

AN EXPERIMENTAL INVESTIGATION OF THE
RESPIRATORY AND ASSIMILATORY FORMS OF NITRATE REDUCTASE
IN ESCHERICHIA COLI STRAIN B.

by

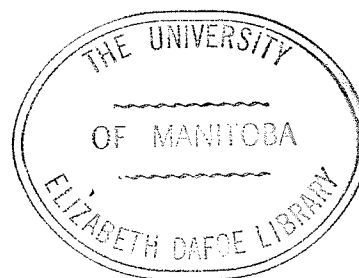
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ABSTRACT

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The properties of the respiratory (dissimilatory) form of nitrate reductase and the assimilatory of the same enzyme from E. coli cells were investigated. It was shown that each enzyme form was attached firmly to cellular particulate material. Although each enzyme is reported in the literature to differ in it's electron donor requirements, no method for differential assaying could be established.

Immunochemical investigations showed that the specific antibody against the highly purified respiratory enzyme form could cross react with the assimilatory enzyme form. Induction studies indicated that as the assimilatory form of nitrate reductase was induced the cellular protein content increased along with the antigenic capacity. These similarities between the respiratory and assimilatory form of nitrate reductase would indicate that little, if any, differences exist between the two forms.

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HISTORICAL

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General

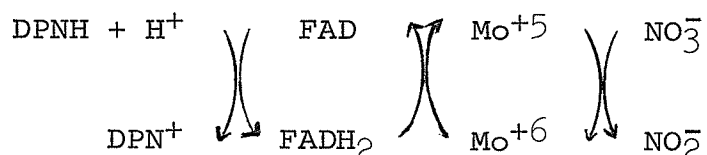
Escherichia coli is classed with a group of micro-organisms known as aerobic or facultatively anaerobic (1). This ability to grow under conditions of either high or low oxygen tension coupled with a rather unusual metabolic utilization of nitrate nitrogen have been the object of much experimental investigation. Although the biological reduction of nitrate was observed as early as 1868 by Schonbein and somewhat later by Quastel et al (2), the enzyme that catalyzed this reduction was not isolated in cell free extracts until 1934 when Green, Stickland and Tarr (3) demonstrated the presence of a nitrate reductase (NaR) in E. coli. The ability of this organism to reduce nitrate was subjected to further studies under aerobic as well as anaerobic conditions and it eventually became apparent that a multifunctional system existed involving two distinct metabolic processes. Under aerobic conditions nitrate nitrogen was reduced stepwise to the level of ammonia and was eventually incorporated into cellular

organic material. Fewson and Nicholas (4) proposed that this process be referred to as nitrate assimilation. The second process was described by Sato (5) as nitrate respiration and involved the use of nitrate as a terminal electron acceptor under conditions where oxygen, the normal acceptor, was absent.

Nitrate Assimilation

The first step in this process is catalyzed by an inducible sulfhydryl-molybdo-flavoprotein, initially characterized from Neurospora crassa by Nason and Evans (6). Later a similar inducible enzyme was demonstrated to occur in E. coli strain B (7) under aerobic growth conditions in a minimal medium containing NaNO_3 . The enzyme was purified 15 fold and was shown to require flavin-adenine dinucleotide (FAD) or flavin mononucleotide (FMN) and molybdenum as cofactors in order to catalyze a reduced pyridine nucleotide dependent reduction of nitrate. The enzyme was specific for reduced diphosphopyridine nucleotide (DPNH), although reduced triphosphopyridine nucleotide (TPNH) also reacted to a limited extent. The first product of nitrate reduction

proved to be nitrite (8). The sequence of electron transfer in the reduction of nitrate was postulated by Nicholas and Nason (7) to be as follows:



Soluble nitrate reductase was also prepared by Itagaki and Taniguchi (9) from cells grown aerobically on the minimal medium of Nicholas and Nason (7) containing a yeast extract was also more dependent upon DPNH as the electron donor than TPNH, but inhibitor studies indicated that the system apparently contained cytochrome b_1 .

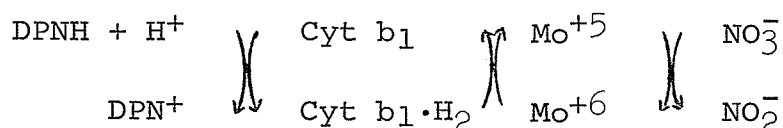
Nitrate Respiration

This nomenclature was originally proposed by Sato (5) who prepared cell free extracts of E. coli grown under anaerobic conditions on a complex medium containing nitrate for enzyme induction. The nitrate reductase in the cell free extracts was found to be associated with particulate material in a relatively insoluble form. In the particulate extract he demonstrated a partial oxidation of cytochrome b_1 concomitant to the reduction of nitrate. With the aid of

advanced spectroscopic techniques he was able to demonstrate the oxidation of this respiratory pigment within the living cell when nitrate was added to cellular suspensions.

Specific inhibition of cytochrome b_1 by 2-heptyl 4 hydroxyquinoline N oxide prevented the demonstration of electron transfer to nitrate in systems containing Sato's enzyme extract (10).

Taniguchi, Sato and Egami (11) prepared cell free extracts of E. coli grown anaerobically on a peptone broth-agar medium containing nitrate. These extracts possessed a particulate nitrate reductase capable of reducing nitrate to nitrite. Later Taniguchi and Itagaki (12) prepared similar extracts which contained cytochrome b_1 as an intermediate electron carrier from formate or DPNH to nitrate. Physiological electron donors were shown to be much less efficient than the artificial donors, methyl and benzyl viologen. The enzyme proved to have a molecular weight of one million with atoms of iron and molybdenum bound to it. Their proposed scheme of electron transfer is outlined below:



INTRODUCTION

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Although a wealth of scientific data exists concerning the reduction of nitrate to nitrite by E. coli, many aspects of this reduction remain unanswered. Foremost of these is the nature of the enzyme. Does the same enzyme catalyze both the assimilatory and the respiratory reactions or do two different forms of nitrate reductase exist, each dependent upon the oxygen tension during growth?

In chemical constituents nitrate reductase appears to be a sulfhydryl-molybdo-protein, these structures being identified in both the assimilatory and respiratory forms. The presence of an iron containing heme portion seems apparent in the anaerobic form but of doubtful existence in the aerobic one. Although both enzymes have similar electron donor requirements (DPNH and benzyl viologen (BVH) are effective in either system while TPNH is not) a discrepancy exists in their electron carrier requirements. While the aerobic form requires FAD or FMN and no cytochromes (7), the anaerobic form demands the opposite (12). It would, therefore, appear that independent assay systems

could be established for each form.

With independent assay systems it would then be possible to show a conversion of the enzyme from one to the other form during anaerobic to aerobic transition if such were indeed the case. If anaerobic cultures were incubated for 18 hours a differential measurement of nitrate reductase activity would give an indication of the total anaerobic form present while basal amounts of the aerobic form could be demonstrated. The cultures could be aerated vigorously and the formation of the aerobic form followed by differential assaying.

It has been reported that the aerobic form of nitrate reductase is soluble (7) (9), while the anaerobic form is particulate in nature (12). The notion of solubility with respect to the aerobic form has not been well defined. The anaerobic form, however, can be precipitated by centrifugation at $40,000 \times g$ (12). If the particulate form could be solubilized it would indeed be interesting to compare the properties of the two enzyme forms when both were in the same state.

As a high degree of specificity has been demonstrated for precipitin reactions (13) it would also seem probable

that immunological methods could be used to demonstrate similarities or differences between the two enzyme forms. If a highly specific antibody could be produced against one pure form of the enzyme it would then be interesting to determine the ability of that antibody to react with the other enzyme form.

In an effort to obtain additional information about the two forms of nitrate reductase it was decided to conduct conversion studies, solubilization experiments and immunochemical investigations into the nature of this enzyme.

MATERIALS AND METHOD

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Enzyme Source

Escherichia coli strain B was used as the biological source of nitrate reductase. The organism was grown under one of three possible conditions:

- i) aerobic on a minimal medium.
- ii) aerobic on a complex medium.
- iii) microaerophilic on a complex medium.

