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NITRITE REDUCTION IN

CLOSTRIDIUM PASTEURIANUM

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF THE

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By

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DEDICATION

To my loving wife, whose interest and understanding were a continuing source of encouragement throughout this investigation.

ACKNOWLEDGEMENT

To the National Institutes of Health, U.S. Public Health Service, for financial support during the period 1963-1965.

To Dr. Howard F. Mower, for suggesting the problem and offering helpful advice and assistance during the study of it.

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NITRITE REDUCTION IN

CLOSTRIDIUM PASTEURIANUM

By

Gordon Clide Edwards

A thesis submitted to the Graduate School of the University of Hawaii

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

ABSTRACT

The mechanism of nitrite reduction in plants and bacteria has not been well defined. The identification of a new, low molecular weight, non-heme iron protein, ferredoxin, as an electron carrier in C. pasteurianum, led to the discovery of a closely associated nitrite reductase activity in this organism (Mortenson, L. E., et al., Biochem. and Biophys. Research Communs., 7, 448 (1962)). This nitrite reductase system was chosen for a detailed study as an example of a ferredoxin-dependent reaction.

Studies with the cell-free extract from which the ferredoxin had been removed, called the phosphoroclastic enzyme system, substantiated the reaction sequence: H_2-hydrogenase-ferredoxin-nitrite reductase-NO_2^--NH_3 proposed earlier (Valentine, R. C., et al., J. Biol. Chem. 238, 856 (1963)). Nitrite was stoichiometrically converted to ammonia. The six-electron reduction occurred as one complete reaction, and no intermediates were detected. The Michaelis constant for the overall reaction, K_m = 9.1 μM.

(ABSTRACT, continued)

Experiments with reduced flavin and pyridine nucleotides added to reaction mixtures or generated *in situ* demonstrated that these nucleotides do not participate in nitrite reduction in this organism. Ferredoxin apparently mediates the electron transfer by coupling directly with the nitrite reductase system.

With sodium dithionite as the electron source, benzyl viologen mediated the direct supply of electrons to nitrite reductase, but not to hydrogenase. This system provided a means of isolating the nitrite reduction activity from other cellular systems and of studying it independently of hydrogenase and ferredoxin activities. Substitution of other artificial dyes for benzyl viologen indicated the enzyme could accept electrons only from sources that had electrode potentials similar to the hydrogen electrode (E_o' = -0.35 to -0.45 v at pH 7). Nitrite reductase was inhibited reversibly by CO. Established sulfhydryl and iron inhibitors were also effective inhibitors of this enzyme system. The enzyme is apparently a metalloprotein containing iron. The K_m = 25 µM for the benzyl viologen-mediated reaction.

Purification studies utilizing most of the common methods of protein fractionation were unsuccessful in obtaining a purified preparation of nitrite reductase. The studies implied that the reductase activity is expressed by a multi-component enzyme complex which is highly oxygen labile.

Results of a comparative study of several other clostridia indicate that nitrite reductase activities are present only in those clostridial strains which also contain an active hydrogenase and nitrogenase system.

INTRODUCTION

The reduction of the most highly oxidized, stable form of nitrogen, nitrate, to the most highly reduced form, ammonia, was suggested in 1894 to involve a metabolic pathway of four steps of two electron reduction in living organisms (20):

$$NO_3^- \longrightarrow NO_2^- \longrightarrow N_2O_2^= \longrightarrow NH_2OH \longrightarrow NH_3$$

Evidence to support this general pathway for nitrate reduction was obtained by early investigators (27). In recent years a series of reductases were assigned to each step of the reaction sequence. The terms nitrate reductase, nitrite reductase and hydroxylamine reductase are now common in nitrogen metabolism discussions.

Much interest has been shown in the past fifteen years in characterizing these reductase enzymes and learning the details of the reaction mechanisms. The first step of the reaction scheme, involving nitrate reductase, is now well understood. Investigations on a wide variety of plant and bacterial systems have led to the conclusion that a two-electron reduction of nitrate is the first step, and nitrite is the reaction product. The enzyme is a sulfhydryl molybdoflavoprotein, and requires a reduced pyridine nucleotide as the immediate electron donor (28).

The remaining six-electron reduction from nitrite to ammonia has received less attention. The enzymes which mediate this reaction sequence are apparently not as widely distributed among plants and microorganisms, as are nitrate reductases (28). However, preparations containing nitrite reductase activity have now been obtained from several sources, as shown in Table I.

Table I

Properties of Nitrite Reductases

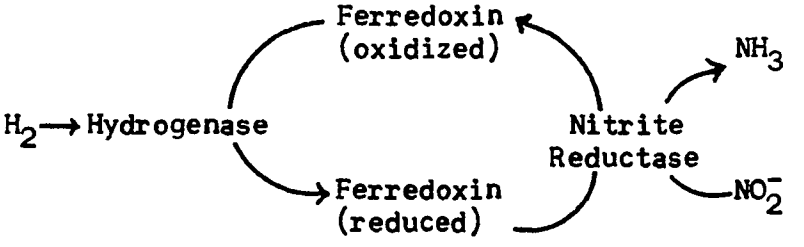
Source	Cofactor Required	Metal	Reaction Product	Degree of Purification	Reference
P. aeruginosa	FMN, FAD	Cu	NO gas	600 X	(53)
Halotolerant 203	FAD	Yes[a]	NH_2OH	8 X	(46)
N. crassa	FMN, FAD NAD, NADP	Fe, Cu	None reported	50 X	(29,30)
E. coli Bn	NADP	None	NH_3	100 X	(16)
E. coli K_{12}	NAD	Yes[a]	None detected	30 X	(26)
Soybeans	NAD, NADP	Mn	None detected	70 X	(40)
Cucurbita pepo	NADP[b]	None	NH_3	None	(12)
Spinach	None (ferredoxin)	None	NH_3	None	(33)
Tomatoes	None	None	NH_3	None	(42)

[a]Specific metal not determined.

[b]Active only in presence of catalytic amounts of benzyl viologen.

It was immediately apparent from Table I that some information had been obtained about the properties of nitrite reductase, but many conflicting points remained. Furthermore, recent workers (12,42), questioned much of this earlier evidence as being inconclusive. They postulated that some investigators may have attributed the effects of associated reactions to those of nitrite reductase. The inconsistencies in cofactor requirements and reaction products were particularly confusing. It was felt that a detailed study should be made of nitrite reductase.

During investigations of a new low molecular weight, non-heme iron protein, named ferredoxin, from Clostridium pasteurianum, evidence was obtained that the organism contained a nitrite reductase (25). It was shown that ferredoxin was necessary to provide an active reducing system when hydrogen was the electron donor. The mechanism shown below was subsequently proposed for this reaction (50):



This mechanism inferred that six electrons or three moles of hydrogen would be required to reduce one mole of nitrite and produce one mole of ammonia. The physiological role of such a system in clostridia was not known, although it may have been associated with nitrogen fixation (28).

It was felt that Clostridium pasteurianum would be an excellent organism to utilize in undertaking a comprehensive study of the nitrite reductase enzyme from a bacterial system. Procedures for obtaining large quantities of the organism were available (2). Investigations would be initiated to: A-define the physiologically-active reaction parameters for nitrite reduction, B-isolate the nitrite reductase activity as an independent activity from other cellular processes, and C-attempt to purify and characterize the enzyme.

MATERIALS AND METHODS

Materials

The compressed gases used in these investigations were obtained from Gaspro, Ltd., Hawaii, except The Matheson Company, California, supplied the carbon monoxide. CM Sephadex and Sephadex G-25 was purchased from Pharmacia Fine Chemicals, New York. Carl Schleicher and Schuell Company, New Hampshire, was the source of the diethylaminoethyl cellulose. Mann Research Laboratories, New York, supplied the benzyl viologen, methyl viologen, pyocyanine perchlorate, methylene blue and 2,6-dichlorophenolindophenol.

Diphosphopyridine nucleotide, triphosphopyridine nucleotide, calcium phosphate gel and alumina C_γ gel were obtained from Nutritional Biochemicals Corporation, Ohio. The enzymes alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, and isocitric dehydrogenase were purchased from Sigma Chemical Company, Missouri.

Cultures of C. pasteurianum (6013), C. butyricum (859) and C. felsineum (13160) were obtained from American Type Culture Collection, Washington, D.C. A culture of C. acidiurici was kindly provided by Dr. J. Rabinowitz, University of California, Berkeley. A culture of a putrefactive anaerobe, PA 3579h, believed to be a strain of C. sporogenes, was obtained from Dr. H. Frank, University of Hawaii, Honolulu.

All other chemicals used were standard laboratory reagents.

Methods

Growth of Bacteria. Cultures of Clostridium pasteurianum were maintained as sand stocks by the Department of Biochemistry and Biophysics, University of Hawaii. Large quantities of the organism were obtained by a batch process. A 40 l culture was used as inoculum for a 150 l fermenter. The bacteria were grown on the medium of Carnahan and Castle (2):

400 g sucrose	4 mg p-aminobenzoic acid
20 g ammonium sulfate	20 ml stock A solution
50 g calcium carbonate	40 l tap water
4 mg biotin	

The stock A solution consisted of 40 g anhydrous magnesium sulfate, 40 g sodium chloride, 4 g sodium molybdate, and 20 g manganese sulfate, in 8 l water.

The 40 l flask of medium was autoclaved at 121° for 30 min, and immediately placed in the 37° incubating chamber. Nitrogen purging was initiated to achieve anaerobic conditions. When the medium had cooled to 37°, the sand stock culture was quickly added, along with 100 ml 1 M potassium phosphate buffer, pH 7.0, which had been autoclaved separately, and 10 ml ferrous sulfate-ferrous citrate solution (25 g Fe SO_4 + 25 g Fe citrate per 1 H_2O) which had been sterilized by ultrafiltration.

When in the activity gassing stage, about 48 h, the 40 l culture was transferred to the 150 l fermenter which was charged with medium containing ten times the composition of that shown above for the 40 l flask. The fermenter was fitted with nitrogen purging equipment, a mechanical stirrer, a thermostated heating coil, and an automatic pH-stat which continuously monitored the pH of the culture. The pH was

controlled by automatic addition of 1 M NaOH to maintain pH 5.5. The 150 l culture was incubated about 8 h at 37°, then harvested with a Sharples supercentrifuge. The yield was 150-200 g cell paste.

The cells were washed in cold water to remove metabolic products and unmetabolized nutrients. They were resuspended in 15 l water at 4°, stirred, and recentrifuged. The washed cell paste was dried *in vacuo* using Rinco evaporators, as described by Carnahan, et al., (3). The dried cells were stored at -10° until used.

Preparation of Cell-free Extracts. The dried cells were autolyzed by suspension in ten volumes (ml/g) water (3) or 0.01 M phosphate buffer, pH 7.0. They were shaken under a hydrogen atmosphere for 40 min at room temperature. The resulting mixture was cooled to 4° (all subsequent steps were carried out in the cold, 0-4°), and centrifuged 15 min at 30,000 X g. The resulting supernatant, referred to as the crude extract, was sometimes dried *in vacuo* and stored at -10°. For most experiments, the ferredoxin was removed from the crude extract by separation on a DEAE cellulose column (i.e., 2 X 6 cm for 200 ml extract), which had previously been equilibrated with 0.01 M Tris-HCl buffer pH 7.3 (25). The solute which passed through the column was dried *in vacuo* or lyophilized. This material, called phosphoroclastic extract (25), represented 90-95% of the original protein and possessed several metabolic activities, including hydrogenase, pyruvic dehydrogenase, transacetylase, NADP reductase and nitrite reductase. This clastic extract was stored frozen. Subsequent activity studies indicated that the crude extracts and clastic extracts prepared by this procedure retained full nitrite reductase activity from the crude cell suspension.

Preparation of Ferredoxin. When the solute from the DEAE cellulose column had been obtained (above), the column was developed with 0.2 M NaCl in 0.1 M Tris-HCl buffer, pH 7.3. The brown band at the top of the column was eluted as a red-brown solution. This material was dialyzed overnight (6-8 h) against nitrogen-sparged water in the cold. The resulting dialyzate was designated crude ferredoxin (25) and stored frozen. This material was purified further for some experiments by the method of Mortenson (24).

Preparation of Adsorbents. The preparation of DEAE cellulose was adapted from the method of Peterson and Sober (36). The dry powder was allowed to settle into a large volume of 1 M NaOH + 0.5 M NaCl. After standing for several hours, the suspension was filtered on a large filter funnel under low vacuum. The filter cake was washed several times with 2-3 volumes of the NaOH/NaCl solution, until the filtrate became colorless. It was then washed several times with distilled water, and resuspended in 1 M HCl. The acidic suspension was immediately filtered off, and the filter cake washed with distilled water until the filtrate became almost neutral (pH 6.5-7). The filter cake was suspended once again in 1 M NaOH + 0.5 M NaCl, and the basic solution filtered off. This was followed by washing with distilled water until the filtrate became neutral to test paper (pH 7). The washed DEAE cellulose was then equilibrated by suspension in the starting buffer of the particular experiment, filtering, repeating with another buffer wash, and finally settling in 2-3 volumes of the starting buffer. Fines were decanted off prior to using the adsorbent.

After use, the DEAE cellulose was regenerated by washing with 1 M NaOH + 0.5 M NaCl, filtering, and repeating until the filtrate became colorless. The filter cake was then washed with distilled water until the filtrate tested neutral, and finally equilibrated with the appropriate buffer, as above.

The CM Sephadex was prepared as recommended by the manufacturer. The new material was allowed to settle in a large volume of water. When the resin was completely wetted, the water was removed by filtration on a large filter funnel, with low vacuum. The filter cake was washed in sequence with 2-3 volumes of 0.5 M NaOH, distilled water, 0.5 M HCl, and finally with distilled water, until the filtrate tested neutral to test paper. The resin was then equilibrated with the appropriate starting buffer by repeated washing until the filtrate had the same pH as the buffer. The equilibrated filter cake was suspended in 2-3 volumes of starting buffer and allowed to settle. Fines were decanted off prior to use. The same procedure was used to regenerate used material.

Fresh Sephadex G-25 was taken up in a large volume of 0.5 M NaCl, allowed to settle, and then transferred to a large filter funnel. The NaCl solution was removed under low vacuum, and the filter cake washed several times with 2-3 volumes of starting buffer, until the filtrate had the same pH as the buffer. The filter cake was then resuspended in a large volume of starting buffer, and allowed to settle. The fines were decanted off prior to use. The resin was regenerated by the same process.

Protein Fractionation with Ammonium Sulfate. A saturated solution of $(NH_4)_2SO_4$ in water was used for some protein purification experiments.

The solution was added dropwise underneath the surface of the protein mixture, while the mixture was being agitated with a magnetic stirrer. A stream of nitrogen was directed on the surface of the mixture to maintain anaerobic conditions. When addition of the saturated salt solution had been completed, the resulting mixture was stirred for 10 min. Then it was centrifuged for 10 min at 30,000 X g. The supernatant was decanted off for later use. The precipitate was resuspended in 0.01 M phosphate buffer, pH 7.0, and dialyzed to remove endogenous ammonia. All operations were carried out in the cold (0-4°).

