954). Partial purification and properties of bacterial luciferin and luciferase. J. Bacteriol., 67:402-8.

McElroy, W. D. and Spencer, D. (1955). Normal pathways of assimilation of nitrate and nitrite, p. 137-152.

In W. D. McElroy and H. B. Glass (eds) "Inorganic Nitrogen Metabolism." Johns Hopkins Press, Baltimore, Md.

Nason, A. and Evans, H. J. (1953). Triphosphopyridine nucleotide-nitrate reductase in Neurospora. J. Biol. Chem., 202:655.

Nicholas, D. J. D. and Nason, A. (1954). Molybdenum and nitrate reductase. 11. Molybdenum as a constituent of nitrate reductase. J. Biol. Chem., 207:353-360.

Nicholas, D. J. D., Nason, A. and McElroy, W. D. (1954a). Molybdenum and nitrate reductase. 1. Effect of molybdenum deficiency on the Neurospora enzyme. J. Biol. Chem., 207:341-351.

Nicholas, D. J. D. and Nason, A. (1954b). Mechanisms of action of nitrate reductase from Neurospora. J. Biol. Chem., 211:183-197.

Nicholas, D. J. D. and Nason, A. (1955). Diphosphopyridine

nucleotide-nitrate reductase from Escherichia coli. J. Bact., 69:580-583.

Nicholas, D. J. D. and Nason, A. (1955). Role of molybdenum as a constituent of nitrate reductase from soy bean leaves. Plant Physiol., 30:135-38.

Nicholas, D. J. D. (1957). Effect of Mo, Cu and Fe on some enzymes in Neurospora crassa. J. Gen. Microbiol., 17:699-707.

Nicholas, D. J. D. (1958). Metabolism of inorganic nitrogen and its compounds in plants. Fourth Int. Cong. Biochem. Vienna, Vol. Xlll. Colloquia on Topical Questions in Biochemistry, p. 307-331.

Pollock, M. R. (1946). Adaptation of "nitratase" in washed suspensions of coliform bacteria. Brit. J. Expt'l Pathol., 27:419.

Quastel, J. H., Stephenson, M., and Whetham, M. D. (1925). Some reactions of resting bacteria in relation to anaerobic growth. Biochem. J., 19:304-317.

Sadana, J. C. and McElroy, W. D. (1957). Nitrate reductase from Achromobacter fischeri. Purification and properties: Function of flavines and cytochrome. Arch. Biochem., 67:16.

Silver, W. S. and McElroy, W. D. (1954). Enzyme studies on nitrate and nitrite mutants of Neurospora. Arch. Biochem. Biophys., 51:379-394.

Silver, W. S. (1957). Pyridine nucleotide-nitrate reductase from Hansenula anomala, a nitrate reducing yeast. J. Bact. 73:241-246.

Smith, L. (1954). A study of some oxidative enzymes of Baker's yeast. Arch. Biochem. Biophys., 50:285-98.

Snell, F. D. and Snell, C. (1949). In Colorimetric Methods of Analysis, 3rd ed., Vol. 2, D. Van Nostrand Co., New York.

Taniguchi, S., Mitsui, H., Toyoda, J., Yamada, T., and Egami, F. (1953). The successive reduction from nitrate to ammonia by cell-free bacterial enzyme

systems. J. Biochem. (Japan) 40:#2, 175-185.

Taniguchi, S. and Itagaki, E. (1960). Nitrate reductase of nitrate respiration type from E. coli. Biochim. Biophys. Acta., 44:263-79.

Virtanen, A. I. and Routanen, N. (1952). Nitrogen assimilation. The Enzymes, J. B. Sumner and K. Myrback (eds) Vol. 2, 1089-1130.

Vogel, H. J. (1957). Repression and induction as control mechanisms of enzyme biogenesis: the "adaptive" formation of acetylornithinase, p. 276-289. In W. D. McElroy and H. B. Glass (eds), The Chemical Basis of Heredity, Johns Hopkins Press, Baltimore, Md.

Yagi, K., Ozawa, T. and Okada, K. (1959). Mechanism of inhibition of D-amino acid oxidase. Biochim. Biophys. Acta., 35:102-10.

Yagi, K. and Ozawa, T. (1959). Binding of the sulphydryl group in D-amino acid oxidase APO-protein with

flavin adenine dinucleotide. Nature, 184:1227-28.

Yamagata, S. (1938). Nitrate reduction by Bacterium coli. Acta Phytochim. (Japan) 10:283-95.

Yamagata, S. (1939). Nitrate reductase and a new nitrite reductase from Bacillus pyocyaneus. Acta Phytochim. (Japan) 11:145-57.

Yates, R. A. and Pardee, B. (1957). Control by uracil of formation of enzymes required for orotate synthesis. J. Biol. Chem. 227:677-692.