The minimal medium was that used by Nicholas and Nason (7) with an added enzyme inducer or ammonia. The constituents of the medium were:

MgSO ₄ ·7H ₂ O	0.2 gm/l
NaCl	0.5 gm/l
KH ₂ PO ₄	3.0 gm/l
Na ₂ HPO ₄	6.0 gm/l
Dextrose	4.0 gm/l
NH ₄ Cl	3.0 gm/l
KNO ₃	1.0 gm/l
(enzyme inducer)	

Bacto Penassay Broth (Difco Antibiotic Medium 3) was used

as the complex medium with added KNO_3 for optimal enzyme induction. This medium, which is very similar to Taniguchi's (12) complex medium, is composed of:

Beef extract	1.50 gm/l
Yeast extract	1.50 gm/l
Peptone	3.00 gm/l
Dextrose	1.00 gm/l
NaCl	3.50 gm/l
K_2HPO_4	3.68 gm/l
KH_2PO_4	1.30 gm/l
KNO_3	1.00 gm/l
	(enzyme inducer)

Standard inocula for each growth condition were prepared from cultures maintained in vegetative states under parallel conditions. Inocula were standardized with the aid of the McFarland Nephelometer. Two ml of inoculum containing approximately 10^9 cells were added to each litre of medium. Aerobic cultures (500 ml per litre flask) were shaken briskly on a rotary shaker while microaerophilic cultures were grown without shaking in three litre flasks that were filled to the top and stoppered with a metal cap in such a way that no air was trapped under it. The tops

were then sealed with parafilm prior to incubation.

After inoculation, the cultures, regardless of medium or oxygen tension, were incubated at 28°C for 18 hours. Upon completion of incubation the cells were collected by centrifugation and washed in 0.85% NaCl until washings gave a negative nitrite test. The cells were frozen at -15°C for 24 hours prior to their disruption and enzyme extraction.

Reagents

1. Electron donors - Reduced DPNH was obtained from the Sigma Chemical Company.

- Benzyl viologen in the oxidized form was procured from the Mann Research Laboratories.

Reduced benzyl viologen was prepared in a nitrogen equilibrated Thunberg tube containing 3 ml of the dye in the main chamber and 6 mg of sodium hydrosulphite in the side arm. The reduced dye (0.1 ml) was transferred into assay tubes with the aid of a calibrated, long tipped Pasteur pipette.

2. Electron carrier - Flavin adenine dinucleotide (FAD) was obtained in the oxidized state from the Sigma Chemical Company.

3. Substrate - Reagent grade NaNO_3 , free from nitrite, was used as the enzyme substrate.

4. Wetting agent - Sodium lauryl sulphate (Duponol C) was obtained from the Dupont Chemicals Department. This reagent was shown to be nitrite free when prepared in the form of a saturated solution at room temperature.

Nitrite Determination

The measurement of nitrite was based on the formation of a red azo compound (14). This involved first the reaction in acid solution of a primary amine (sulphanilamide) with any nitrite present so that a diazonium salt was formed. The latter was then coupled to an aromatic amine (N (1 naphthyl) ethylenediamine hydrochloride) to yield the red azo dye whose concentration was then determined in a colorimeter.

Protein Determination

When assaying for nitrate reductase activity the protein content of cell free extracts was determined by using the Folin-Phenol reagent in accordance with the method of Lowry et al (15). Protein determinations on the insoluble antigen-antibody complexes were made using the ultraviolet absorption technique of Gitlin (16).

In both nitrite and Lowry protein determinations color densities were read with the aid of a Klett-Summerson Colorimeter and correlated to standards of NaNO_2 and Bovine Serum Albumin (BSA) respectively.

Demonstration of Nitrate Respiration and Assimilation

Eighteen 50 ml Erlenmeyer flasks containing 25 ml of complex medium with inducer were all inoculated at zero time with the same number of cells that had been grown on minimal medium without inducer. The flasks were then divided into two groups, the first group was subjected to shaking, i.e., aerobic conditions, while in the second group each flask was filled, sealed and incubated without shaking

to maintain microaerophilic conditions. Both groups were incubated at 28° C. After various time intervals a flask from each group was analysed for nitrite content of the media and number of cells per mg dry weight. The results are shown in Table 1.

Definitions

1. Enzyme units - these were defined as the number of millimicromoles (μmoles) of nitrite produced per ml of extract in one hour.

2. Antienzyme units - the amount of antibody required to neutralize one unit of nitrate reductase.

3. Specific activity - this was expressed in terms of enzyme units per mg of protein in one ml of enzyme extract.

Enzyme Assay

1. BVH method - the assay was carried out in open tubes containing 0.4 ml of 0.1 M NaNO_3 , 0.1 ml of 1×10^{-4} M BVH, 0.1 M phosphate buffer, pH 7.2, and samples of the enzyme extract so that the final volume was 1.0 ml. The

reaction was started by the addition of BVH to a preincubated system of the remaining constituents. The mixture was incubated at 28°C , and the reaction was terminated after the required length of time by oxidizing the remaining BVH (merely by shaking). In order to separate the protein from nitrite formed, 0.5 ml of wetting agent was added. Nitrite was determined in the manner previously described.

2. DPNH method - aerobic enzyme extracts exhibited a strong DPNH oxidase system and hence the assay was conducted under anaerobic conditions in Thunberg tubes. Microaerophilic extracts did not possess this oxidase system and, therefore, assaying was done in open tubes. The reaction system was composed of 0.4 ml of 0.1 M NaNO_3 , 0.1 ml of 1×10^{-4} M FAD, 0.08 ml of 4.4×10^{-3} M DPNH, 0.1 M phosphate buffer, pH 7.2, and enzyme extracts to make a final volume of ml. In each type of tube the reaction was started by the addition of DPNH, allowed to progress for the required length of time and then stopped by oxidizing any remaining DPNH with the addition of pyruvic acid and lactic acid dehydrogenase. Before determining nitrite produced,

0.5 ml of sodium lauryl sulphate was added.

In each of these assay methods the following control systems were always utilized:

- i) boiled extract substituted for normal extract.
- ii) complete system minus substrate.
- iii) complete system minus enzyme extract.

Preparation of Crude Extract

Cells grown under conditions of nitrate reductase induction were ground in a mortar with twice their weight of acid washed levigated alumina powder and 5 volumes of 0.1 M K_2HPO_4 buffer at pH 7.2. Cysteine hydrochloride, neutralized before use, at a final concentration of 1×10^{-4} M was incorporated into the extraction buffer. Initially the cells and alumina were ground for 10 minutes and then small volumes of buffer were added over a period of continual grinding lasting 20 minutes. The resulting slurry was then centrifuged at $2,000 \times g$ for 20 minutes, in order to precipitate the alumina and any remaining whole cells. After centrifugation the supernatant was collected and used as a crude extract for further work. This entire

extraction procedure was executed at 4° C.

Demonstration of Enzymic Activity

The enzyme in cells grown under aerobic or micro-aerophilic conditions was assayed after having established the following conditions:

i) Linearity with respect to time - the optimal time for incubation was determined by using a fixed volume of enzyme (0.04 ml) in the normal BVH assay system and incubating the reaction tubes for varying time intervals. Figures 1 and 2 refer.

ii) Effect of increasing enzyme concentration - after the optimal reaction time was established, further BVH assay was done in order to determine the effects of increasing volumes of enzyme extract. The optimal volume of extract to be used in future assays was then determined from Figures 3 and 4.

Sedimentation of Nitrate Reductase from Cell Free Extracts

Crude extracts containing nitrate reductase were prepared from aerobic cultures grown on minimal medium and

microaerophilic cultures grown on complex medium. The initial BVH-NaR activity was determined in each crude extract, then 10 ml volumes from each extract were centrifuged at increasing speeds for 15 minute intervals. The activity of each precipitate was then calculated. Figure 5 shows the effect of increasing centrifugal forces on the sedimentation of nitrate reductase of each crude extract.

Enzyme Purification Procedures

A crude extract, prepared from cells grown under microaerophilic conditions was centrifuged at 35,000 x g for 45 minutes. The pellet was resuspended in half of the original volume of phosphate buffer (0.1 M K_2HPO_4 , pH 7.2), containing 1×10^{-3} M cysteine and then heated in a water bath for 6 minutes at 57° C. The extract was immediately cooled in an ice bath and the coagulated protein was centrifuged off (2,000 x g for 20 minutes). The nitrate reductase activity was retained in the supernatant solution. The enzyme was precipitated with the particulate material by centrifugation of the supernatant solution at 35,000 x g

for 45 minutes.

The enzymatically active precipitate was suspended in a small volume of sodium bicarbonate buffer (0.02 M, pH 8.3) containing 0.85% NaCl and 1×10^{-3} M cysteine. The enzyme was solubilized according to the method of Morton (17). For each 25 ml of bicarbonate suspension, 7.5 ml of chilled n-butyl alcohol was added, the mixture was stirred gently for eight minutes, and then centrifuged at $35,000 \times g$ for 45 minutes. This centrifugation resulted in the formation of four visible layers within the centrifuge tube. The top layer, approximately 1 mm thick, had a solid consistency with a density of less than 0.81; the next layer was apparently pure n-butyl alcohol which could be decanted off. The third layer was shown to contain the enzyme while the fourth layer consisted of an inactive precipitate. The third layer was routinely collected and concentrated by lyophilization. The lyophilized enzyme could then be stored for periods of at least 5 months without any loss of activity. A summary of purification steps is presented in Table 2.