Protein Fractionation with Ethanol. The purification of protein solutions with ethanol was carried out in small insulated baths containing ethanol. These baths were maintained at 0°, -10°, -18° and -25°, by addition of powdered dry ice (CO_2). A protein solution was placed in a small Erlenmeyer flask and cooled to about 1°. Then ethanol at -10° was added dropwise with stirring. The sample was transferred to the -10° bath when it contained about 5% ethanol. The addition of cold ethanol continued until 10-15% of the mixture was ethanol. The mixture was stirred and maintained at -10° for 10 min. It was then centrifuged at the same temperature at 30,000 X g for 10 min. The supernatant was treated with additional ethanol to obtain a 25% ethanol cut (maintained at -18°). The procedure was repeated again to obtain a 50% ethanol cut (maintained at -25°). The precipitates from each fractionation step were suspended in 0.01 M phosphate buffer pH 7.0, and frozen until used in further studies.

Protein Fractionation with Gel Adsorbents. Calcium phosphate gel and alumina C_γ gel were used in aged form as supplied by the

manufacturer. The given protein solution was placed in a beaker surrounded by an ice bath, and stirred with a magnetic stirrer. A stream of nitrogen was directed on the surface of the mixture. pH electrodes were placed in the solution, and ice-cold 0.1 M acetic acid was added until the pH was lowered to pH 5.5. The suspension of pre-weighed gel in a small amount of cold water was added dropwise under the surface of the solution. The mixture was stirred occasionally over a 10 min period, then centrifuged at 2000 X g for 10 min.

The resulting supernatant solution was decanted off and frozen. The protein which had adsorbed to the precipitated gel was recovered by resuspending the gel in 2-3 ml 0.1 M phosphate buffer pH 7.0. The suspension was stirred intermittently for 10 min, then recentrifuged. This process was repeated for a total of three times, and the eluates were combined.

Protein Fractionation with Protamine Sulfate. The pre-weighed sample of protamine sulfate was placed in a test tube with 1-2 ml water. When the salt was completely in solution, dilute NaOH was added until the solution was neutral (pH 6.5-7) to test paper. The clastic extract sample was placed in an erlenmeyer flask on a magnetic stirrer. Nitrogen gas was utilized to maintain an anaerobic atmosphere within the flask. The solution of protamine sulfate was added to the protein sample dropwise. The resulting mixture was stirred 10 min, then centrifuged 10 min at 30,000 X g. The supernatant was decanted off for further use. The precipitate was discarded. All operations were carried out in the cold (0-4°).

Starch Block Electrophoresis. Preparative electrophoresis experiments were performed as adopted from the method of Kunkel and Slater (15). 350 g purified potato starch was washed twice with 2 volumes distilled water and twice with 2 volumes 0.05 M Veronal buffer, pH 8.6 (2.8 g barbituric acid + 20.6 g sodium barbital per 1, diluted with 1 part water before use). The washed starch was stored in buffer until used. Prior to use, the excess buffer was decanted off, and a small amount of fresh buffer (ca. 5 ml) was added to make a very thick starch paste. The paste was mixed thoroughly and poured into a 40 X 13 X 0.5 cm plexiglass tray, which had been lined with plastic wrap. The poured starch block was blotted with paper toweling until the surface appeared dry. A 2 ml (ca. 50 mg protein) sample was made into a thick paste with dry starch. The sample paste was poured into a narrow trough cut across the starch block at the longitudinal midpoint. Wicks of cloth toweling were placed at each end of the block. The starch was covered with plastic wrap and a plexiglass cover was placed tightly over the tray. The wicks were placed in electrolyte (Veronal buffer) compartments.

The electrophoresis was carried out at 20 ma constant current (450-600v) for 12 hours in the cold. At the completion of the experiment, the block was cross-sectioned into strips 1 cm wide. The protein was separated from the starch by homogenizing each strip in 3 ml buffer and centrifuging 15 min at 3000 X g. The eluted protein fractions were stored at 4°C.

Starch Gel Electrophoresis. Electrophoresis on starch gel, using the method of Ashton and Braden (1), was utilized for some protein characterization studies. The pH 8.0 buffer contained 4.8 g tris + 1.6

g citric acid monohydrate per 1. The electrolyte consisted of 11.8 g boric acid + 0.44 g lithium hydroxide per 1, pH 7.8. A mixture of 10.4 g Connaught hydrolyzed starch in 10 ml electrolyte + 90 ml buffer, was heated to boiling in a filter flask, and immediately aspirated to remove entrapped air. The viscous liquid was then poured into a 17 X 17.5 X 0.3 cm plexiglass tray. Once the gel had begun to set (ca. 15 min), the tray was placed in the cold. It was cooled and ready for use in about one hour.

A concentrated protein sample (30-100 mg/ml) was prepared and a small amount (0.06-0.07 ml) adsorbed on a 0.2 X 3 cm piece of Whatmann #3 filter paper. The wetted paper was carefully introduced into a slit at the anode end of the gel, in a position for the final gel path to be 12 cm long. When all samples were in place, flat sponge wicks were attached to each end of the gel, and arranged into divided cell compartments containing electrolyte. The gel was covered with plastic wrap and weights were placed over the sponge wicks to assure continuous contact between the wick and gel surface.

The electrophoresis was carried out with a constant current power supply, at 40 ma (500-900 v) for about 3 h in the cold. A fan provided continuous air cooling of the plate. The progress of the run was followed visually, since the discontinuous buffer system utilized produced a distinguishing band at the moving electrophoretic boundary. At the termination of the run, the gel was removed in strips and stained for protein using a saturated solution of amido black in 50 methanol : 50 water : 10 glacial acetic acid. The stain was removed after 30 sec, and the gel strips were washed repeatedly with the above solvent mixture, and finally left overnight in the solvent. The final gel color was light blue, with dark blue stained bands of separated protein.

Nitrite Reductase Assays. Two methods were utilized to determine the activity of a given preparation for nitrite reduction. Experiments in which carbon monoxide gas, or a mixture of gases, were used in the gas phase of the reaction vessel, were carried out in 25 ml erlenmeyer flasks. These flasks were modified by the addition of a side arm on the vertical edge of the flask and a two-way stopcock on the top. A vacuum manifold was constructed in a vented chemical hood which would accept six reaction vessels simultaneously through ground glass ball joints. The manifold was fitted with sources of hydrogen, nitrogen, helium, nitrous oxide and carbon monoxide.

The reaction vessels were prepared for the experiment and placed on the manifold, degassed with the vacuum line, and flushed and evacuated several times with the desired gas. The stopcocks were then closed and the vessels placed in a New Brunswick rotary incubator. They were pre-incubated by shaking for ten min, then substrate or electron donor was tipped into the vessel from the side arm, and the reaction carried out.

Manometric studies and other assays were conducted with the use of standard Warburg reaction flasks, fitted with two side arms, and degassing stopcocks. A manifold system, furnished with nitrogen, helium and hydrogen was utilized to flush the flasks to remove all air (oxygen) prior to initiation of the experiment. The standard procedure used in all experiments was to flush with the desired gas for 15 min at ambient temperature, then place the flasks in the constant temperature incubating bath and continue flushing for an additional 15 min with shaking. The reaction was initiated by closing off the gas system, and tipping in substrate or electron donor from the side arm. Changes in manometric

pressure were read from an open end manometer connected to each flask. The center wells contained 0.2 ml of 14% NaOH to adsorb CO_2.

All experiments were carried out at 37°, unless otherwise noted. Samples of the reaction mixtures were removed at the termination of the experiment for appropriate analyses.

Reduced Pyridine Nucleotide Sources. $NADH_2$ and $NADPH_2$ were formed from their oxidized derivatives in the reaction mixture for some experiments. This was accomplished by using the dehydrogenase generating systems shown in Table II. These systems were used when it was desirable to provide small amounts of the reduced pyridine nucleotide throughout the incubation period, rather than a large amount of the reduced compound at the initiation of the experiment.

Table II

Reduced Pyridine Nucleotide Generating Systems

$NADH_2$ System	Symbol	Reference
Alcohol Dehydrogenase (Yeast)	ADH	(38)
5 μmoles NAD		
130 μmoles ethanol		
30 μmoles phosphate buffer pH 7.0		
80 units[a] alcohol dehydrogenase		
$NADPH_2$ Systems		
Glucose-6-Phosphate Dehydrogenase (Yeast)	G-6-PDH	(14)
0.15 μmoles NADP		
30 μmoles glucose-6-phosphate		
20 μmoles Mg^{++}		
30 μmoles phosphate buffer pH 7.0		
100 units[a] G-6-P dehydrogenase		
Isocitric Dehydrogenase (Bovine Heart)	IDH	(32)
0.45 μmoles NADP		
1.8 μmoles isocitric acid		
3.0 μmoles Mn^{++}		
100 μmoles Tris-HCl buffer pH 7.3		
50 units[a] isocitric dehydrogenase		

[a] 1 unit of activity = $\Delta O.D._{340}^{37^\circ} = 0.001/min = 4.8 \times 10^{-4}$ μmoles $NADH_2$ or $NADPH_2$ formed per min in 3 ml.

Analytical Methods. The assay for nitrate ion was carried out using 4-methylumbelliferone as described by Skujins (43). The concentration of nitrite ion was determined according to Nicholas and Nason (31) from the color complex formed with N-(1-naphthyl)-ethylenediamine. The method of Frear and Burrell (11) was employed to determine the concentration of hydroxylamine, from its reaction with 8-quinolinol.

Protein concentration was estimated by the method of Lowry, et al. (19). The spectral absorption characteristics of column eluates were determined in a Beckman Model DB spectrophotometer.

Determination of Ammonia. Ammonia was separated from the reaction mixture by a modification of the method of Mortenson (22). Using standard micro-conway dishes, 1.0 ml saturated potassium carbonate was placed in the outer compartment, and 1.0 ml 2% boric acid in the inner compartment. A sample containing 1 - 20 µg ammonia in a total of 1.0 ml was placed in the outer compartment. The lid was placed on the dish, and the sample and potassium carbonate were carefully mixed. The dish was incubated at 37° for 1 h then the cover was removed and 0.8 ml of the solution from the center well was taken for ammonia determination.

The concentration of ammonia in the sample removed from the micro-conway dish was determined by its reaction with ninhydrin. The ninhydrin reagent consisted of 10 g ninhydrin, 1.5 g hydrandantoin, 375 ml methyl cellosolve (peroxide free), and 125 ml 1 M sodium acetate buffer, pH 5.6. This mixture was stored under nitrogen pressure and remained active for 3 - 4 months. One-half ml ninhydrin reagent was added to the 0.8 ml ammonia sample, and placed in a boiling water bath for 15 min. The sample was removed, allowed to cool, and diluted with 4.0 ml 1/1 ethanol/

water, v/v. The absorption of the purple complex was determined at 540 mμ in a Klett colorimeter.

The precision of the method, as determined according to Mills (21), was ± 0.7 μg at the 10 μg level, and ± 1.1 μg at the 20 μg level. This corresponds to ± 0.5 μmoles in 10 μmoles total, and ± 1.0 μmole in 20 μmoles total ammonia in the original reaction mixture. This precision was considered acceptable for this investigation, since only differences greater than 1 umole would be considered of metabolic significance.

This was the only method of several studied where other materials in the reaction mixture did not interfere with the determination. Other workers had shown that under the conditions described, the ammonium ion was the only form of nitrogen distilled over into the center well (4).

RESULTS

Physiological Reaction Parameters

The data summarized below confirmed the existence of a ferredoxin-dependent, nitrite reductase in C. pasteurianum. The various parameters of the reaction are presented.

Time Course of the Reaction. The formation of ammonia from nitrite was studied as a function of time in both nitrite reductase assays, to establish the length of incubation period for all future studies. These studies were carried out using hydrogen gas as the electron source, ferredoxin as the electron mediator, and the complete clastic extract (which contained the hydrogenase and nitrite reductase activities).

In the 25 ml flask assay (Figure 1), the formation of ammonia proceeded almost linearly for the first 2 h, then gradually leveled off. The reaction had ceased after 4 h. The loss of nitrite was also determined (Figure 1). It followed the same general kinetics as ammonia formation, as expected. No further nitrite was consumed after 2h. From these data it was decided to incubate the 25 ml flask assays for a 2 h period.

The time course for the Warburg flask studies was linear for the first 20 min (Figure 2). Ammonia production then leveled off by 30 min. All subsequent Warburg flask assays were conducted for a 30 min incubation period.

Clastic Extract Concentration Response. The relation between nitrite reduction and clastic extract concentration was studied by the Warburg flask assay. With hydrogen gas as the electron donor, and 0.05

mg crude ferredoxin as the electron carrier, the data indicated that a threshold level of about 1.5 mg clastic extract was required before nitrite reduction occurred (Figure 3). The activity rapidly increased with increasing clastic extract concentration, to a maximum of 4-5 mg extract. The accompanying curve of hydrogen utilization followed the same general pattern.

Ferredoxin Concentration Response. The effect of ferredoxin concentration on nitrite reduction was determined in the Warburg flask assay. Hydrogen gas was the electron source. Pure ferredoxin was used in conjunction with the clastic extract. The results (Figure 4) show that very small amounts of ferredoxin are required for activity. Significant activity was obtained with 0.05 mg of the pure iron protein. The optimum ferredoxin concentration for the system utilized here (H_2 + 6 mg clastic extract) was about 0.7 - 0.8 mg ferredoxin. Hydrogen consumption was also followed, and gave the same general curve as ammonia formation.

Endogenous Nitrite Reductase Activity. The amount of nitrite reductase activity due to the presence of endogenous substrate and electron source in the clastic extract was determined by replicate Warburg flask assays where one set of flasks were incubated with hydrogen in the gas phase, and the duplicate set under nitrogen. The data, presented in Table III, indicate that no activity was present in the absence of the electron donor, hydrogen.

Table III

Endogenous Nitrite Reductase Activity[a]

Gas Phase	μmoles Gas Utilized	μmoles NH_3 Formed
N_2	0.0	0.2
H_2	14.0	7.3

[a]Reaction vessels contained 7 mg clastic extract, 0.05 mg crude ferredoxin, 20 μmoles nitrite, 25 μmoles phosphate buffer pH 7.0, in 3 ml total. Warburg flask assay for 30 min at 37°.

pH Profile. The activity of C. pasteurianum nitrite reductase as a function of pH was determined in the 25 ml flask assay. Using hydrogen as the electron source, and 25 mg crude extract, a broad pH optimum was obtained (Figure 5). The system exhibited maximum activity in the neutral pH region, pH 6.5 - 7.5.

Stoichiometry of the Reaction. The relation between nitrite consumed and ammonia formed was determined by varying substrate concentration, with the 25 ml flask assay. The stoichiometry of the reaction was good (Table IV).