Demonstration of Enzyme Purity

Disc electrophoresis studies, similar to those conducted by Reisfeld et al (17), were done on lyophilized extracts resuspended in phosphate buffer. Electrophoresis was done in a vertical glass tube, 4 cm long and 0.4 cm internal diameter, containing a specially processed acrylamide-methylene bis acrylamide gel marketed by Canalco Company. After the electrophoretic run the gel was stained with bromophenol blue and then destained in 7.5% acetic acid. A control system consisting of four known proteins was also subjected to this technique.

Immunization Scheme

Mature albino rabbits that had not been used previously for immunological investigations were injected subcutaneously in the nuchal region with purified nitrate reductase from microaerophilic cultures. The enzyme was emulsified prior to injection using the adjuvant techniques of Freund (19) and Cohn (20). Four separate emulsified injections were given, each seven days apart. The total protein content of

each injection was between 1.8 and 2.3 mg.

Prior to the series of injections a sample of normal rabbit serum (NRS) was collected, also before each injection a fresh serum sample was subjected to a ring test in order to obtain a rough indication of the animal's response to the antigen. This test was done by layering 0.2 ml of undiluted antigen (enzyme) over 0.2 ml of concentrated serum. The reaction system, contained in a small fermentation tube, was observed for the appearance of a turbid area at the interfacial zone. The velocity of the formation of the turbid zone was taken as an indication of antibody potency.

After seven days from the last injection the rabbits were deprived of food and water for 18 hours and subsequently exsanguinated. The blood was collected and incubated at 37° C for one hour, ringed, and then allowed to stand at 4° C for 12 hours, before clarification by centrifugation. Two ml aliquots of the serum were distributed in sterile tubes which were then stored at -15° C. No preservatives were added to the serum.

'In vitro' Reactions

Before each 'in vitro' testing, the enzyme and anti-serum were centrifuged at $8,000 \times g$ for 30 minutes to remove any particulate matter. Unless otherwise stated all saline solutions consisted of 0.15 M (0.85%) NaCl.

Glassware used during 'in vitro' testing was washed in a potassium dichromate-sulphuric acid cleaning solution. A standard incubation routine of 30 minutes at 37°C followed by 12 hours at 4°C was observed. Throughout these tests the pH of enzyme extracts was constant at 7.2. At the end of the incubation period the reaction mixtures were centrifuged for 20 minutes at 3,000 r.p.m. in a Servall Angle centrifuge before precipitates were read using a Fisher-Kahn Viewer.

Serum Dilution Titre

As the quantity of antigen present in a precipitin reaction system is critical owing to its inhibitory effect at high concentrations, it was essential that initial testing be done to determine a workable antigenic range. For this the serum dilution technique of Martin (21) was followed.

Various dilutions of antiserum were each titrated with antigen and after incubation the reaction systems were observed for the formation of precipitates, Table 4 refers. The end point of the system occurred where a visible precipitate was formed in the tube containing the highest dilution of antiserum and the least amount of antigen. Three controls were included with the experimental system:

- i) antigen plus saline.
- ii) antigen plus NRS plus saline.
- iii) antigen plus unrelated antiserum plus saline.

The unrelated antiserum was prepared from rabbits using sonicated extracts of Lactobacillus plantarum as the antigenic source.

Establishment of Accurate Titre

From the initial serum dilutions it was apparent that the antiserum titre was between 160 and 320, hence further serum titration was done to establish the accurate titre. One fifth of a ml of optimal concentration of antigen was used in each reaction tube with dilutions of antiserum ranging from 1:120 to 1:320 and decreasing by an

additive factor of 1:20. The formation of precipitates is recorded on Table 5.

The Effect of NRS and BSA

NRS and BSA were tested for any inactivating effect upon the enzyme and also for any non specific cross reaction. The system was set up as shown in Table 3, the antigen was used in optimal dilutions while the final dilution of all sera was 1:20. In addition to the normal controls, extras were used in order to compensate for enzyme denaturation throughout the incubation period. After incubation the system was observed for the formation of precipitates and the supernatants from each tube were assayed for nitrate reductase. Inactivation of the enzyme by non specific sera could then be determined by mathematical calculation.

The precipitates from control tubes C1 and C2 were carefully washed twice in cold distilled water and finally resuspended in buffer. This suspension was substituted for an enzyme sample in the standard BVH-NaR assay. It was possible in this manner to demonstrate that the insoluble

antigen-antibody complex possessed definite enzymic activity.

Establishment of Neutralization Time

The reaction system as shown in Table 6 was set up in order to determine the exact time required to complete phase one of the precipitin reaction, i.e., the non visible union of antigen and antibody. Sufficient controls were included to compensate for enzyme denaturation at each step of this timed reaction and also to correct for serum interference in the nitrite determinations. A disappearance of nitrate reductase activity in the reaction systems, independent of enzyme denaturation, indicated a non visible combining of antigen and antibody.

Demonstration of Equivalence Zone

Preliminary testing had given only a rough indication of optimal conditions required for precipitation reactions, it was therefore essential that the entire precipitin curve be determined. To do this increasing units of antigen were added to a constant amount of antibody, two identical systems were established (Table 7). After incubation the

reaction tubes were centrifuged and the supernatant solutions carefully separated. In the first reaction system the supernatant was tested for remaining nitrate reductase activity while those of the second system were divided into two fractions. To the first fraction (one third of the reaction volume) an excess of antigen was added and to the second fraction (the remainder of the reaction volume) an excess of antibody was added. The systems were incubated and then precipitate formation was noted.

The insoluble precipitate from each tube was washed gently twice in cold distilled water and finally dissolved in 5.0 ml of 0.25 N acetic acid. The Optical Density (OD) of each precipitate-acetate solution was measured in a Beckman DB Spectrophotometer at a wavelength of 277 mμ. The same method was used to determine the OD of antigen concentrations identical to those used in the reaction tubes. By using the method of Gitlin (16) it was possible to determine the amount of antibody protein in each precipitate by substituting in the following formula:

$$M = \frac{D_{sp} - D_x}{E_{1cm}^{1\%} (AB)} \cdot V \cdot \frac{1000}{100}$$

where: M = the amount of antibody protein (mg) in the precipitate.

D_{sp} = OD of specific precipitate.

D_x = OD of the antigenic protein used in the reaction systems.

V = the volume of acetate used to dissolve the specific precipitates.

$E_{1cm}^{1\%} (AB)$ = the extinction coefficient of rabbit antibodies (14.4).

The entire precipitin curve was presented graphically by plotting increasing enzyme units on the abscissa and μ gm of antibody protein on the left ordinate. To demonstrate further the zone of antigen excess, remaining enzyme units were plotted on the right ordinate against the same abscissa (Figure 6).

Calculating Anti-enzyme Units

In order to determine the amount of antibody required to neutralize one unit of nitrate reductase a system as shown in Table 8 was prepared. This consisted of antibody

titrated against a constant amount of antigen present in optimal quantities. Sufficient controls were included to correct for increasing quantities of antibody.

Purification of Antiserum

Disc electrophoresis testing had shown that a mixture of two proteins was present in purified nitrate reductase extracts. Therefore it was possible that the antiserum was not of a homologous nature and hence absorption of any contaminants was essential. Crude extracts were prepared from cells grown on minimal medium without enzyme inducer (nitrate). Only basal amounts of nitrate reductase activity could be shown in these extracts. From equivalence zone studies the optimal amount of antigen protein required in a 2.0 ml reaction system was 0.038 mg. As a fairly large amount of absorbed antiserum was required a system as shown in Table 9 was set up in one litre flasks.

All four litre flasks were incubated under the standard conditions. Precipitates were determined after centrifugation. The supernatant from flask 1 was subjected

to fractional precipitation with 33% ammonium sulphate in order to precipitate the globulin content and hence the antibody to nitrate reductase. The precipitate was suspended in 0.85% NaCl and stored for further 'in vitro' testing.

Titration of the Assimilatory Form of Nitrate Reductase

Varying concentrations of the assimilatory form of nitrate reductase were added to an optimal amount of absorbed antiserum. Negative controls were established to correct for this increasing amount of enzyme and also a positive control containing the respiratory enzyme and its specific antibody was used. Table 10 refers.

Kinetics of Enzyme Synthesis

Cultures of E. coli were grown on minimal medium without added nitrate and after standard incubation the cells were collected and washed aseptically. The washed cells were suspended in a solution containing 0.1 M NaNO_3 and 0.1 M K_2HPO_4 buffer (pH 7.2) and then shaken briskly. At the end of two hour time intervals a sample of the

suspension was removed and the cells collected from it. The induced nitrate reductase was extracted from each sample of cells and assayed for enzyme units and protein content. Precipitation systems were then set up so that the number of enzyme units precipitated from optimal amounts of antigen could be determined. The results are represented graphically in Figure 7.

RESULTS

RESULTS

Demonstration of Nitrate Respiration and Assimilation

Under different conditions of growth the cell yield is three times greater in aerobic cultures compared to that obtained in microaerophilic cultures. This is clear from the data presented in Table 1. It is noteworthy that the amount of nitrite excreted into the media per mg dry weight of cells under microaerophilic conditions is greatly in excess of that accumulated under aerobic conditions. This seems to be due to the increased nitrate reductase content of the cells grown under a severe limitation of oxygen.

Enzyme Concentration and Reaction Time

The optimal time of incubation for both the respiratory and assimilatory forms of nitrate reductase is 5 minutes, (Figures 1 and 2). The optimal enzyme concentration of the assimilatory form is 0.15 ml (Figure 3), while that of the respiratory form is 0.07 ml (Figure 4).

This would again indicate that the enzyme concentration is higher in cells grown under microaerophilic conditions.

Sedimentation of Nitrate Reductase from Cell Free Extracts

Crude extracts of the assimilatory and respiratory forms of nitrate reductase were subjected to differential centrifugation. The amount of enzyme precipitated in each case showed a constant increase with increasing centrifugal force. When the data is presented graphically a parallel relationship between the two forms is apparent (Figure 5). It is clear that nitrate reductase, under both aerobic and microaerophilic conditions, is associated with particulate material of the cells.

Enzyme Purification

A summary of the purification procedure for the respiratory form of nitrate reductase is outlined in Table 2. The combined effects of differential centrifugation, heat denaturation and alcohol solubilization made possible a 325 fold purification. Disc electrophoresis studies on purified extracts indicated the presence of two protein

bands. The enzyme preparation thus seems to be contaminated with a minor unrelated protein fraction.

Table 1

A comparison of cell yield and nitrite excretion
under aerobic and microaerophilic conditions

Time (hours)	Cells Grown Aerobically			Cells Grown Microaerophilically		
	$\mu\text{moles of NO}_2$ excreted per ml	Dry weight of cells (mg/ml)	$\mu\text{moles of NO}_2$ excreted per mg dry weight	$\mu\text{moles of NO}_2$ excreted per ml	Dry weight of cells (mg/ml)	$\mu\text{moles of NO}_2$ excreted per mg dry weight
0	0	0	0	0	0	0
2	0.003	0.9	0.003	0.03	0.21	0.14
4	0.014	2.8	0.005	0.46	1.40	0.32
6	0.480	5.6	0.087	2.00	2.80	0.72
8	1.10	7.0	0.160	4.60	4.20	1.10
10	2.55	11.2	0.228	6.10	4.62	1.32
12	2.60	11.6	0.228	6.90	5.11	1.35
18	2.80	12.6	0.228	7.70	5.60	1.36
24	3.05	8.4	0.360	8.40	3.85	2.11
30	3.10	8.4	0.370	8.40	2.80	3.00

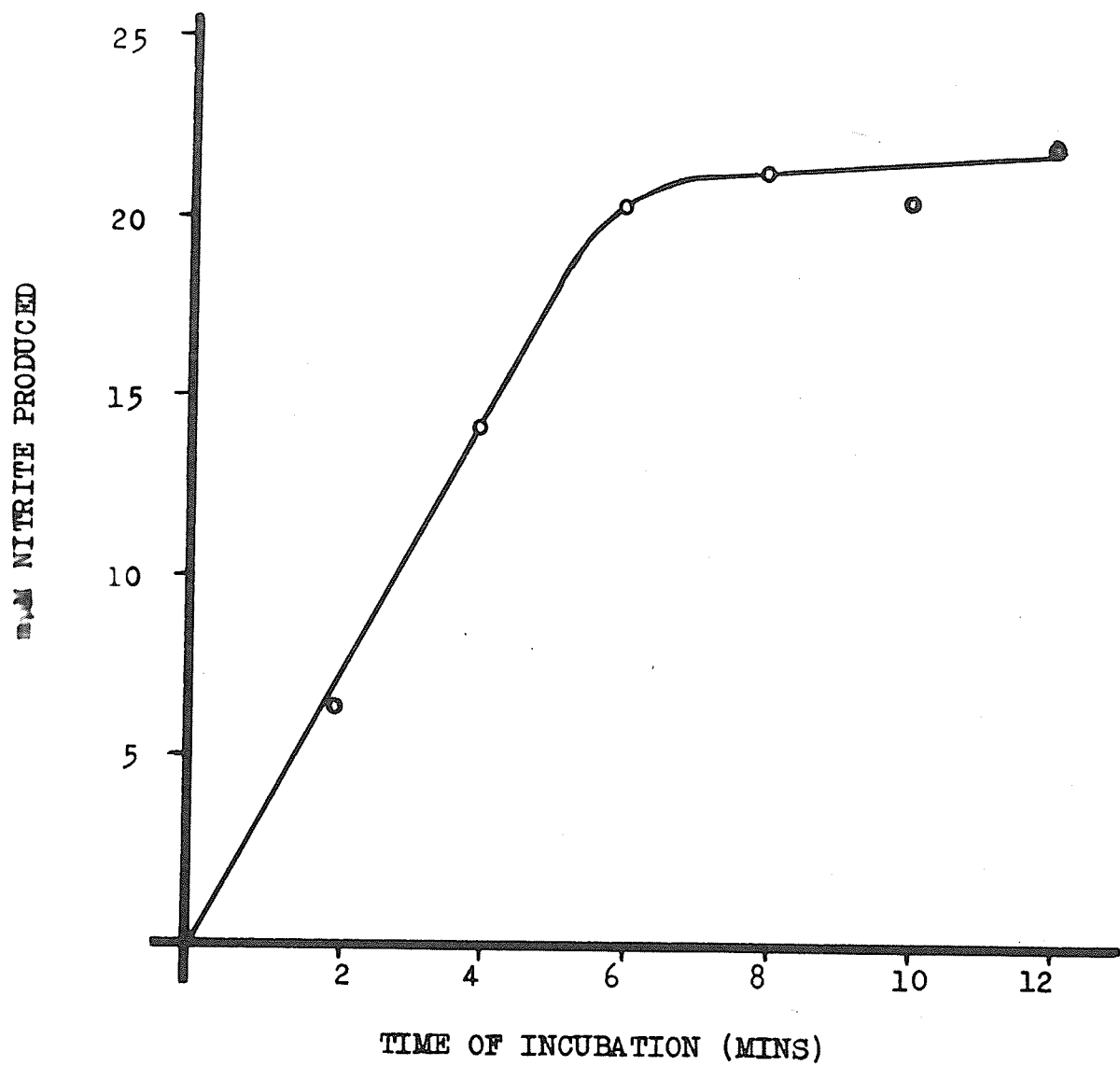


Figure 1. The effect of increasing incubation time on the activity of nitrate reductase from aerobic extracts.