Table IV

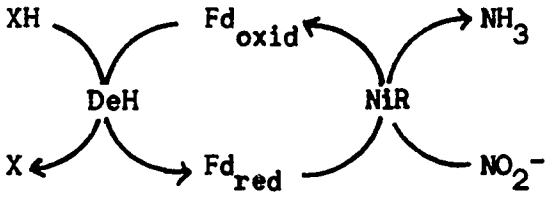
Stoichiometry of Nitrite Reduction[a]

μmoles NO_2^- Added	μmoles NO_2^- Lost	μmoles NH_3 Formed
0	0.0	0.0
5	4.5	5.5
10	8.2	7.4
20	9.0	9.4

[a]Reaction vessels contained 24 mg clastic extract, 0.08 mg ferredoxin, 50 μmoles phosphate buffer pH 7.0, in 5 ml total. 25 ml flask assay with hydrogen gas. 2 h at 37°.

The kinetic constants of the enzyme reaction were determined with a Lineweaver-Burk plot (8) of the data (Curve A, Figure 6). Extrapolation of the plot provided a value of maximum velocity, V_m = 15.9 μmoles NH_3/2 h. The Michaelis constant, K_m = 9.1 μM.

Natural Electron Donors for Nitrite Reduction. A generalized scheme for electron flow from a donor (XH), through a dehydrogenase (DeH) to ferredoxin (Fd), and on to nitrite reductase (NiR), could be represented as follows:



The studies summarized thus far have utilized only one natural electron donor, hydrogen (gas), which couples with ferredoxin through hydrogenase. Valentine (47) has summarized the evidence in micro-organisms for other natural electron donors which will also reduce ferredoxin through appropriate dehydrogenases, in accordance with the scheme outlined above:

Organism Studied	Electron Donor
Several clostridia and other M/O	Pyruvate
C. acidiurici	Formate
M. lactilyticus	∝-ketoglutarate
C. acidiurici, C. cylindrosporum, M. lactilyticus	Hypoxanthine

Also, Carnahan, et al., (3) noted that ∝-ketobutyrate could substitute for pyruvate in a nitrogen-fixing system from C. pasteurianum.

The ability of each of these electron donors to couple with the ferredoxin-nitrite reductase system of C. pasteurianum was determined. These experiments were carried out with a high concentration (25 mg) of cell-free extract (containing ferredoxin), so any endogenous activity for these donors would be detected. The data, Table V, show that only pyruvate and ∝-ketobutyrate, substrates for the phosphoroclastic enzyme system found in C. pasteurianum and many other micro-organisms (25), were successful electron donors for nitrite reduction. They were about 60-80% as effective as hydrogen in this experiment. The other substrates provided only token reduction, and would not be considered physiologically important.

Table V

Comparison of Electron Donors for Nitrite Reduction

Electron Donor	μmoles NH_3 Formed[a]	% Nitrite Reduced[b]
Pyruvate	12.5	62.5
α-ketobutyrate	15.9	79.5
Formate	0.5	2.5
α-ketoglutarate	0.7	3.5
Hypoxanthine	0.5	2.5
Hydrogen (gas)	19.3	96.5

[a]Reaction mixtures contained 25 mg crude extract, 20 μmoles nitrite, 50 μmoles electron donor, 100 μmoles phosphate pH 7, in 3 ml. Warburg flask assay with helium gas (except for hydrogen donor), 30 min at 37°.

[b]Based on 20 μmoles NH_3 = 100% nitrite reduced.

Independent Nitrite Reductase Activity

Once the basic parameters of nitrite reduction in C. pasteurianum were known, it was desirable to obtain a system in which the reductase activity could be studied separately from other activities present in the crude extract. It had been shown previously that sodium dithionite (hyposulfite) could reduce ferredoxin and evolve hydrogen from hydrogenase (25). Furthermore, earlier workers had shown that low-potential dyes (such as methyl and benzyl viologen), reduced with dithionite, would couple with the hydrogenase from C. pasteurianum to evolve hydrogen (34).

It thus became evident that the replacement of H_2-hydrogenase by sodium dithionite, and ferredoxin by an artificial dye, could provide a non-physiological electron source for nitrite reductase. This combination would effectively isolate the nitrite reducing system from its dependence upon hydrogenase, an enzyme which has not been purified from this system.

Replacement of Ferredoxin with Benzyl Viologen. Hageman, et al., (12) recently reported the use of reduced benzyl viologen as an electron donor for a cell-free extract from vegetable marrow. Benzyl viologen was substituted satisfactorily for ferredoxin in the C. pasteurianum nitrite reductase system (Table VI). This dye was able to replace ferredoxin in the transfer of electrons from the H_2-hydrogenase system to nitrite reductase. Benzyl viologen was also effective in the transfer of electrons from sodium dithionite to nitrite reductase in an inert atmosphere.

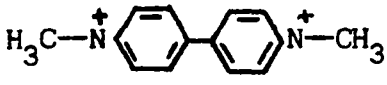
Table VI

Nitrite Reduction with Ferredoxin and Benzyl Viologen[a]

Electron Carrier	μmoles NH_3 Formed	
	H_2 gas	He gas$^+$ 100 μmoles S_2O_4 =
0.1 mg ferredoxin	4.9	3.7
25 μmoles benzyl viologen	7.9	5.6

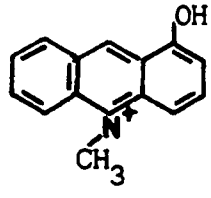
[a]Reaction vessels contained 12 mg clastic extract, 12 μmoles nitrite, 200 μmoles phosphate buffer pH 7.0, in 5 ml total. 25 ml flask assay. 2 h at 37°.

Nitrite Reductase Activity with Artificial Dyes. To determine the range of electrode potentials which would couple efficiently to nitrite reductase, several artificial dyes were employed as electron carriers. They represented an E_o' range of -0.440 to +0.217 v (Table VI). The structures of these dyes are shown below:

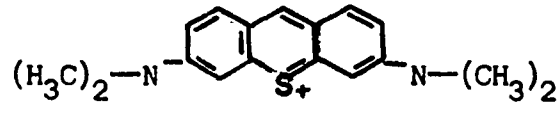


Methyl Viologen

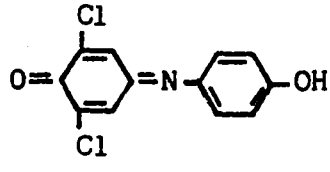
Benzyl Viologen



Pyocyanine



Methylene Blue



2,6-dichlorophenolindophenol

Table VII

Activity of Artificial Dyes with Nitrite Reductase

Dye	E_o' at pH 7.0[a]	μmoles NH_3 Formed[b]
Methyl viologen	-0.440	1.5
Benzyl viologen	-0.359	2.5
Pyocyanine	-0.034	0
Methylene blue	+0.011	0
2,6-dichlorophenolindophenol	+0.217	0

[a]Data source - Mann Research Laboratories, New York. Reference Guide and Price List No. 129.

[b]Reaction vessels contained 25 mg clastic extract, 12 μmoles nitrite, 100 μmoles dithionite, 200 μmoles phosphate buffer pH 7.0, in 5 ml total. 25 ml flask assay with helium gas. 2 h at 37°.

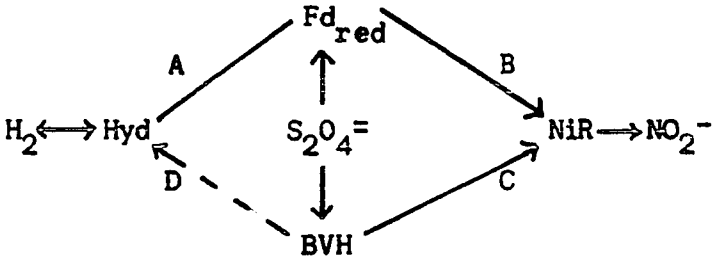
Reduced methyl and benzyl viologen were the only dyes which provided an electron source for nitrite reductase (Table VII). These were the only compounds which had electrode potentials similar to the potential of ferredoxin (E_o' = -0.417 v) (45). Benzyl viologen was utilized for the remaining studies, since it provided the best artificial electron source for nitrite reduction (Table VI).

Hydrogen Evolution with Benzyl Viologen. Although reduced benzyl viologen provided an independent means of studying nitrite reduction, it was important to determine the extent to which this artificial electron carrier coupled with other metabolic activities in the clastic

extract, particularly with hydrogenase. More useful information could be obtained with the nitrite reductase system if other activities were not in significant competition for the electron source.

Replicate experiments with benzyl viologen and ferredoxin were carried out in Warburg flask assays. Sodium dithionite was the electron donor, and three clastic extract concentrations were used. The results (Figure 7) show that benzyl viologen couples much less effectively with hydrogenase than does ferredoxin. The ferredoxin-hydrogenase system produced up to 10 μmoles of hydrogen in 30 min at the highest clastic extract concentration (10 mg). In contrast, benzyl viologen-hydrogenase evolved only 1 μmole at this protein level. The difference is more evident when the ratios of ammonia formation to hydrogen formation are plotted (Figure 8). Benzyl viologen gave approximately 10-fold higher ratios than the ferredoxin-mediated system.

Consider the diagram below, representing electron flow from reduced ferredoxin (Fd_{red}) or reduced benzyl viologen (BVH) to hydrogenase (Hyd) and nitrite reductase (NiR). The data presented above indicates that electron flow for pathways A and B are essentially equivalent from reduced ferredoxin. However, from reduced benzyl viologen, pathway C predominates greatly, at the expense of little electron flow along pathway D. Thus, the reduction of nitrite was essentially isolated from hydrogenase activity by use of the dithionite-benzyl viologen combination as the electron source.



Carbon Monoxide Inhibition of Nitrite Reductase. Carbon monoxide has been shown to inhibit a number of enzymes (13), including hydrogenases (37,41,48). The effect of carbon monoxide on nitrite reduction in C. pasteurianum was studied. Mixtures of CO and H_2 were made using the manifold described earlier. Studies were made using 0 - 100% CO in the gaseous portion of the reaction vessel. Duplicate experiments were conducted, with identical flasks containing clastic extract and either benzyl viologen or ferredoxin. Sodium dithionite was added to all flasks to assure reducing conditions throughout the incubation period.

The data show that nitrite reduction was inhibited by carbon monoxide (Figure 9). The extent of inhibition increased with increasing CO concentration. Essentially complete inhibition of ammonia formation was obtained with CO concentrations greater than 25%. The inhibitory pattern was identical for both the natural electron pathway, involving reduced ferredoxin, and the artificial pathway, using reduced benzyl viologen. It is evident that the nitrite reductase system is the principle enzyme system being inhibited by CO. The inhibitory action cannot be attributed to the iron-protein, hydrogenase, since hydrogenase is not involved in electron flow from reduced benzyl viologen to nitrite reductase (Figures 7,8).

Studies with hydrogenase had shown that this enzyme is inhibited reversibly by carbon monoxide (37,41). It was of interest to determine

if the inhibition of nitrite reductase was also of a reversible nature. This was determined by using the clastic extract-benzyl viologen reaction mixture, with sodium dithionite as the electron source. Duplicate flasks were filled with 10% CO and 90% He. After two minutes of preincubation, one of the flasks was re-evacuated and filled with 100% He. Both flasks were then assayed for nitrite reduction in the usual manner, along with controls. As shown in Table VIII, the inhibition was reversible. The flask which had been re-evacuated and filled with helium had the same activity as the control containing helium initially. The duplicate flask containing 10% CO throughout the incubation period formed only about half as much ammonia, as would be predicted from Figure 9.

Table VIII

Reversible Inhibition of Nitrite Reductase with CO

Flask No.	Gas Phase	μmoles NH_3 Formed[a]
1	10% CO + 90% He	1.1
2	Same as (1) for 2 min, then replaced with 100% He	3.0
3	100% He	2.8
4	100% CO	0.1

[a]Reaction vessels contained 12 mg clastic extract, 12 μmoles nitrite, 25 μmoles benzyl viologen, 100 μmoles dithionite, 200 μmoles phosphate buffer pH 7.0, in 5 ml total. 25 ml flask assay incubated for 2 h at 37°.

Chelator Inhibition of Nitrite Reductase. The inhibiting effects of several sulfhydryl- and metallo-enzyme inhibitors were determined. The inhibitors were evaluated at 10^{-3}M concentration, in 25 ml flask assays. The results, Table IX, indicated that the nitrite reductase from C. pasteurianum is a metalloenzyme, since the general metal chelating agent, EDTA, significantly inhibited the enzyme. This enzyme may also contain sulfhydryl groups as part of the active configuration. Two -SH inhibitors, $NaAsO_2$ and PCMB, imparted appreciable inhibition of nitrite reductase activity.

Significant inhibition was observed only with the iron chelators. Chelating compounds for copper, magnesium and nickel did not lead to significant inhibition. These studies indicate that the enzyme is a metalloprotein containing iron.

Kinetics of Nitrite Reduction with Benzyl Viologen. The reaction kinetics for the benzyl viologen-mediated reduction of nitrite were determined. The Lineweaver-Burk plot of the relationship between reaction velocity and substrate (nitrite) concentration is shown in Curve B, Figure 6. The maximum velocity constant, V_m = 5.6 μmoles NH_3/30 min, and K_m = 25 μM.

A similar kinetic study with benzyl viologen as the substrate-limiting variable lead to the Lineweaver-Burk plot shown in Figure 10. The Michaelis-Menten constants were V_m = 2.2 μmoles NH_3/30 min, and K_m = 0.57 μM. A straight-line relationship for benzyl viologen as substrate could be approximated only for low dye concentrations (1-10 μmoles). At higher concentrations, the points were scattered. This may be attributed to the reversible oxidation-reduction nature of the dye,

Table IX

Chelator and SH Group Inhibition of Nitrite Reductase[a]

Chelating Agent	% Inhibition[b]	Inhibitor Specificity[c]
Disodium ethylenediamine-tetracetate	71	general
$NaAsO_2$	60	$<^{SH}_{SH}$
p-chloromercuribenzoate	41	SH
2,2'-bipyridine	86	Fe^{+2}
Tiron (disodium-1,2-dihydroxy-benzene-3,5-disulfonate)	37	Fe^{+3}, Ti
Bathophenanthroline (4,7-diphenyl-1,10-phenanthroline)	30	Fe^{+2}
KF	22	Fe^{+3}, Mg
KN_3	0	Fe^{+3}, Cu
Diethyldithiocarbamate	6	Cu
Bis-cyclohexanoneoxaldihydrazone	0	Cu
Potassium ethylxanthate	0	Cu
Cuprione (2,2'-biquinoline)	0	Cu
1-allyl-2-thiourea	0	Cu
Salicylaldoxime	0	Cu, Ni
o,o'-dihydroxyazobenzene	28	Mg
Erichrome Black T	0	Mg

[a]Reaction vessels contained 25 mg clastic extract, 12 μmoles nitrite, 25 μmoles benzyl viologen, 100 μmoles dithionite, 200 μmoles phosphate buffer pH 7, 10^{-3} M inhibitor, in 5 ml total. 25 ml flask assay with helium gas. 2 h at 37°.

[b]Calculated as $\frac{(\mu\text{moles } NH_3 \text{ w/o inh.} - \mu\text{moles } NH_3 \text{ w inh.})}{\mu\text{moles } NH_3 \text{ w/o inh.}}$ times 100%.