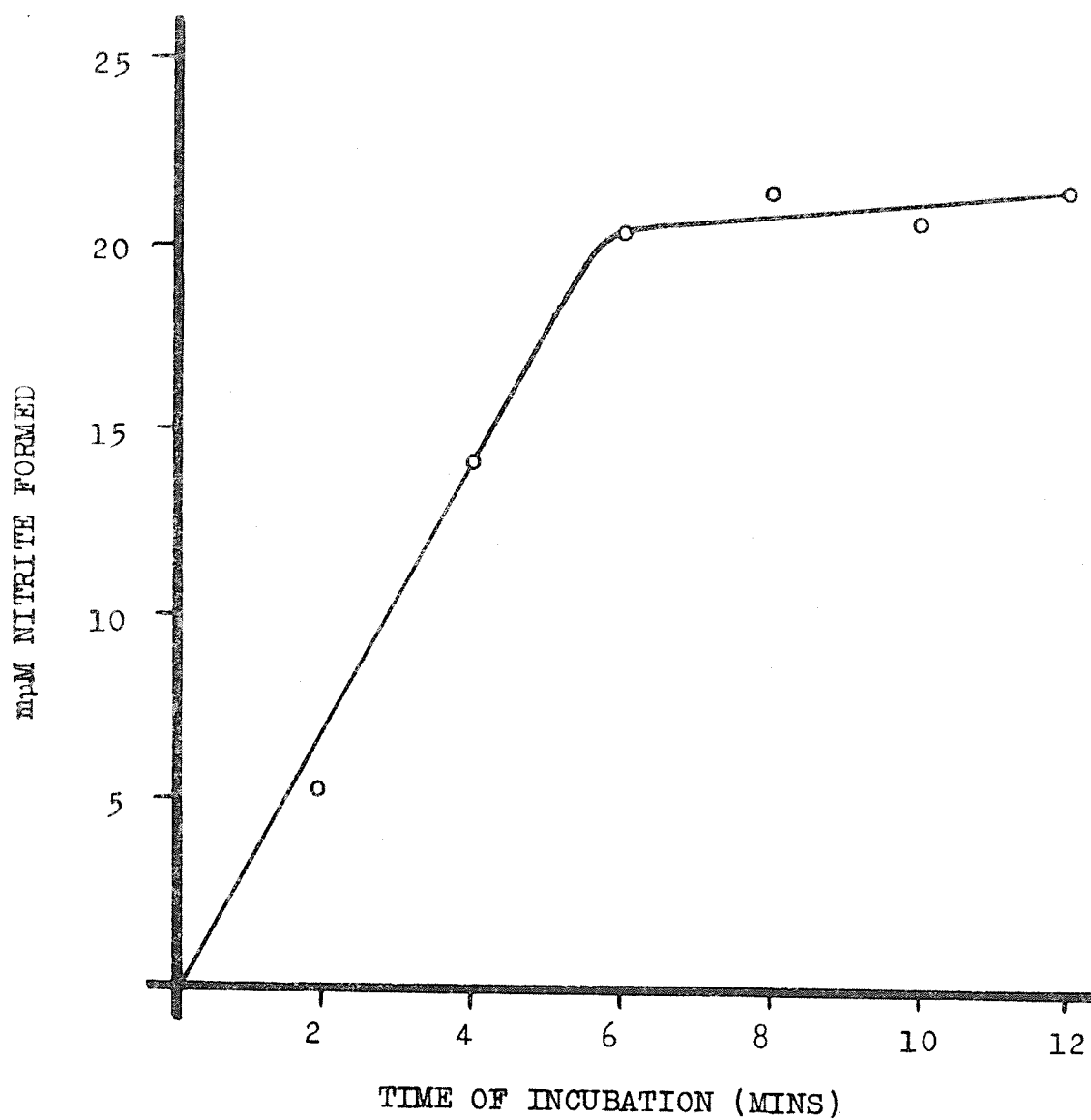


Figure 2. The effect of increasing incubation time on the activity of nitrate reductase from microaerophilic extracts.

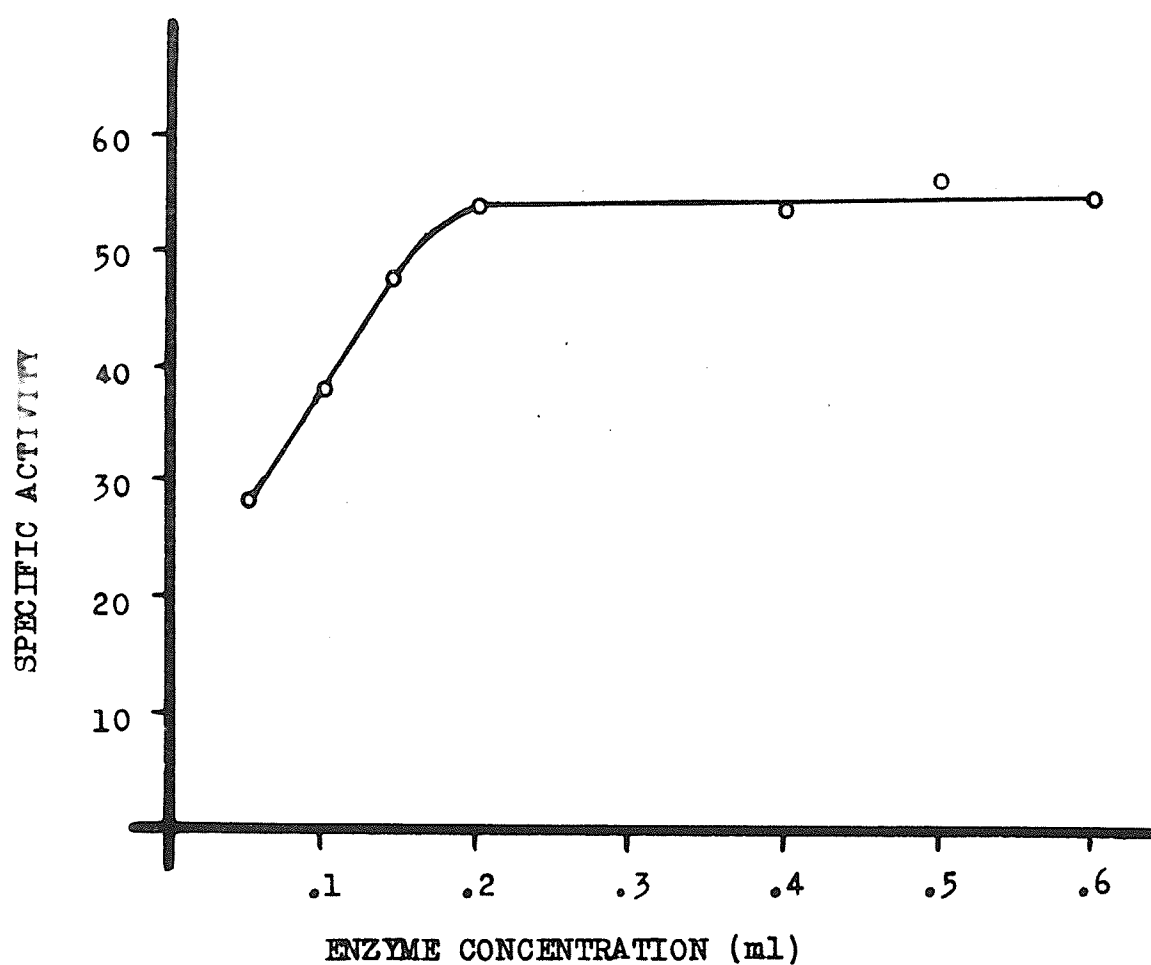


Figure 3. The effect of enzyme concentration on the activity of nitrate reductase from aerobic cultures.

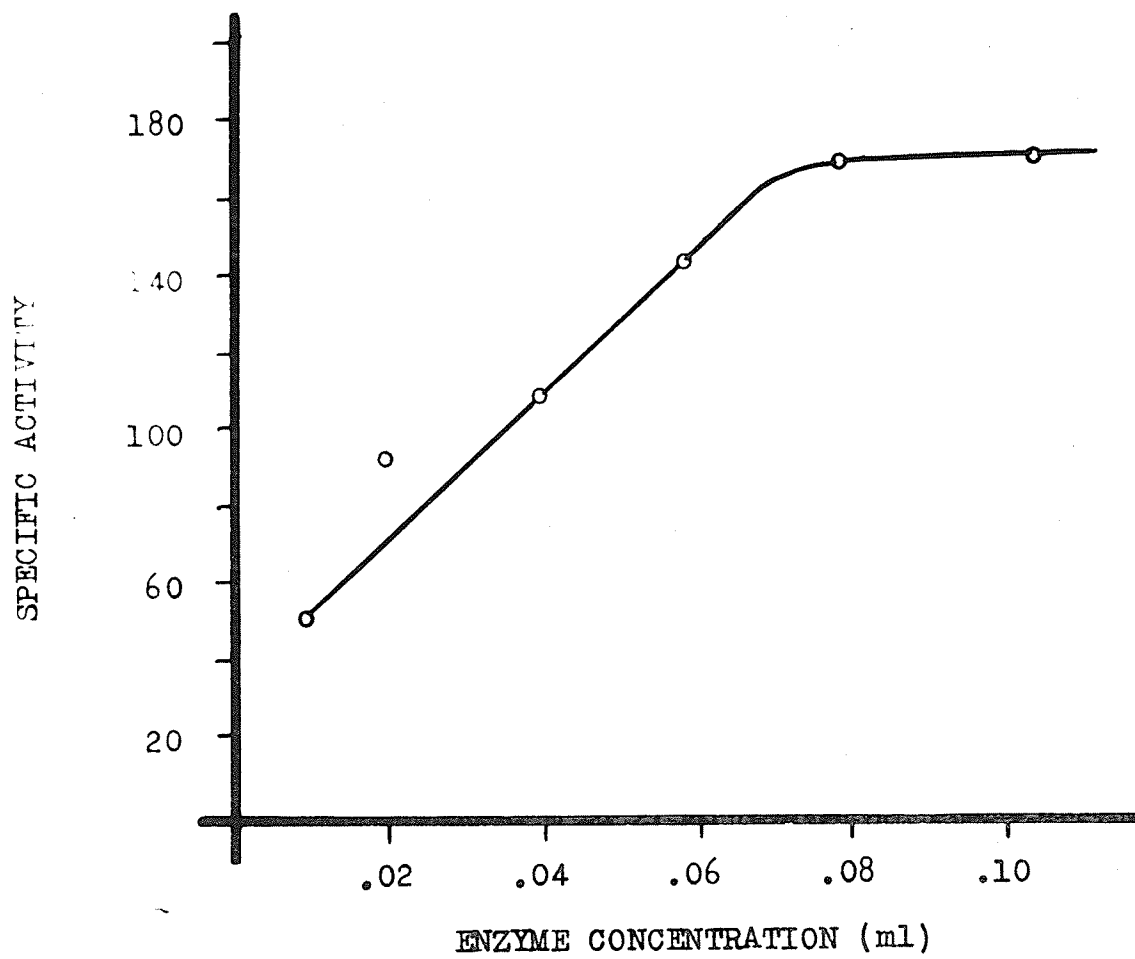


Figure 4. The effect of enzyme concentration on the activity of nitrate reductase from microaerophilic cultures.

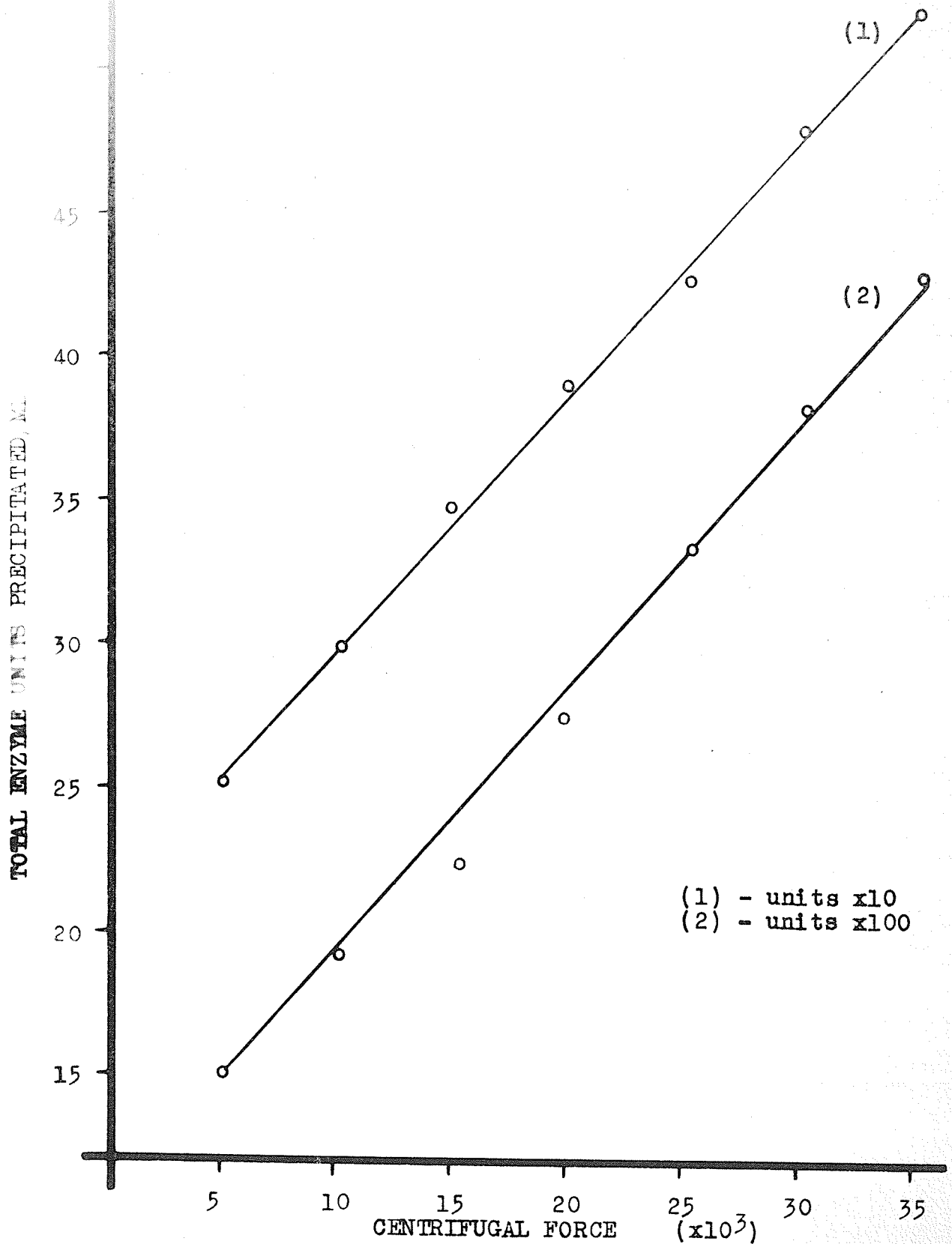


Figure 5. The effect of increasing centrifugal acceleration upon the sedimentation of nitrate reductase from crude extracts prepared from cells grown microaerophilically on complex media (2) and aerobically on minimal media (1).

Table 2

A summary of the purification procedures for nitrate reductase

Step	Total Volume	Total Protein	Protein (mg/ml)	Units (per ml)	Specific Activity	Purification
1. Crude extract from 47.3 gms of cells (wet weight)	340 ml	3026.0 mg	8.9	4800.0	.54 x 10 ³	-
2. Precipitate after 35,000 x g	170 ml	204.0 mg	1.2	8700.0	7.3 x 10 ³	13.5
3. Active precipitate heating and re- centrifuging at 35,000 x g	170 ml	110.5 mg	0.65	15600.0	24.0 x 10 ³	44.4
4. After solubilization (third layer)	85 ml	4.08 mg	0.048	8400.0	175.5 x 10 ³	325.0

Immunochemical Reactions

Controls used during 'in vitro' testing failed to show any non specific cross reaction with BSA, NRS and antiserum against an unrelated antigen. Reaction tubes containing enzyme plus saline and antienzyme plus saline did not form precipitates (Table 3). It was demonstrated further that BSA and NRS did not inactivate enzyme activity, as enzymic activity remaining in systems containing these sera was essentially the same as that remaining in systems possessing no sera (Table 3). Positive reaction systems were judged firstly on the basis of precipitate formation and secondly on the disappearance of the enzyme from the incubation mixture. Insoluble precipitates possessed the ability to reduce nitrate enzymatically. All enzymic activity disappearing from supernatants could be relocated in the insoluble precipitates.

Initial serum dilutions following the method of Martin (21) indicated the rough antienzyme titre to be between 160 and 320 when the final dilution of the enzyme was 1:160 (Table 4). An additional titration system

(Table 5) containing constant enzyme at a dilution of 1:160 and decreasing quantities of antienzyme indicated that the accurate titre of the antiserum is 220. Phase one of the precipitin system, i.e., the invisible combination of enzyme and antienzyme, was completed after 30 minutes of incubation at 37° C (Table 6). Some denaturation of the enzyme occurred during this period, therefore additional incubation was carried out at 4° C.

In order to further characterize the precipitation system the entire precipitin curve was determined (Table 7 and Figure 6). Constant amounts of antienzyme (final dilution of 1:200) were added to increasing amounts of enzyme. After incubation any enzyme remaining in the supernatant was determined immunologically and enzymatically; remaining antienzyme being determined solely by the former method. It was thus possible to show that increasing enzyme concentration inhibited the formation of specific precipitates, while excesses of antienzyme depleted all of the added enzyme. With the establishment of the equivalence zone it was possible to show that one antienzyme unit, i.e., the amount of undiluted antiserum required to neutralize



one unit of nitrate reductase, was 1.9×10^{-5} ml (Table 8).

A titration system consisting of optimal amounts of absorbed antienzyme (Table 9) and increasing quantities of the assimilatory form of nitrate reductase showed that the antibody against the respiratory form combined with the assimilatory form of the enzyme to yield an insoluble precipitate. The disappearance of the assimilatory enzyme from the supernatants in the reaction tubes was shown (Table 10).