[c]Taken from references 5,6,7,13.

which was consequently not under substrate limiting conditions at the higher concentrations employed.

pH Profile with Benzyl Viologen. The activity of the benzyl viologen-mediated system as a function of pH was determined in 25 ml flask assays. The results (Figure 5) show a narrow optimum in the pH range 6.5-7.5, dropping off sharply in either direction. The pH values for the study shown here represent the pH of the buffer with sodium dithionite added, since the dithionite lowers the pH of the buffered solution. The pH profile for the independent nitrite reductase system is much more limited than for the overall system involving hydrogenase and ferredoxin (Figure 5).

Reduction of Other Substrates. The ability of the nitrite reductase system to reduce three other forms of nitrogen: nitrate, hydroxylamine and nitrous oxide, were determined in 25 ml flask assays. The results, Table X, showed that the system could readily reduce hydroxylamine, but not nitrate or nitrous oxide. Hydroxylamine was reduced much more completely than nitrite, as might be expected, since the formation of ammonia from this compound requires only a two-electron reduction per mole. This data indicates that no active nitrate reductase is present in C. pasteurianum when the organism is grown on ammonia as the nitrogen source.

The isolation of nitrous oxide as an intermediate in nitrite reduction by several aerobic bacteria has been reported (9). The above data indicate that it is not an intermediate in the system under study here. Not only can it not be utilized as a substrate for the C. pasteurianum nitrite reductase enzyme, it does not inhibit the reduction of

Table X

Activity of Other Substrates

Substrate	μmoles NH_3 Formed[a]
NO_3^-	0
NO_2^-	2.5
NH_2OH	10.5
N_2O (gas)[b]	0

[a]Reaction vessels contained 25 mg clastic extract, 25 μmoles benzyl viologen, 100 μmoles sodium dithionite, 200 μmoles phosphate buffer pH 7.0, 12 μmoles substrate, in 5 ml total. 25 ml flask assay with helium gas. 2 h at 37°.

[b]Studied as 10, 25, 50 and 100% mixtures with helium gas.

nitrite. Inhibition experiments were carried out with a series of 25 ml flasks containing 0, 5, 10, 25 and 100% N_2O in helium, respectively. All flasks reduced equivalent amounts of nitrite to ammonia when incubated for 2 h at 37°.

Cofactor Requirements for Nitrite Reduction. Valentine and Wolfe (52) reported previously that ferredoxin could not mediate the reduction of nitrite with $NADPH_2$. This observation was confirmed in the current studies. $NADH_2$ and $NADPH_2$ were supplied to the reaction mixture containing crude extract, by appropriate generating systems (Table II).

The results, Table XI, show that the system readily reduced nitrite from sodium dithionite but not from either reduced pyridine nucleotide. In another study, 25 μm of riboflavin, FMN, FAD, NAD, or NADP were added separately to flasks containing the reaction mixture as shown in Table X, except 25 mg clastic extract and 100 μm sodium dithionite were included instead of crude extract. None of these reduced cofactors provided an electron source for nitrite reduction, since no ammonia was formed from any of the reaction mixtures.

Table XI

Cofactor Participation with Nitrite Reductase

Electron Donor	μmoles NH_3 Formed[a]
None	0.0
100 μmoles $S_2O_4^=$	9.7
80 units ADH--$NADH_2$[b]	0.2
100 units G-6-PDH--$NADPH_2$	0.1
50 units IDH--$NADPH_2$	0.0

[a]Reaction vessels contained 25 mg crude extract, 20 μmoles nitrite, 100 μmoles phosphate buffer pH 7.0, electron donor as indicated, in 3 ml total. Warburg flask assay with helium or nitrogen gas. 30 min at 37°.

[b]Complete generating systems given in Table II.

Purification of Nitrite Reductase

The ultimate goal in most detailed studies of a given enzyme is to purify the protein, and determine as many of its inherent characteristics as possible. In this study, it was desirable to have a highly purified preparation to study more closely the stoichiometry of the reaction, to determine the specific metal involved and its role in the reaction being mediated, and to learn the physical and chemical parameters of the enzyme or enzymes involved.

Several methods were used in attempts to prepare a purified preparation of the nitrite reductase from C. pasteurianum. The results of these studies are summarized below. In these experiments, specific activity was defined as μmoles NH_3/mg protein/30 min, and total units of activity as specific activity multiplied by the total mg protein in the sample.

Protamine Sulfate Precipitation. Protamine Sulfate (PS) is commonly used in protein purification studies to remove inactive protein and nucleic acid material from a preparation. Its effectiveness in purification of clastic extract was determined in an experiment where the extract was treated with 1 mg PS/10 mg protein. The characteristics of the two preparations are shown in Table XII. In addition to producing a much clearer preparation, the protamine sulfate treatment increased the specific activity by 32%.

A study was undertaken to determine the effect of PS concentration on the ratio of optical absorption at 260 mμ (nucleic acid absorption maximum) and 280 mμ (protein absorption maximum). This was done to find the

optimum PS concentration for use with clastic extract preparations. The results of this study (Figure 11) indicated that the most useful range of PS concentration would be in the 4-7 mg protein/mg PS ratio, where the absorbency ratios (A_{260}/A_{280}) had decreased from about 1.9 to 1.4-1.6. The addition of larger amounts of PS did not lead to much greater decreases in the absorbency ratio (i.e., lower the nucleic acid concentration).

Table XII

Properties of a Protamine Sulfate Preparation

Fraction	Appearance	Protein (mg/ml)	μmoles NH_3 Formed[a]	Specific Activity[b]	Total Activity[c]
Clastic extract	cloudy, brown	14.0	6.8	1.36	572
PS cut[d]	clear, lt. brown	10.0	9.0	1.80	504

[a]Reaction vessels contained 5 mg protein, 20 μmoles nitrite, 25 μmoles benzyl viologen, 100 μmoles dithionite, 100 μmoles phosphate buffer pH 7, in 3 ml total. Warburg flask assays with helium gas. 30 min at 37°.

[b]Expressed as μmoles NH_3/mg protein/30 min.

[c]Expressed as specific activity X total mg protein in fraction.

[d]1 mg PS/10 mg protein.

Protamine sulfate was used in conjunction with other methods of purification as described below. Unfortunately, the relationships between PS concentration, protein recovery and activity recovery did not

remain constant throughout the studies. It was obvious that in some of the results (i.e., Tables XIV, XV following) significant activity was lost during PS treatment. It appeared that PS behavior varied with the batchof cells, or the age of the cells or some other factor that was not readily apparent. Eventually, it was found that a preliminary PS concentration study had to be carried out prior to the initiation of each experiment, to determine the optimum PS concentration to use for that particular preparation.

Ammonium Sulfate Precipitation. The use of ammonium sulfate in the purification of an enzyme is one of the most widely used methods in enzymology. The method was used in these studies but was not very satisfactory. A 0-67% $(NH_4)_2SO_4$ fraction was obtained from clastic extract. The material was dialyzed overnight against 0.1 M phosphate buffer pH 7.0, with nitrogen purging, to remove the ammonia. However, the preparation still contained about 1 µmole NH_3/mg protein. This was considered excessive for the type of experiments being planned.

The purified material was active in reducing nitrite with either benzyl viologen or ferredoxin, when sodium dithionite was the electron source (Table XIII). The hydrogenase present in the purified fraction was apparently inactivated during dialysis, since no ammonia was formed and no hydrogen was consumed when hydrogen was used as the electron donor.

An attempt was made to remove more of the endogenous reaction product, ammonia, from the purified nitrite reductase fraction by use of a molecular sieve bed. Since this technique would be less time consuming than dialysis, it was felt that the enzyme might remain more active. Duplicate samples of a freshly prepared 0-67% $(NH_4)_2SO_4$ fraction were

Table XIII

Nitrite Reduction with an $(NH_4)_2SO_4$ Fraction[a]

Electron Donor	μmoles NH_3 Formed	μmoles H_2 Change
100 μmoles $S_2O_4^=$ (He gas)		
+ 25 μmoles benzyl viologen	7.0	0
+ 0.2 mg ferredoxin	1.7	+11.2
H_2 Gas		
+ 25 μmoles benzyl viologen	0	0
+ 0.2 mg ferredoxin	0	0

[a]Reaction vessels contained 4.4 mg protein, 20 μmoles nitrite, 100 μmoles phosphate buffer pH 7, electron donor as indicated, in 3 ml total. Warburg flask assay for 30 min at 37°. Data shown are averages of two assay experiments.

compared after one was dialyzed as before, and the other passed through a 5 X 22 cm Sephadex G-25 medium column, equilibrated with 0.01 M Tris-HCl buffer pH 7.3. However, the dialyzed fraction contained much less ammonia (0.76 μmoles NH_3/mg protein) than the Sephadex-treated fraction (10.0 μmoles NH_3/mg protein).

In another study, the dialysis buffer was changed after three hours of a six hour dialysis period. The resulting 0-67% $(NH_4)_2SO_4$ precipitate fraction had only 89 units of activity, compared to 2395 units of nitrite reductase activity in the clastic extract (assayed using the same dithionite and benzyl viologen system as described for Table XIII).

Further studies were made using a heat treatment (60° for 15 min, under H_2), or protamine sulfate precipitation prior to ammonium sulfate precipitation, but these were not of value in enhancing the separation of a more active reductase fraction. Conversely, it was noted that during heat treatment, some unstable form of low molecular weight material apparently decomposed, as the ammonia level increased 8-fold over the pretreatment level.

Ethanol Precipitation. Selective precipitation of proteins by ethanol at very low temperatures has proven of value in many purification schemes. This type of purification was investigated to determine if selective recovery of nitrite reductase could be achieved. The results of the best ethanol precipitation experiment are summarized below.

A clastic extract sample was treated with protamine sulfate (1 mg PS/9 mg protein) and the resulting supernatant was treated with ethanol to give 0-15% EtOH ppt, 15-50% EtOH ppt, and 50-67% EtOH ppt. The precipitates were resuspended in N/150 phosphate buffer pH 7.0, and assayed the same day. The results of the study are shown in Table XIV. Although about 33% of the total activity was recovered in the 15-50% ethanol fraction, the specific activity was only slightly higher. Repeated attempts to improve on the specific activity and total yield gave similar results. Thus, it was decided that ethanol precipitation would not be a suitable purification procedure for these investigations.

Purification with Gel Adsorbents. Calcium phosphate and alumina C_γ gels have proven useful in certain purification procedures. Experiments were performed to determine their usefulness in the purification of nitrite reductase.

Table XIV

Nitrite Reductase Activity of Ethanol Precipitates

Fraction	Total mg Protein	µmoles NH_3 Formed[a]	Specific Activity[b]	Total Activity[c]	% Total Activity
Clastic Extract	968	8.4	3.36	3255	(100)
PS Supernatant	673	9.1	3.64	2450	75
---					---
0-15% EtOH ppt	25	7.1	2.84	71	2
15-50% EtOH ppt	215	9.5	3.80	818	25
50-67% EtOH ppt	19	1.2	0.48	9	0.3

[a]Reaction vessels contained 2.5 mg protein, 20 µmoles nitrite, 100 µmoles dithionite, 100 µmoles phosphate buffer pH 7, 25 µmoles benzyl viologen, in 3 ml. Warburg flask assay with helium gas. 30 min at 37°.

[b]Expressed as µmoles NH_3/mg protein/30 min.

[c]Expressed as specific activity X total mg protein in fraction.

Preliminary experiments with calcium phosphate gel indicated that at low concentrations (0.5-1 mg gel/mg protein) the gel would not adsorb significant amounts of the nitrite reductase. The specific activity of the supernatant was not increased. A further experiment was completed to determine if larger concentrations of the gel would prove more satisfactory. A clastic extract was first treated with 1 mg protamine sulfate per 5 mg protein. The pH of the resulting supernatant was lowered to pH 5.5 with dilute acid, and divided into four equal samples. Initial experiments indicated that essentially no reductase activity was lost when

the adsorption was carried out at pH 5.5. Three portions of the acidified protein solution were treated with calcium phosphate gel, to give gel to protein ratios (by wt) of 2:1, 5:1, and 10:1. The fourth sample was treated with Alumina C_γ gel at a 2:1 ratio.

After recovery of the supernatant and resuspension of adsorbed protein from the gels, the nitrite reductase activities of all fractions were determined. The results are summarized in Table XV. The data indicate that no significant separations of nitrite reductase activity from clastic extract were made by treatment with either gel, at any of the ratios studied. The highest increase in specific activity was found in the protein adsorbed by the calcium phosphate at the 2:1 gel:protein ratio. However, this was only a two-fold increase, and represented only about 14% of the initial activity. These studies indicated that these gels would not be useful in purification of nitrite reductase.

Purification on DEAE Cellulose. A preliminary experiment had indicated that nitrite reductase would bind tightly to DEAE cellulose at pH 8, and would be eluted only under conditions of high ionic strength and pH 7.3. This information was utilized to determine if an active fraction which would reduce nitrite could be obtained by column chromatography separation of a protamine sulfate fraction.

A sample of clastic extract was treated with protamine sulfate (1 mg PS/5 mg protein), the mixture was centrifuged for 30 min at 144,000 X g, and 142 mg of the supernatant was placed on a 2 X 15 cm column of DEAE cellulose which had been equilibrated with 0.01 M Tris-HCl pH 8.0. The column was developed in succession with 150 ml 0.01 M Tris-HCl pH 7.3, 200 ml 0.01 M Tris-HCl pH 7.3 + 0.1 M NaCl, and finally

Table XV

Activity of $CaPO_4$ and Alumina C_γ Gel Fractions

Fraction	Total Mg Protein	µmoles NH_3 Formed[a]	Specific Activity[b]	Activity[c] Cut	Activity[c] Total	% Initial Activity
PS Sup[d]	60.0	2.7	1.18	---	71.4	(100)
2/1 CP Sup	55.0	2.3	0.92	51.0		
2/1 CP Ppt	4.4	5.8	2.30	10.1	61.1	86
5/1 CP Sup	40.7	0.9	0.36	14.6		
5/1 CP Ppt	18.4	0.9	0.36	6.5	21.1	30
10/1 CP Sup	31.0	1.4	0.56	17.4		
10/1 CP Ppt	28.6	1.8	0.72	20.6	38.0	53
2/1 Al Sup	54.1	1.4	0.56	30.3		
2/1 Al Ppt	5.2	4.1	1.63	8.5	38.8	54

[a]Reaction vessels contained 2.5 mg protein, 10 µmoles benzyl viologen, 20 µmoles nitrite, 100 µmoles dithionite, 100 µmoles phosphate buffer pH 7, in 3 ml total. Warburg flask assay with helium gas. 30 min at 37°.

[b]Expressed as µmoles NH_3/mg protein/30 min.

[c]Expressed as specific activity X total protein in fraction.

[d]Values shown are for 25% of total PS supernatant, to facilitate direct comparison with each treatment below.

with a gradient of 300 ml 0.1 M NaCl to 300 ml 0.5 M NaCl, in 0.01 M Tris-HCl pH 7.3. The elution pattern is shown in Figure 12.