Table 3

The effect of NRS, BSA and antiserum on nitrate reductase

Tube	1	2	3	4	5	6	C1	C2	C3	C4	C5	C6
BSA (ml)	0.2	0.2	0.2	-	-	-	-	-	-	-	-	-
NRS (ml)	-	-	-	0.2	0.2	0.2	-	-	-	-	-	-
Antiserum (ml)	-	-	-	-	-	-	0.2	0.2	-	-	0.2	0.2
Enzyme (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	-	-
Saline (ml)	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.6	1.6	1.8	1.8
Precipitate	-	-	-	-	-	-	+	+	-	-	-	-
Enzyme Units in Supernatant	91.	90.	90.5	90.	90.	91.	24.0	24.5	90.	90.	0	0

Table 4

Determination of the rough antiserum titre using a serum dilution technique (21) involving the titration of antigen against various dilutions of antiserum

Antiserum Dilution	Antigen Dilution						
	1:5	1:10	1:20	1:40	1:80	1:160	1:320
1:10	+	+	+	+	+	+	-
1:20	+	+	+	+	+	+	-
1:40	+	+	+	+	+	+	-
1:80	-	-	-	+	+	+	-
1:160	-	-	-	-	-	+	-
1:320	-	-	-	-	-	-	-

Table 5

The establishment of an accurate titre using an optimal amount of antigen, specific activity 1.75×10^5 , with decreasing concentrations of antiserum

Tube	1	2	3	4	5	6	7	8	C1	C2	C3
Enzyme (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	-	0.2
Antiserum dilution	1:120	1:140	1:160	1:180	1:200	1:220	1:240	1:260	-	1:120	1:120 (NRS)
Saline (ml)	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.8	1.6	1.4
Precipitate	+++	+++	++	++	+	+	-	-	-	-	-

Table 6

The establishment of neutralization time in enzyme-antienzyme systems.
Enzyme preparation used had a specific activity of 1.75×10^5 .

Tube	1	2	3	4	5	6	C1	C2	C3	C4	C5	C6	C7	C8
Incubation time (minutes)	10	20	30	60	90	120	10	20	30	60	90	120	120	120
Enzyme (ml)	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	-	-
Antienzyme (ml)	0.2	0.2	0.2	0.2	0.2	0.2	-	-	-	-	-	-	0.2	0.2
Saline (ml)	1.4	1.4	1.4	1.4	1.4	1.4	1.6	1.6	1.6	1.6	1.6	1.6	1.8	1.8
Klett Reading	78	71	67	67	66	64	64	63	63	62	62	60	18	18
Correction for serum (-18)	60	53	49	49	48	46								
Neutralized enzyme units	9.5	24.5	34.5	32	34.5	36								

Table 7

Reaction system used to construct a precipitin curve

Tube	1	2	3	4	5	6	7	C1	C2	C3
Antigen (units)	16.5	82.5	165.0	247.5	330.0	412.5	495.0	495.0	-	495.0
Antibody (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	-	0.2	0.2 (NRS)
Saline (ml)	1.79	1.75	1.7	1.65	1.6	1.55	1.5	1.8	1.8	1.5
Precipitate (total system)	+	+	++	+++	+++	+++	++	-	-	-
Precipitate (1/3 system)	+	+	-	-	-	-	-	(excess antigen)		
Precipitate (2/3 system)	-	-	+	+	+	+	+	(excess antibody)		

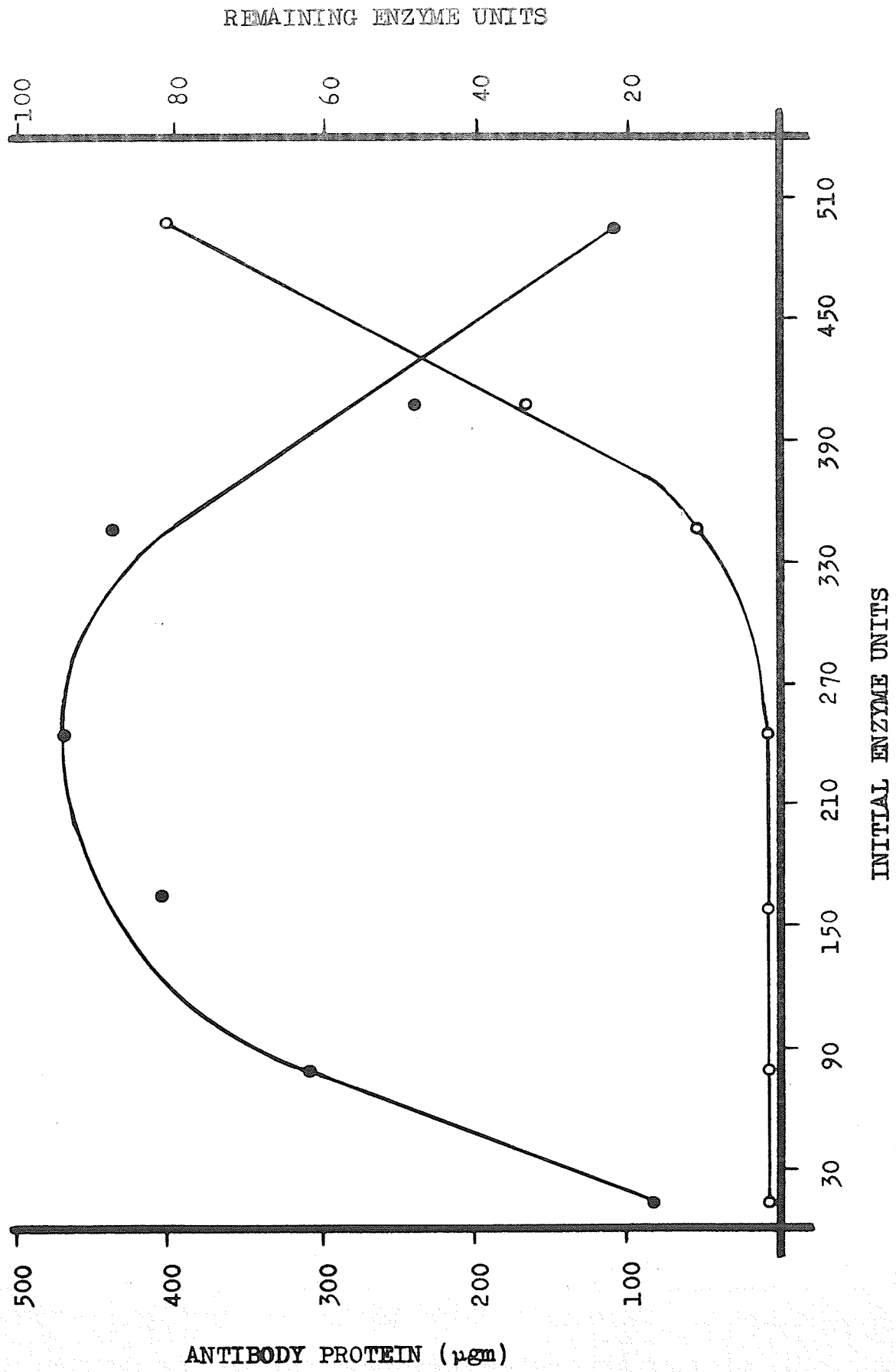


Figure 6. The precipitin curve (1) and remaining enzyme units.(2).

Table 8

The system used to determine anti-enzyme units

Tube	1	2	3	4	C1	C2	C3	C4	C5	C6
Antigen (ml)	0.2	0.2	0.2	0.2	-	-	-	-	0.2	0.2
Antibody (ml)	0.1	0.2	0.3	0.4	0.1	0.2	0.3	0.4	-	-
Saline (ml)	1.5	1.4	1.3	1.2	1.7	1.6	1.5	1.4	1.6	1.6
Precipitate	+	++	+++	++++	-	-	-	-	-	-
Klett units*	36	32	28	27	21	23	25	27	30	30
Corrected Klett units	15	9	3	0						
Enzyme units	186	96	30	0					330	330

*Nitrite determinations, controls C1 to C4 enabled a correction to be made for the varying amounts of antibody in each reaction tube.

Table 9
System for absorbing antiserum contaminants

	Flask 1	Flask 2	Flask 3	Flask 4
Antibody (ml)	10.0	-	-	10.0
NRS (ml)	-	-	10.0	-
Antigen (ml)*	-	-	-	7.5
Protein extract (ml)**	0.33	0.33	0.33	-
Saline (ml)	82.5	92.5	82.5	75.33
Precipitate	+	-	-	++++

*Purified respiratory form of nitrate reductase.

**Prepared from non induced E. coli cultures.

Table 10

The titration system used to determine the ability of the assimilatory form of nitrate reductase to react with the antibody to the respiratory form of the same enzyme

Tube	1	2	3	4	C1	C2	C3	C4	C5	C6	C7
Antigen (ml)*	0.05	0.1	0.15	0.2	0.05	0.1	0.15	0.2	0.15	0.15	-
Antibody (ml)	0.62	0.62	0.62	0.62	-	-	-	-	0.62	0.62	0.62
Saline (ml)	1.33	1.28	1.23	1.18	1.95	1.9	1.85	1.8	1.23	1.23	1.38
Precipitate	+	++	++	++	-	-	-	-	+++	+++	-
Enzyme units remaining	0	0	44	115	60	105	165	230			

*The antigenic source in tubes 1 to C4 inclusive was the assimilatory form of the enzyme, while in tubes C5 and C6 the respiratory form of the enzyme was used.