As expected, the nitrite reductase activity was in the last peak off the column (E). It had a specific activity of 2.2, a 3-fold increase over the PS-treated fraction (0.8). Although the total recovery of protein was good, 83%, the recovery of nitrite reductase activity was poor, 16 units out of 114 units activity initially. (Some reductase activity may actually have been present in one or more of the other fractions but was lost during storage at 4° for several hours prior to assay at the completion of the separation.) This purification method was not considered satisfactory for these studies.

Purification on CM Sephadex. A sample of clastic extract was treated with protamine sulfate (1 mg PS/8 mg protein) and centrifuged at 144,000 X g for 45 min. The supernatant was dialyzed four hours against 0.01 M phosphate buffer pH 6.0 with nitrogen purging. 400 mg protein was placed on a 1.6 X 12 cm column of CM Sephadex, which had been equilibrated with 0.01 M phosphate buffer pH 6.0. The column was developed as follows: a linear gradient of 200 ml 0.01 M phosphate pH 6.0 to 200 ml 0.01 M phosphate pH 7.0, 150 ml 0.1 M NaCl in 0.01 M phosphate pH 7.0, and finally 200 ml 0.5 M NaCl in 0.01 M phosphate pH 7.0. The elution pattern is shown in Figure 13.

The activity of the eluted fractions is summarized in Table XVI. The largest portion of the nitrite reductase activity, 36%, was recovered in fraction A. The specific activity was increased only 2-fold from the PS fraction. 20% of the original protein placed on the column was eluted at the completion of the experiment. Elution with saturated

NaCl in 0.01 M phosphate pH 7.0 did not yield additional protein. The higher salt concentration caused aggregation of the gel bed and the column was rendered useless.

Table XVI

Nitrite Reduction with CM Sephadex Fractions

Fraction	μmoles NH_3 Formed[a]	Specific Activity[b]	Total Activity[c]	% Initial Activity
PS Fraction	2.3	0.92	368	(100)
CMS Fraction A	4.8	1.96	131	36
CMS Fraction B	2.5	1.00	7	---
CMS Fraction C	1.3	0.53	2	

[a]Reaction vessels contained 2.5 mg protein, 20 μmoles nitrite, 10 μmoles benzyl viologen, 100 μmoles dithionite, 100 μmoles phosphate pH 7, in 3 ml total. Warburg flask assay with helium gas. 30 min at 37°.

[b]Expressed as μmoles NH_3/mg protein/30 min.

[c]Expressed as specific activity X total protein in fraction.

Further studies were conducted with fraction A to determine if its activity could be increased. Using the same assay as given for Table XVI, the following observations were made:

(1) Lyophilization caused 60% loss of nitrite reductase activity compared to direct freezing of the sample.

(2) $NADPH_2$ added directly to the reaction mixture did not enhance activity.

(3) Addition of 4 mg "boiled extract" supernatant (clastic extract heated 45 min under H_2 in boiling water bath, then centrifuged 30,000 X g for 15 min) did not enhance activity.

The effect of pH on CM Sephadex behavior was then determined. Three identical 1.6 X 18 cm columns of the gel were prepared, equilibrated at pH 6, 7, and 8, respectively. Identical samples of 49.5 mg clastic extract (sp. act. = 1.2) were placed on each column. Each column was developed with its starting buffer. Large protein peaks came off each column immediately, containing 14.1, 25.2 and 29.2 mg protein, respectively, which were immediately frozen. Additional elution with buffer containing 0.5 M NaCl did not remove additional protein from any of the three columns. The specific activity of the three eluates was identical, 0.71, which was lower than the initial clastic extract. This represented total activity recoveries of 17, 30 and 35%, respectively.

No further studies were made with CM Sephadex as a purification method.

Purification by Starch Block Electrophoresis. Broad separation of protein mixtures by electrical charge using starch as supporting medium has been a successful tool in some purification studies. This technique was employed in an attempt to obtain an active nitrite reductase system from C. pasteurianum clastic extract.

A 60 mg sample of clastic extract (specific activity = 1.1, 660 units total activity) was separated by starch block electrophoresis as outlined in Methods section. The separated proteins were recovered by elution in 3 ml 0.1 M phosphate buffer pH 7.0, which contained 5 μmoles benzyl viologen reduced by 10 μg sodium dithionite + 10 μg sodium

bicarbonate. The protein eluates were maintained under reduced conditions by addition of the dithionite-bicarbonate mixture as needed to maintain the deep blue color of the reduced benzyl viologen dye.

The protein concentrations of the eluted fractions are plotted in Figure 14. Eleven major peaks were obtained from this separation. These fractions were recombined and represented 55% of the original protein sample. This recombined protein mixture exhibited no nitrite reductase activity. The long exposure of the proteins to aerobic conditions (12 hours) during the electrophoretic run, apparently inactivated the reductase enzyme system.

This experiment demonstrated the usefulness of starch block electrophoresis in effecting the separation of protein mixtures in good yield. 86% of the applied protein was recovered in the total eluates.

Purification under Reduced Conditions. Recent investigations have shown that the enzymatic activity of highly labile nitrogen-metabolizing enzymes could be maintained if the entire purification scheme was kept under highly reduced conditions (18). This technique was utilized in an attempt to purify the nitrite reductase system of C. pasteurianum. All steps were carried out under reduced benzyl viologen. All buffers and other solutions contained 10 μmoles benzyl viologen, 20 μg sodium dithionite and 20 μg sodium bicarbonate per 100 ml initially. The dye was maintained in the reduced (dark blue) state by addition of dithionite-bicarbonate as needed. 0.1 M Tris-HCl buffer, pH 7.3, containing 10^{-3} M cysteine, plus 10^{-4} M reduced benzyl viologen was used throughout the studies. The preparations were thus handled under reduced conditions from the time the cell-free extract was prepared, until the

final fractions were obtained. The final purified cuts were dialyzed overnight against the "reduced" Tris-HCl buffer.

A. $(NH_4)_2SO_4$ Fractionation and Calcium Phosphate Adsorption.

A clastic extract sample was subjected to several purification steps under reduced conditions. Initially, ammonium sulfate cuts were taken at 42%, 67% and 90% saturation. In contrast to earlier studies, the resulting fractions retained active nitrite reductase activity (Table XVII). About one-half of the original activity was found in the 42-67% fraction. However, the specific activity of this fraction was slightly lower than the original clastic extract. A definite fractionation of the total clastic extract protein mixture had been obtained, as was shown by the starch gel electrophoresis patterns obtained on the materials (Figure 15). Major portions of a fast moving material, $R_f' = 0.97$, were separated in the 0-42% fraction. All of a major band of $R_f' = 0.68$ was precipitated into the 67-90% cut. This latter material was apparently not required for activity, since both the 42-67% cut and the 67-90% cut had the same specific activity.

The 42-67% $(NH_4)_2SO_4$ fraction was subjected to additional purification steps. 8 ml (308 mg protein) of the cut was treated with 0.1 M acetic acid until the pH of the mixture was lowered to pH 5.0. The pH 5 precipitate was recovered by centrifugation, and the pH 5 supernatant was treated with calcium phosphate gel (2 mg $CaPO_4$/mg protein). The resulting supernatant and precipitate were recovered.

The nitrite reductase activity of the purified fractions was determined (Table XVIII). The pH 5 precipitate contained about one-third of the reductase activity. The specific activity of this fraction was 40% higher than that of the starting preparation. However, the total

Table XVII

Activity of $(NH_4)_2SO_4$ Fractions
Prepared under Reduced Benzyl Viologen

Fraction	Total Mg Protein	µmoles NH_3 Formed[a]	Specific Activity[b]	Total Activity[c]	% Initial Activity
Clastic Extract	622	1.2	2.4	1490	(100)
0-42% A.S.P.[d]	10	--	--	--	--
42-67% A.S.P.	404	1.0	2.0	808	54
67-90% A.S.P.	143	1.0	2.0	286	19

[a]Reaction vessels contained 0.5 mg protein, 20 µmoles nitrite, 10 µmoles benzyl viologen, 50 µmoles dithionite, 100 µmoles Tris-HCl, pH 7.3, in 3 ml total. Warburg flask assay with helium gas. 30 min at 37°.

[b]Expressed as µmoles NH_3/mg protein/30 min.

[c]Expressed as specific activity X total protein in fraction.

[d]A.S.P. = $(NH_4)_2SO_4$ precipitate.

nitrite reductase activity in this final pH 5 precipitate represented only about 14% of the total activity of the original clastic extract, and exhibited essentially the same specific activity (2.4 vs 2.6). Tables XVII and XVIII). Starch gel electrophoresis of the pH 5 precipitate fraction gave essentially the same pattern as that shown for the 42-67% (NH_4) SO_4 precipitate in Figure 15. Although no purified

Table XVIII

Activity of Purified Fractions
Prepared under Reduced Benzyl Viologen

Fraction	Total Mg Protein	µmoles NH_3 Formed[a]	Specific Activity[b]	Total Activity[c]	% Initial Activity
42-67% A.S.P.[d]	308	1.0	2.0	616	(100)
pH 5 Ppt	79	1.3	2.6	205	33
$CaPO_4$ Ppt	7	0.8	1.6	15	2
$CaPO_4$ Supernatant	51	0.7	1.4	71	12

[a]Reaction vessels contained 0.5 mg protein, 20 µmoles nitrite, 10 µmoles benzyl viologen, 50 µmoles dithionite, 100 µmoles Tris-HCl, pH 7.3, in 3 ml total. Warburg flask assay with helium gas. 30 min at 37°.

[b]Expressed as µmoles NH_3/mg protein/30 min.

[c]Expressed as specific activity X total protein in fraction.

[d]A.S.P. = $(NH_4)_2SO_4$ precipitate.

reductase fraction was obtained through the use of these purification steps under reduced conditions, this study did demonstrate that dialysis of an active preparation could be performed without the complete loss of nitrite reductase activity, by including reduced benzyl viologen in the dialyzing medium.

As part of these experiments, the feasibility of using starch gel for obtaining a purified nitrite reductase system was determined. In

several studies where varying amounts of protein were placed on starch gel, it was shown that only relatively small amounts of protein (10-15 mg) could be separated by this method at one time. Protein recovery was also poor. In the best experiment, only 12% (1.6 mg) total protein was recovered from a 13 mg initial sample, after freezing and thawing the final gel protein bands, and eluting the protein by homogenization and centrifugation in buffer. Consequently, this method was not considered satisfactory for protein purification.

B. Chromatography on Sephadex G-200.

Since the above data indicated that better recovery or retention of nitrite reductase activity could be achieved under reduced conditions, it was of interest to determine if improved separations of reductase activity could be made with column chromatography under reduced conditions. In an effort to learn something of the approximate molecular size of the nitrite reductase system, a column chromatography experiment with Sephadex G-200 was carried out. Sephadex G-200 fractionates materials of roughly 5,000 - 200,000 molecular weight. A 1 X 17 cm column of this material was prepared and equilibrated overnight with the reduced Tris buffer described above. By following elution of a reduced dye band, the holdup volume of the column was determined to be 5 ml.

A sample of 65 mg protein clastic extract was suspended in reduced buffer and placed on the top of the column. The column was then eluted with 28 ml reduced Tris buffer, followed by 20 ml reduced Tris buffer + 0.1 M NaCl, and finally with 18 ml reduced Tris buffer containing 0.5 M NaCl. The eluate was collected in 5-7 ml portions, maintained in

reduced form with dithionite, and immediately frozen after withdrawing 0.5 ml for protein determinations. The protein concentration of the eluted fractions is shown in Figure 16. 54% of the material was eluted as a large initial peak, A. Three other distinguishable peaks were also obtained, labelled B, C and D; representing 12%, 6% and 6% of the total material applied, respectively. 91% of the total applied protein was recovered in all of the eluted fractions.

The nitrite reductase activity of the eluted fractions is shown in Table XIX. These data indicate that 36% of the total nitrite reductase activity was recovered in the initial fraction, A. The addition of 0.5 mg protein portions of each of the fractions B, C and D to 5 mg protein A, did not enhance the reductase activity. Rather, the combined fractions showed essentially no activity, for some unknown reason.

The results of this experiment indicate that the nitrite reductase activity is expressed by a protein or protein-complex of high molecular weight. The protein fraction containing the recovered reductase activity was eluted in the initial eluate, indicating that its molecular weight was relatively large (100,000 - 200,000). Later fractions of lower molecular weight exhibited no reductase activity, nor did they enhance the activity of the main fraction, A, suggesting that no low molecular weight material is directly involved in the reductase system. Furthermore, the recovery of nitrite reductase activity under reduced conditions was identical to that obtained under normal conditions with CM Sephadex (36%) (Table XVI). This suggests that some irreversible inactivation of the reductase activity occurs when it is placed on the column, which is not directly due to oxidation of the initial sample.

Table XIX

Nitrite Reductase Activity of Sephadex G-200 Fractions Prepared under Reduced Conditions

Fraction	Total Mg Protein	µmoles NH_3 Formed[a]	Specific Activity[b]	Total Activity[c]	% Initial Activity
Clastic Extract	65	6.0	1.2	78	(100)
A	35	4.0	0.8	28	36
A+B+C+D[d]	48	0.4	0.06	0.3	--

[a]Reaction vessels contained 5 mg protein, 20 µmoles nitrite, 10 µmoles benzyl viologen, 50 µmoles dithionite, 100 µmoles Tris-HCl buffer pH 7.3, in 3 ml. Warburg flask assay with helium gas. 30 min at 37°.

[b]Expressed as µmoles NH_3/mg protein/30 min.

[c]Expressed as specific activity X total mg protein in fraction.

[d]Assayed as 5 mg fraction A + 0.5 mg each of fractions B, C. D.

Multiple Ferredoxin Proteins

During the elution of ferredoxin from a DEAE cellulose column with dilute NaCl, it was sometimes noted that the dark brown ferredoxin band would separate into two or more distinct bands. Some darkly-colored material would not be eluted if the NaCl concentration was 0.2 M or lower. Other workers had also noted the occasional observance of multiple ferredoxin bands. It was felt that perhaps several ferredoxin-like proteins were present in a cell-free extract of C. pasteurianum. If this were true, they might vary in their electron-mediating capacity with nitrite reductase, and other cellular activities. This possibility was investigated.

Isolation of Ferredoxin Proteins. During the preparation of pure ferredoxin, the partially purified material was eluted from a column of DEAE cellulose by a linear gradient of 0-0.2 M NaCl (24). At the completion of this elution, a dark brown band of polar material remained at the top of the column. This material could be removed by increasing the eluant concentration to 0.4 M NaCl. In initial experiments, it was found that several ferredoxin-like proteins could be identified from this polar material. Preliminary analyses showed that these proteins varied in their electron-mediating capabilities. However, larger quantities of each protein were needed to confirm these observations.

A large quantity of dark brown polar material was obtained following the large-scale preparation of pure ferredoxin from 2 kg of C. pasteurianum cells. This polar material was recovered from the DEAE cellulose columns by elution with saturated NaCl. The briny solution

was dialyzed against cold water with nitrogen purging overnight, to remove the salt. The brown solution was then carefully placed on a 2.2 X 13 cm column of DEAE cellulose which had been equilibrated with 0.01 M Tris-HCl pH 7.3. It formed a tight dark brown band about 1 cm wide at the top of the column.