Induction of Nitrate Reductase

Studies on the kinetics of 'in vivo' enzyme synthesis showed that as enzyme units increased due to induction, the cellular protein content also increased. By plotting the increases of enzyme activity against increases of total protein during induction, a linear curve was obtained (Figure 7). Such graphs show that the increase in activity is not due to non specific activity increases of the enzyme due to the presence of nitrate, but to quantitative increases of the amount of nitrate reductase synthesized in the cells (22). This contention is further supported by the fact that an increase in antigenic activity occurs in extracts prepared from cells subjected to increased induction periods under conditions of aeration. This increased antigenic activity parallels the increase in enzyme units (Figure 7). Crude extracts prepared from non induced cultures were void of any material capable of reacting with the antibody to the respiratory form of nitrate reductase.

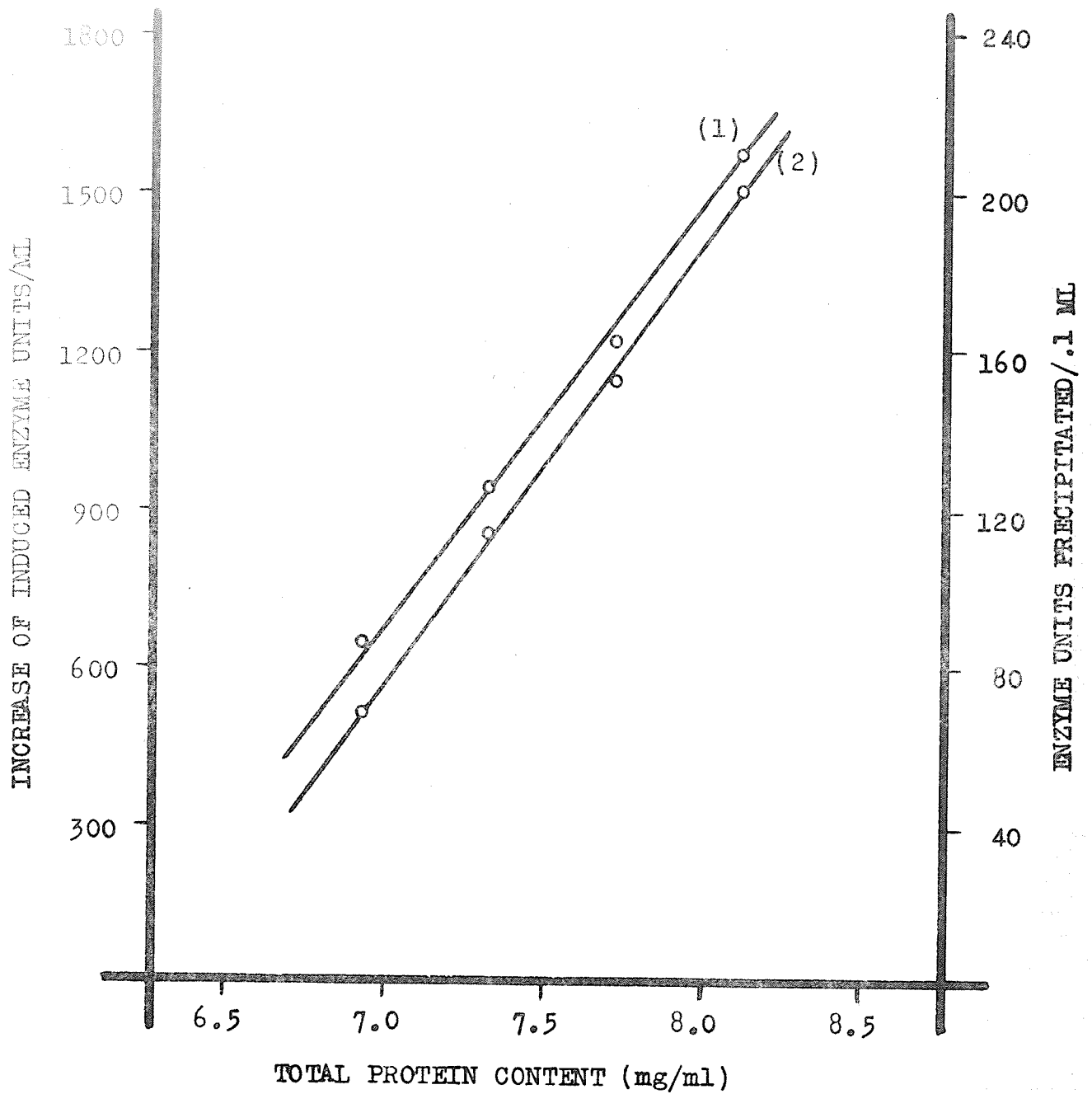


Figure 7. The kinetics (1) and antigenicity (2) of the induced assimilatory form of nitrate reductase.

DISCUSSION

DISCUSSION

The need for the investigation presented here arose from the conflicting reports available in the literature concerning the presence of quantitatively different kinds of nitrate reductases depending on the growth conditions of the cells. Escherichia coli grown under micro-aerophilic conditions produces a nitrate reductase (dissimilatory or respiratory enzyme) which appears to require a ferricytochrome system for the reduction of nitrate (9) (10) (11). Nitrate reductase produced in cells grown under aerobic conditions, (assimilatory enzyme) however, does not seem to require the cytochromes (4) (7). There are other differences reported in the literature between the dissimilatory and the assimilatory enzymes, notably, that the assimilatory enzyme occurs in a soluble form while the dissimilatory form exists in association with particulate material (4) (23).

The nitrate reductase system of E. coli thus resembles the lactic dehydrogenase system of bakers yeast. The enzyme produced under anerobic conditions does not

react with ferricytochrome c, while the enzyme produced under aerobic conditions does so readily. Slonimski and coworkers (24) (25) have presented evidence for the fact that the two forms of lactic dehydrogenase are distinct, and during transition from the anaerobic to aerobic state one form of the enzyme is converted to the other.

In view of these conditions in yeast, attempts were made to determine, (a) whether the nitrate reductases present under various growth conditions in E. coli are different from one another, and if the answer to this question were yes, (b) would it be possible to show the conversion of one nitrate reductase to another during transition from anaerobic to aerobic growth conditions?

It was first of all essential to show that nitrate respiration (dissimilation) and nitrate assimilation occurred in E. coli strain B under different growth conditions. In Table 1 it is demonstrated clearly that although the cellular yield from aerobic cultures is three times as great as in the microaerophilic cultures, a comparatively small amount of nitrite is excreted into the medium, i.e., under aerobic conditions nitrite is indeed

assimilated while under microaerophilic conditions no further use is made of the nitrite and it is excreted into the medium.

Many efforts were expended to establish a differential assay technique for the two enzyme forms. The reduction and oxidation of mammalian cytochrome could not be demonstrated, also flavins and flavin inhibitors were used to no avail. A differential assay method could not be established; all assaying tended to show that the two enzyme forms had basically identical demands. The failure to establish a differential assay technique forced an abandonment of any attempts to carry out conversion studies.

Studies on the optimal reaction time and concentration of each enzyme form indicate clearly that enzymic reactions did occur. It is interesting to note that the optimal reaction time for each enzyme form is essentially the same. Sedimentation experiments show that both the assimilatory and respiratory forms are bound firmly to particulate matter, i.e., an additional similarity is apparent. Attempts to solubilize the respiratory form of nitrate reductase

were successful only with comparatively harsh treatments, milder treatments were of no avail.

Initial immunochemical investigations served merely to characterize the precipitation system. The use of a total of five negative controls would appear to eliminate any possibility of a non specific cross reaction. The enzyme-antienzyme system acted in the manner of a typical precipitation system; an excess of antigen inhibited while antibody in excess caused the reaction to go to completion. The precipitin curve (Figure 6) showed three definite zones; one of antibody excess on the left, a central equivalence zone and an antigen excess zone on the right. The accurate titre of the antiserum was 220. However, final tests were done under conditions of slight antibody excess (dilution of 1:200). The assimilatory form of nitrate reductase combined immunologically with the antibody to the respiratory form of nitrate reductase, i.e., although the antibody exhibited a high degree of specificity the assimilatory form of the enzyme could cross react with it. Studies on the kinetics of enzyme synthesis by immunological means showed that the induction of nitrate reductase

resulted not only in increases in enzyme activity but this increase also paralleled the appearance and increase of a serologically specific protein. It is to be noted that this serologically specific protein is the assimilatory form of nitrate reductase while the antibody is against the respiratory form of the same enzyme.

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