The column was developed by elution with a linear gradient of 500 ml 0.1 M Tris-HCl pH 7.3 to 500 ml 0.2 M NaCl in 0.1 M Tris-HCl pH 7.3. This was followed with another linear gradient of 500 ml 0.2 M NaCl to 0.4 M NaCl, in 0.1 M Tris-HCl pH 7.3. The elution pattern of the separation is shown in Figure 17. Both the normal protein absorption maximum at 280 mμ and the ferredoxin absorption maximum at 390 mμ were monitored (17). The absorption maxima were very similar, and indicated the presence of four ferredoxin proteins in the eluate.

Nitrite Reductase Activity. The fractions were assayed for their activity as electron donors for nitrite reductase (Table XX). The data showed that all four fractions were quite active, although the specific activities varied somewhat. The greatest amount of activity was found in fraction A, which probably reflects its similarity to normal ferredoxin.

Phosphoroclastic Reaction Activity. One of the earliest reactions in which ferredoxin was identified as a participant was the phosphoroclastic reaction (25):

$$\text{Pyruvate} + PO_4^{-3} \xrightarrow[\text{CoA,TPP}]{\text{Fd}} \text{Acetyl} \sim P + CO_2 + H_2$$

The relative effectiveness of the ferredoxin proteins in mediating this reaction was determined. (There was not a sufficient quantity of fraction B remaining to determine its activity in this reaction). The

Table XX

Ferredoxin Proteins Activity with Nitrite Reductase

Fraction	μmoles NH_3 Formed[a]	Specific Activity[b]	Total Activity[c]
A	6.8	32.4	356
B	6.3	20.3	18
C	4.3	35.8	161
D	7.9	13.9	71

[a]Reaction vessels contained 12 mg clastic extract, 0.1 ml ferredoxin fraction, 10 μmoles nitrite, 100 μmoles phosphate pH 7, in 3 ml total. Warburg flask assay with hydrogen gas. 30 min at 37°.

[b]Expressed as μmoles NH_3/mg ferredoxin/30 min.

[c]Expressed as specific activity X total protein in fraction.

assay was carried out in Warburg flasks and the evolution of hydrogen was followed manometrically. The results (Table XXI) indicated that all three fractions were active in this reaction. They exhibited somewhat lower specific activities than pure ferredoxin. No outstanding differences in activity among the fractions were shown.

The studies summarized in Tables XX and XXI above indicated that no significant differences existed among the four fractions obtained from the chromatographic separation. Although they varied in their

Table XXI

Ferredoxin Proteins Activity in Phosphoroclastic Reaction

Fraction	μmoles H_2 Formed[a]	Specific Activity[b]
A	2.13	10.1
C	3.15	15.0
D	2.31	8.0
Pure Ferredoxin	1.21	19.9

[a]Reaction vessels contained 6 mg clastic extract, 0.1 ml ferredoxin, 20 μmoles pyruvate, 100 μmoles phosphate pH 7, in 3 ml total. Warburg flasks fitted with open end manometers and 0.2 ml 14% NaOH in center wells to absorb CO_2. Helium gas. 30 min at 37°.

[b]Expressed as umoles H_2/mg ferredoxin/30 min.

affinity to DEAE cellulose, and thus in their overall molecular charge, their physiological functions were essentially identical to normal ferredoxin. Furthermore, no differences could be detected in the complete spectrum of each fraction as determined on a Beckman DK-2a recording spectrophotometer. All showed the characteristic ferredoxin protein trace with a shoulder at 390 mμ (17). Consequently, no further studies were carried out on these materials.

Comparison of Nitrite Reductase Activities in Several Clostridial Strains

Once the general parameters of nitrite reduction had been defined for Clostridium pasteurianum, it was of interest to compare them with the characteristics of other clostridial strains. For this study, two strains were selected which were known to be similar metabolically to C. pasteurianum. They were C. butyricum and C. felsineum. In addition, two strains were also included which were known to be quite different from the above three strains. They were C. acidiurici and C. PA 3579h. These four strains were grown on the medium of Carnahan and Castle (2) (see Methods section), supplemented with 0.25 - 1.0% tryptone or yeast extract. Uric acid was substituted for sucrose in the medium for C. acidiurici. Cultures were grown and harvested, and the cell-free extracts were prepared, as outlined in the Methods section.

Nitrite Reductase Activity. Clastic extracts were prepared from each bacterium to determine the kinetic parameters of nitrite reduction for each strain. Specific activities were determined using reduced benzyl viologen as the artificial electron donor. The activity of the enzyme from C. felsineum was quite similar to that from C. pasteurianum, while that from C. butyricum exhibited a somewhat lower activity (Table XXII). No nitrite reductase activity was detected from extracts of the other two clostridia.

The relationships between reaction rate and substrate (nitrite) concentration were determined for each strain and the results were expressed in typical Lineweaver-Burk plots (Figure 18). The Michaelis-

Table XXII

Kinetic Parameters of Nitrite Reduction

for Several Clostridial Strains

Clostridium Strain	Specific Activity[a]	Michaelis-Menten constants[b]	
		V_{max}[c]	K_m[d]
pasteurianum	1.1	5.6	25
butyricum	0.4	3.5	7.1
felsineum	1.6	12.5	50
acidiurici	0.0	---	---
PA 3579h	0.0	---	---

[a]Expressed as μmoles NH_3/mg protein/30 min. Determined from Warburg flask assays where reaction vessels contained 5 mg clastic extract, 20 μmoles nitrite, 10 μmoles benzyl viologen, 50 μmoles dithionite, 100 μmoles phosphate buffer, in 3 ml. He gas. 30 min at 37°.

[b]Determined from Lineweaver-Burk plots shown in Figure 18.

[c]Expressed as μmoles NH_3/30 min.

[d]Expressed as μM.

Menten constants for the reactions were taken from these plots and are given in Table XXII. The values are all within a 10-fold range, and suggest a close association of the substrate to the enzyme from each active clostridial strain.

Nature of the Enzyme. Some information regarding the nature of the reductase enzyme from each bacterial strain was studied using the

independent activity technique with reduced benzyl viologen, as outlined previously. The inhibitory effects of iron chelators and SH group inhibitors were determined, by addition to reaction mixtures at 10^{-3} M. The results of these assays, shown in Table XXIII, parallel those reported previously for C. pasteurianum when obtained under slightly different conditions (Table IX). The metalloprotein nature of the enzyme was reflected in the effective inhibition by EDTA and iron chelators, and

Table XXIII

Chelator and SH Group Inhibition of Nitrite Reductase in Several Clostridial Strains

	% Inhibition[a]			
Specificity:	General	Iron		Sulfhydryl
Strain of Clostridium	EDTA[b]	a,a'-dipyridyl	bathophenanthroline[c]	$NaAsO_2$
pasteurianum	45	85	31	34
butyricum	74	73	53	42
felsineum	80	93	80	40

[a]Expressed as $\frac{(\mu\text{moles } NH_3 \text{ w/o inh.} - \mu\text{moles } NH_3 \text{ w inh.})}{\mu\text{moles } NH_3 \text{ w/o inh.}}$ times 100%. Determined from Warburg flask assays where reaction vessels contained 5 mg clastic extract, 20 μmoles nitrite, 10 μmoles benzyl viologen, 50 μmoles dithionite, 10^{-3} M inhibitor, 100 μmoles phosphate buffer pH 7, in 3 ml. He gas. 30 min at 37°.

[b]Disodium-ethylenediaminetetraacetate.

[c]4,7-diphenyl-1,10-phenanthroline.

indicate that iron plays an important role in the nitrite reductase activity of all three active strains. The inhibition shown by arsenite suggests that sulfhydryl groups are involved in the active center of the enzyme.

Other Natural Electron Donors. Earlier studies showed that, of several naturally-occurring compounds tested, pyruvate and ∝-ketobutyrate could substitute for hydrogen as an electron source for nitrite reduction in C. pasteurianum (Table V). The ability of these compounds to serve as electron donors with the other clostridial strains under study was determined. Nitrite reductase assays were carried out on crude extracts of each culture, so the endogenous ferredoxin of each strain was present in the reaction mixture. The data, Table XXIII, show that C. butyricum and C. felsineum can utilize the same natural electron donors as does C. pasteurianum. Although the C. butyricum enzyme was able to use the donors in about the same capacity as C. pasteurianum, the C. felsineum preparation provided a significantly lower reaction yield, from each active electron source. No ammonia was formed from crude extracts of either C. acidiurici or C. PA 3579h with any energy source tested.

Table XXIV

Electron Donor Specificity for Nitrite Reduction in Several Clostridial Strains

	μmoles NH_3 Formed[a]				
Clostridium: Electron Donor	pasteurianum	butyricum	felsineum	acidiurici	PA 3579h
Pyruvate	12.5	6.9	3.6	0.0	0.0
α-ketobutyrate	15.9	8.0	8.5	0.0	0.0
α-ketoglutarate	0.7	0.5	0.0	0.5	0.0
Formate	0.5	0.0	0.2	0.0	---
Hypoxanthine	0.5	0.0	0.0	0.7	---
Hydrogen (gas)	19.3	19.5	10.7	0.9	0.0

[a]Reaction mixtures contained 25 mg crude extract, 20 μmoles nitrite, 50 μmoles electron donor, 100 μmoles phosphate buffer pH 7, in 3 ml. Warburg flask assay with He gas (except H_2 gas where indicated). 30 min at 37°.

DISCUSSION

The Reaction. The reduction of nitrite occurs as a six-electron reaction in Clostridium pasteurianum. Ammonia is the only reaction product. The stoichiometry of nitrite loss and ammonia formed reflects the complete reduction of the substrate to product. Repeated attempts to demonstrate the formation of an intermediate compound (i.e., hydroxylamine) or gas (i.e., NO, N_2) were unsuccessful. (Hydroxylamine is readily accepted and reduced to ammonia by the same preparations, however.)

The physiological electron transport mechanism proposed previously (52) has been substantiated:

$H_2 \longrightarrow$ hydrogenase $\longrightarrow$ ferredoxin $\longrightarrow$ nitrite reductase $\longrightarrow NO_2^-$

Ferredoxin couples directly with the reductase system, and is required for activity.

The relationship between hydrogen consumed and ammonia formed does not support a straightforward six-electron per mole ($3H_2/NH_3$) requirement for nitrite reduction. The general observation from the experiments reported here was closer to a 2/1 ratio. (Figures 3 and 4). This is in contrast to the work of Valentine and Wolfe (52) who reported a 3/1 ratio of hydrogen utilized to ammonia formed in their system. No explanation for the discrepancies in these observations is immediately apparent. The lack of any endogenous nitrite reductase activity has been shown (Table III).

The Michaelis constant, K_m, obtained for the overall reaction is similar to that determined for several other preparations. The K_m for C. pasteurianum is 9.1 μM. Other investigators have reported values of 31 μM for P. aeruginosa (53), 60 μM for E. coli B_n (16), 63 μM for

A. vinelandii (44), 7 μM for soybeans (40), and 2.1 μM for tomatoes (42). These values all lie within 10-fold of each other. They reflect a high affinity of the enzyme for the substrate, irrespective of the source of the nitrite reductase enzyme.

The Independent Assay. Studies of nitrite reductase in the past have utilized several methods of determining enzyme activity. All of these methods have depended on the presence of one or more physiologically-dependent enzymes or cofactors, in addition to the nitrite reductase enzyme itself, in the assay procedure. They may have depended on the complete cellular system, such as H_2-hydrogenase-ferredoxin-nitrite reductase. They may have required the presence of a reduced pyridine and/or flavin nucleotide. Or they may have required the complete cell-free extract plus some extra-cellular electron source. In each of these methods, the number and type of cofactors required for activity in addition to the enzyme was not well defined.

The combination of sodium dithionite and benzyl viologen provided a defined electron source for nitrite reduction. This system is an improvement over the existing method of using H_2-hydrogenase-ferredoxin, since hydrogenase is an enzyme that has not been purified, and ferredoxin mediates several other electron-requiring reactions in the clastic extract. Thus the dithionite-benzyl viologen system effectively isolates the nitrite reductase enzyme from other physiologically-dependent factors, and allows an independent investigation to be made on this separate activity. This defined electron source is particularly valuable in studies of enzyme purification, where it is desirable to separate the nitrite reduction activity from other cellular activities, yet still be able to obtain an independent measure of the isolated activity.

The Redox Potential. Comparative studies with ferredoxin and benzyl viologen demonstrated that nitrite reductase and hydrogenase actively compete for electrons when reduced ferredoxin is the electron source. But when benzyl viologen is utilized, the nitrite reductase system is almost completely dominant. Benzyl viologen couples directly with the reductase and does not require hydrogenase for activity.

These results are in contrast to those carried out with cell-free extracts of M. lactilyticus (52). In this system, benzyl or methyl viologen effectively replaced ferredoxin in evolving hydrogen from sodium dithionite. This suggests that the potential of M. lactilyticus hydrogenase is more positive (i.e., less negative) than the C. pasteurianum hydrogenase.

The studies using artificial dyes of varying redox potentials indicated that the operating potential of the C. pasteurianum nitrite reductase is about equal to the hydrogen electrode (E_o' = -0.42 v at pH 7). By contrast, the nitrite reductase from strain 203 bacterium of Taniguchi, et al., (46), would be more positive in nature, since it was active with reduced methylene blue (E_o' = +0.011 v). Also the enzyme from P. aeruginosa can apparently accept electrons from a more positive source, as both reduced pyocyanine (E_o' = -0.034 v) and reduced methylene blue were successful artificial electron donors for this system (53).

These observations of oxidation-reduction potentials with hydrogenase and nitrite reductase suggest that the overall redox systems for C. pasteurianum may operate in a more negative potential range than those of other micro-organisms. This may be a reflection of the anaerobic character of the clostridia.

The Metal. Cu, Fe and Mn have been reported to be associated with nitrite reductase (Table I). The evidence for the presence of Cu and Fe is based on well-established methods of specific metal inhibitor and chelator studies. The presence of one or both of these metals in the organisms reported seems valid. However, the reports concerning Mn have been based on studies of bacteria grown on media of varying metals concentration. Or additions of various metal ions were made to an assay mixture, and the effects on activity determined. It has already been pointed out that such methods lead to misinterpretations of the effects of such metals (12). Side reactions may become important in such instances. With Mn, coincident reactions involving a Mn-dependent peroxidase or a Mn-dependent diaphorase may lead to an erroneous conclusion from the data obtained (12, 42). Unfortunately, no inhibitor or chelating compound is available which will act specifically with Mn.

Mortenson (23) first suggested that iron was involved in nitrite reduction in C. pasteurianum in some manner, in addition to its role in ferredoxin and hydrogenase. The inhibitor studies reported here suggest that iron is present in the reductase system. The evidence obtained with the well known iron chelators support this conclusion. Furthermore, the reversible inhibition demonstrated with CO is very indicative of the presence of iron as an active constituent of the system. The behavior of CO in the current work is typical of the evidence obtained in many studies over the past four decades, where iron has been identified in metalloproteins (13). Since the evidence presented in this study was obtained with the independent nitrite reduction system, the results reflect inhibition on the reductase enzyme, and not on hydrogenase or some other physiologically-dependent protein.

The iron is apparently bound quite tightly to the enzyme, since clastic extract preparations have been dialyzed under inert conditions for several hours without losing activity. More definitive studies of the iron-protein relationship must await further purification of the system.

The Cofactor. The flavin and pyridine nucleotides have been implicated in the electron transport scheme to nitrite reductase of several organisms (Table I). Much of this work has since been severely criticized and discounted by more recent studies. The group led by E. J. Hewitt (12), as well as Sanderson and Cocking (42), have offered several possible explanations for the erroneous implications of the reduced nucleotides in nitrite reduction:

(a) Formation of ammonia through NAD(P) degradation in the micro-conway diffusion dish.

(b) Oxidation of $NAD(P)H_2$ by a Mn-dependent, pyridine nucleotide oxidase which increased the amount of NAD(P) available for (a) above.

(c) Oxidation of nitrite (or hydroxylamine) by a Mn-dependent, FAD-stimulated peroxidase system.

(d) Reduction of a non-biological electron carrier with a $NADPH_2$-dependent diaphorase system.

Any of these associated reactions could provide data which would indicate that reduced nucleotides were participating in nitrite disappearance or ammonia production. These investigators were unable to demonstrate any direct participation of $NADH_2$ or $NADPH_2$ in their studies with several plant nitrite reductase systems.

Hageman, *et al.*, (12) suggested that the benzyl viologen-$NADPH_2$ technique used in their studies proceeded *in vitro* by mechanism (d) above. The $NADPH_2$-dependent diaphorase reduced benzyl viologen which then coupled with nitrite reductase. This artificial electron carrier replaced some unknown factor which performs the same function *in vivo*.

Paneque, *et al.*, (33) have demonstrated that NADP participates in the reduction of nitrite in soybeans only through NADP-ferredoxin reductase, which in turn reduces soybean ferredoxin. (This NADP-ferredoxin reductase may be the unknown factor mentioned by Hageman, *et al.*, above). Furthermore, their studies have supported the earlier observations of Valentine and Wolfe (52), showing that reduced pyridine nucleotides do not participate in the reduction of nitrite with *Clostridium* ferredoxin.

The present studies confirm the recent work summarized above. The evidence obtained indicate that the reduced flavin or pyridine nucleotides do not participate in nitrite reduction in *C. pasteurianum*. The nucleotides were ineffective when added to the reaction mixture initially in the reduced form, or when generated *in situ* by appropriate enzyme generating systems during the course of the reaction. The evidence obtained from this study, together with that obtained by other investigators during the past two years, using bacterial and plant systems, form the basis for the generalization that the only role of reduced pyridine nucleotides in nitrite reduction occurs in plants, where they are indirectly involved by providing a reducing potential for plant ferredoxin.

Enzyme Solubility. Reports of nitrite reductase systems from other sources indicated that some of the reductases are soluble while others are particulate in nature. As part of the initial purification procedures in the current work, precipitates from protamine sulfate, $(NH_4)_2SO_4$, or ethanol treatments were routinely centrifuged at 144,000 X g for 1-4 hours. Although this step was eventually abandoned as an unnecessary pro-oxidative operation, the supernatant fractions did retain nitrite reductase activity after 4 hours. No reductase activity was detected in the precipitates (pellets). These observations suggest that nitrite reductase is a soluble enzyme system.

Attempts were made to confirm this property of the nitrite reductase system by a sucrose density gradient centrifugation. However, when a clastic extract suspension sufficiently concentrated to provide enough protein for subsequent analysis for reductase activity was placed on a 3-20% sucrose gradient, the protein mixture immediately diffused into the lower portion of the tube. It was determined that a sucrose gradient starting at 15% sucrose would be necessary to initially support the clastic extract. Since such an experiment would not be very meaningful, no sucrose gradient was carried out.

Enzyme Purification. Purified preparations of nitrite reductase have been reported from several sources (Table I). A combination of ammonium sulfate precipitations and calcium phosphate gel adsorption provided one purification of 600-fold from P. aeruginosa (53). Other investigators have obtained 10- to 100-fold purifications using similar conventional purification methods.

An acceptable method or combination of methods for obtaining a purified protein preparation must meet the following criteria:

(1) Effective separation of a protein mixture into two or more fractions of less complex protein composition.

(2) Good recovery of initial enzymatic activity in one separated fraction.

(3) Significant increase in specific activity of the protein fraction containing the enzyme being studied.

The methods employed in the present study failed to meet these criteria when utilized in an attempt to obtain a purified preparation of nitrite reductase. No clear-cut separation of the nitrite reductase activity from other cellular proteins was obtained from most of the methods employed. Separations obtained from ethanol precipitation, calcium phosphate gel adsorption and chromatography on CM Sephadex gave a broad spectrum of activity over several fractions. Only in the instance of DEAE cellulose chromatography and Sephadex G-200 chromatography was the major resultant activity concentrated into one protein fraction.

Although high recoveries of total activity were obtained from some calcium phosphate gel separations, the activity was distributed between both resulting components (adsorbate and supernatant) (Table XV). Good recovery of enzymatic activity was also shown in an ammonium sulfate precipitation study, but again the activity was divided between two major fractions (Table XVII). The other methods employed gave poor yields of total enzymatic activity.

No significant increase in specific activity of fractions containing nitrite reductase activity were obtained from any purification method studied. The only increases observed were of the order of 2- to 3-fold, from a calcium phosphate gel precipitate and a fraction separated on a column of DEAE cellulose. Even in these instances, the total nitrite reductase activity represented by the resulting purified fractions was small compared to that of the initial clastic extract (Table XV and page 43).

These results suggest that at least two factors have hindered the purification of nitrite reductase from C. pasteurianum:

(1) The nitrite reductase activity is highly oxygen labile.

(2) The nitrite reductase activity is expressed by a multi-component enzyme complex.

The sensitivity of the reductase system to oxygen is apparent when the results of the ammonium sulfate fractionation studies are considered. In an initial study, 4% of the initial reductase activity was recovered following preparation without exclusion of air, whereas 73% of the activity was retained in a later experiment when prepared under reduced conditions and dialyzed under buffer containing reduced benzyl viologen (page 38 and Table XVII). High recovery of nitrite reductase activity was obtained with protamine sulfate (88%) and in a calcium phosphate gel study (86%) when these entire procedures were performed under nitrogen (Tables XII and XV). The poor recovery of reductase activity in those methods where the preparations were subjected to long exposure of oxidizing conditions; i.e., column chromatography and starch block electrophoresis, lend further evidence to support this conclusion.

There is considerable evidence to support the suggestion that the nitrite reductase activity is expressed by a multi-component enzyme system. The lack of effective separation of the activity into any one fraction by most of the methods employed indicated that the enzyme system is not a simple protein; rather, it is complex in nature and thus distributed itself throughout the separated protein cuts as its various parameters were expressed according to the pH, ionic strength, ionic charge, or other variables of the separation method. This phenomenon was particularly evident from the non-specific activity distributions obtained from ammonium sulfate and ethanol precipitations, and calcium phosphate and alumina C_γ gel fractionations. Only in the fractionations on DEAE cellulose and Sephadex G-200 did the reductase appear in one peak.

The immediate elution of the nitrite reductase activity from the Sephadex G-200 column indicates that the reductase enzyme has a relatively high molecular weight, probably greater than 100,000. The inference of a large molecule which is made by this data compliments the idea of a complex structure for the reductase system.

Consequently the present studies have shown that the conventional approaches to enzyme separation and purification are not applicable to the separation of the nitrite reductase from C. pasteurianum. New methods or alterations of present methods will be needed which will take into account the factors mentioned above--to maintain enzyme stability and at the same time afford a means of preferential enzyme separation, before this enzyme system can be obtained in a purified form.

Multiple Ferredoxins. The demonstration that several proteins possessed physical and chemical characteristics similar to normal ferredoxin led to the initial speculation that several such species may be active with various systems in the bacteria cell metabolism. However, assays of the physiological activity of the proteins indicated they had similar properties. The only significant difference that could be demonstrated among these proteins was their variation in affinity for DEAE cellulose. It now appears that these proteins may be partially degraded species of regular ferredoxin. Or they may have been synthesized by small amounts of contaminating bacteria present in the culture. Their structural differences are apparently of a minor nature, since the active site of electron transport and coupling to electron acceptors has not been significantly altered.

Physiological Role of the Enzyme. During studies of the nitrite reductase enzyme from A. vinelandii, Spencer and co-workers (44) found the enzyme to be constituitive when the organism was grown on N_2, NH_3 or glutamine as sole nitrogen source, but the reductase activity increased about 3-fold when the bacteria were grown on NO_3^-. Only under the latter conditions did the organism require a nitrite reductase activity. Under anaerobic conditions, the respiratory enzyme cytochrome oxidase isolated from P. aeruginosa (55) and pig skeletal muscle (54) have been demonstrated to function as a nitrite reductase. It can accept NO_2^- as a substrate and reduce it to NO, with reduced cytochrome c as the immediate electron donor.

The evidence for the presence or absence of the inter-related activities of nitrite reductase, ferredoxin, and hydrogenase, along with

nitrogen fixation ("nitrogenase"), in the clostridial strains studied in this program, are summarized below:

Strain of *Clostridium*	Enzymatic Activity			
	Nitrite Reductase	Ferredoxin	Hydrogenase	Nitrogenase
pasteurianum	present[a]	present[b]	present[c]	present[d]
butyricum	present[a]	present[b]	present[c]	present[d]
felsineum	present[a]	present[b]	present[a]	present[d]
acidiurici	absent[a]	present[b]	absent[b]	absent[d]
PA 3579h	absent[a]	present[a]	absent[a]	unknown

[a]Determined in current study.

[b]Reference (47).

[c]Reference (35).

[d]Reference (39).

The close association of nitrite reductase, ferredoxin and hydrogenase has now been well established. Comparisons of the data summary above suggest that the ability to fix nitrogen is also present in those strains which metabolize hydrogen and reduce nitrite. That these enzymes are separate activities is known. Procedures have been published for separating hydrogenase from nitrogenase (3) and hydrogenase from nitrite reductase (33) in *C. pasteurianum* preparations. Since the cofactor requirement for nitrogenase activity varies considerably from that of nitrite reductase, these activities are likely expressed by independent enzymes. Thus it appears that although different enzymes are involved in the utilization of H_2, N_2 and NO_2^- in clostridia, their

elaboration may be controlled by the same genetic mechanism. Whether the physiological role of nitrite reductase is, in fact, the reduction of NO_2^-, remains to be established.

SUMMARY

1. A cell-free extract has been obtained from C. pasteurianum which mediates the one-step reduction of nitrite to ammonia. No intermediates have been isolated and ammonia was the sole reaction product.

2. The reaction sequence H_2-hydrogenase-ferredoxin-nitrite reductase-NO_2^--NH_3 has been confirmed. The molar ratio $3H_2/NH_3$ implied by this sequence was not demonstrated in this study.

3. No cofactor requirement could be shown for the reduction of nitrite by the clostridial preparations.

4. The substitution of sodium dithionite and benzyl viologen for H_2-hydrogenase and ferredoxin, respectively, provides an independent method of assay for nitrite reductase where no other physiologically-dependent factors are involved.

5. Inhibitor studies show nitrite reductase is a metalloprotein containing iron.

6. Purification studies indicate that the reductase activity is expressed by a soluble, oxygen sensitive, large protein or protein-complex.

7. Nitrite reductase activity may be distributed among those clostridia which also possess hydrogenase and nitrogen fixing activities.

8. The physiological role of the reductase system has not been established.

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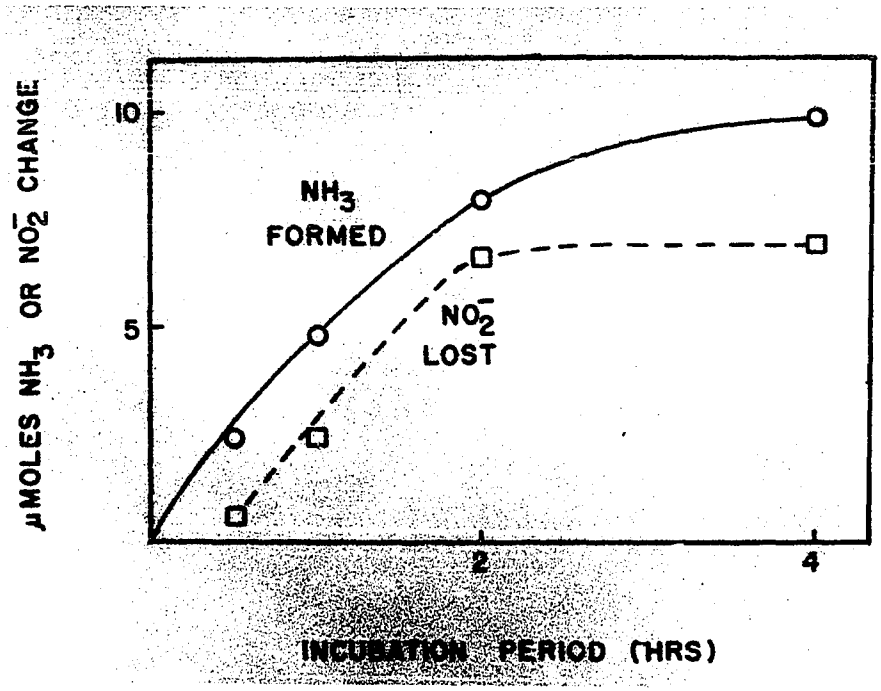

Figure 1. Nitrite Reductase Assay in 25 ml Flasks. 12 mg clastic extract, 0.1 mg ferredoxin, 42 μmoles nitrite, 100 μmoles phosphate buffer pH 7, in 5 ml total. H_2 gas. 37°.

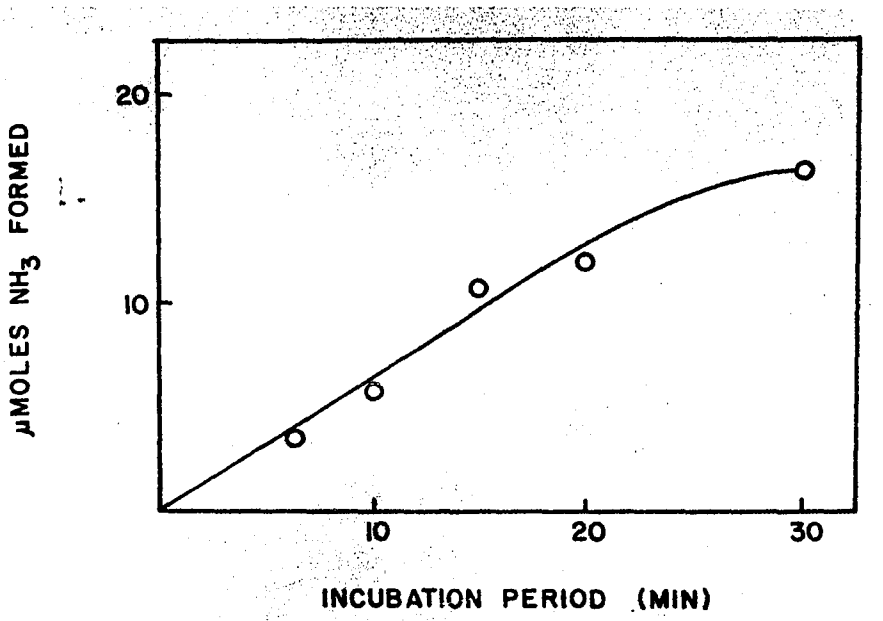

Figure 2. Nitrite Reductase Assay in Warburg Flasks. 12 mg crude extract, 20 μmoles nitrite, 100 μmoles phosphate buffer pH 7, in 3 ml total. H_2 gas. 37°.

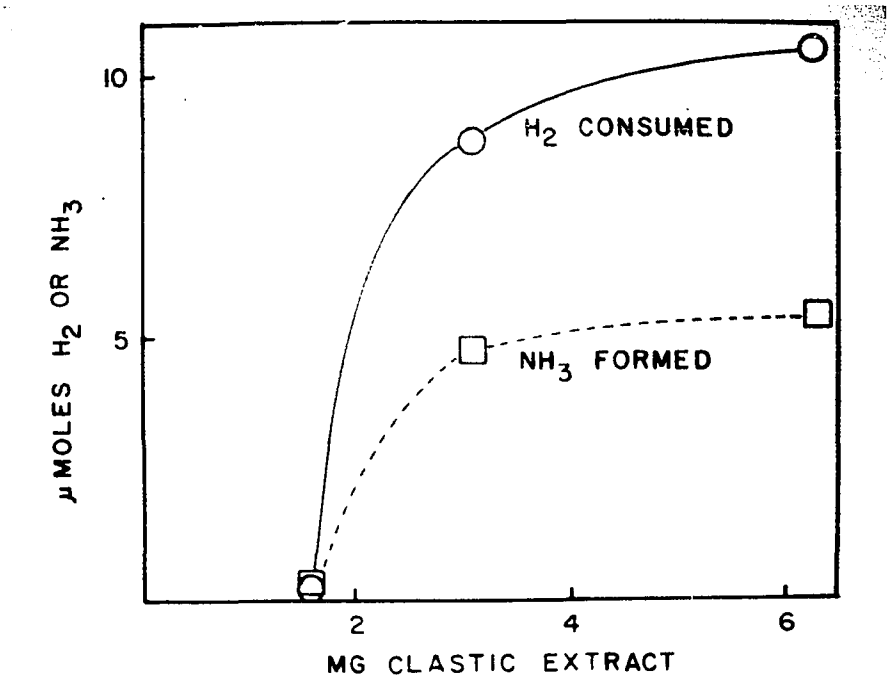

Figure 3. Effect of Enzyme Concentration on Nitrite Reduction. 0.05 mg crude ferredoxin, 20 μmoles nitrite, 25 μmoles phosphate buffer pH 7, in 3 ml total. Warburg flask assay with H_2 gas. 30 min at 37°.

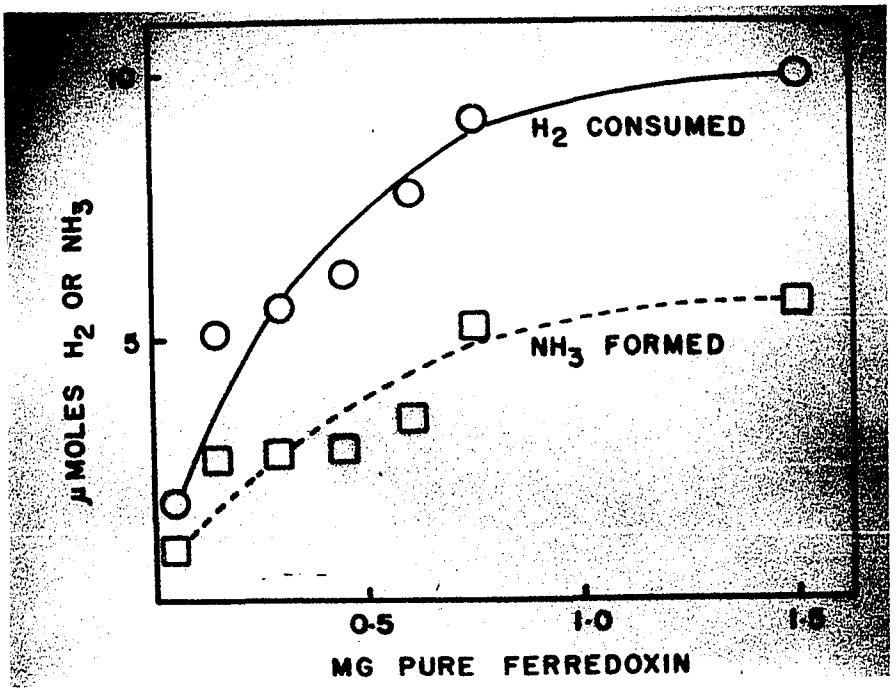

Figure 4. Effect of Ferredoxin Concentration on Nitrite Reduction. 6 mg clastic extract, 10 μmoles nitrite, 100 μmoles phosphate buffer pH 7, in 3 ml total. Warburg flask assay with H_2 gas. 30 min at 37°.

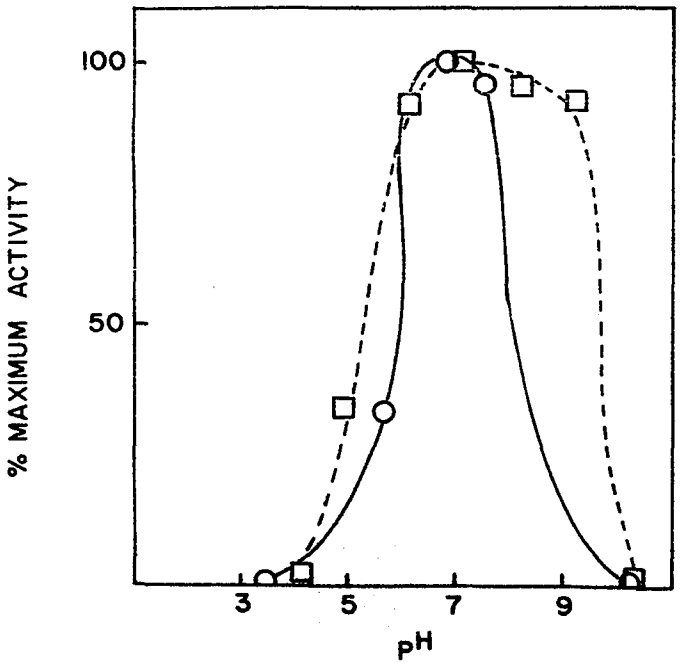

Figure 5. pH Profile of Nitrite Reduction. 100 µmoles dithionite, 400 µmoles phosphate buffer (except carbonate buffer at pH 10.3), in 5 ml total. 25 ml flask assay with He gas.- -□- -□- -25 mg crude extract.——O——O——25 mg clastic extract + 25 µmoles BV.

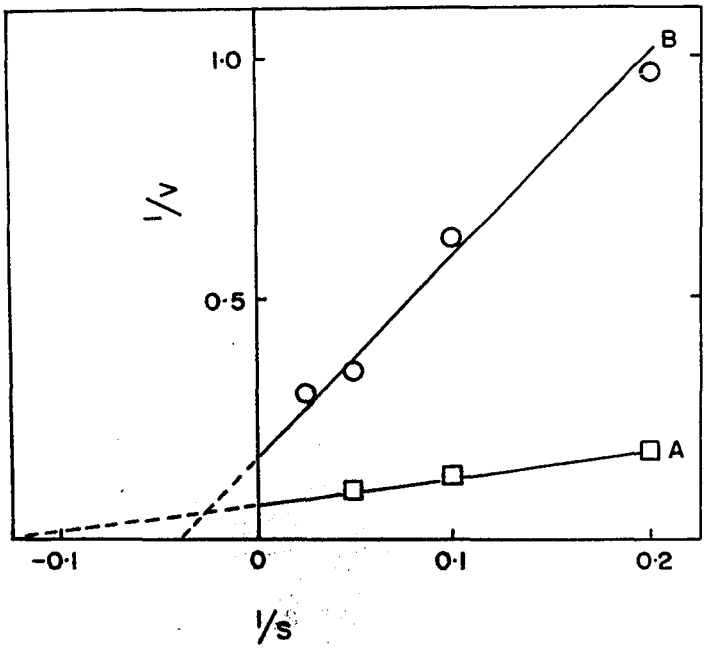

Figure 6. Lineweaver-Burk Plot of Nitrite Reduction. 15 mg clastic extract, 100 µmoles phosphate buffer pH 7, in 3 ml total. Curve A: 0.08 mg crude ferredoxin + H_2 gas. Curve B: 10 µmoles BV + 50 µmoles dithionite + He gas. 1/S = reciprocal µmoles nitrite. 1/V = reciprocal µmoles NH_3 formed in 30 min.

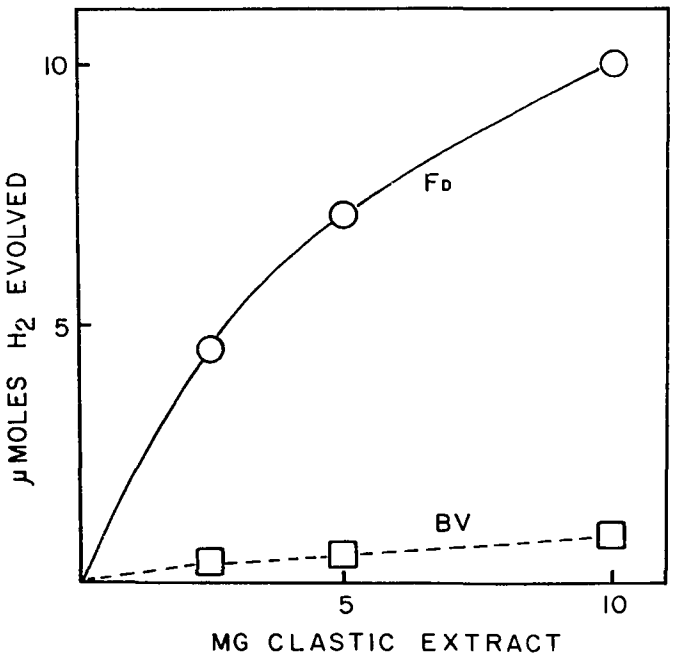

Figure 7. Hydrogen Evolution with Ferredoxin and Benzyl Viologen. 20 μmoles nitrite, 100 μmoles dithionite, 100 μmoles phosphate buffer pH 7, in 3 ml total. Warburg flask assay with He gas.—○—○—0.1 mg pure ferredoxin.- - -□- -□- -25 μmoles benzyl viologen. 30 min at 37°.

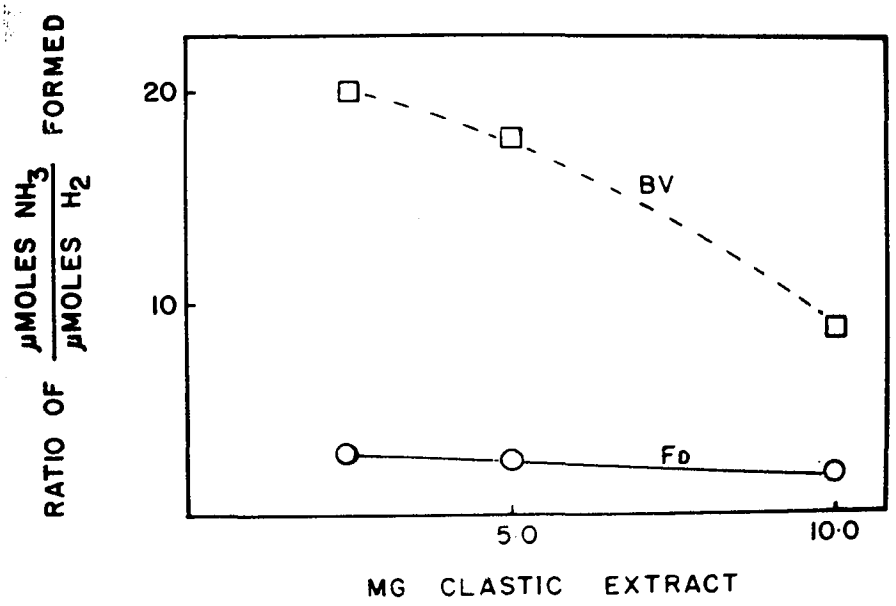

Figure 8. Ratios of Ammonia to Hydrogen Formed by Ferredoxin and Benzyl Viologen. System - same as Figure 7.

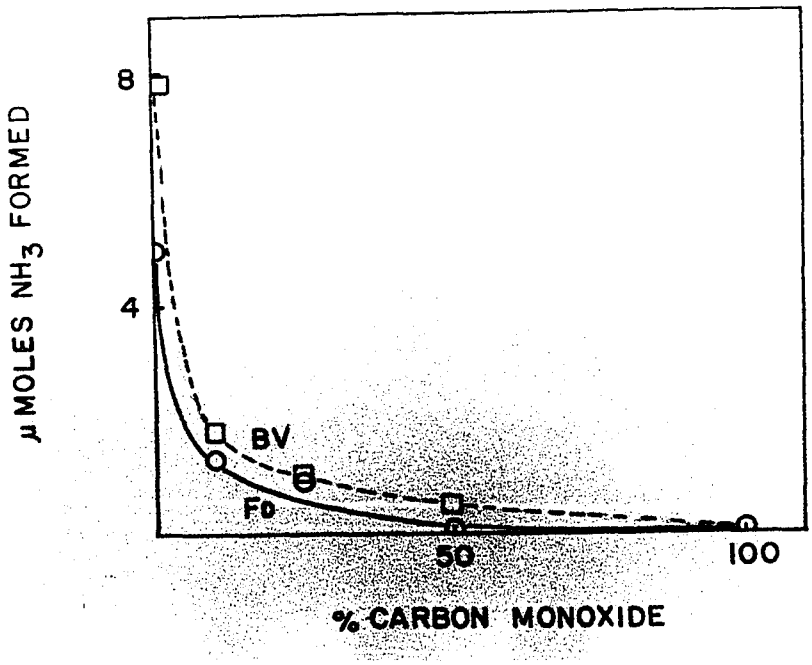

Figure 9. CO Inhibition of Nitrite Reduction. 25 mg clastic extract, 12 μmoles nitrite, 100 μmoles dithionite, 200 μmoles phosphate buffer pH 7, in 5 ml total. 25 ml flask assay. Indicated % CO made up to 100% (1 atm) with H_2. —O—O— 1 mg crude ferredoxin.--□--□-- 25 μmoles benzyl viologen.

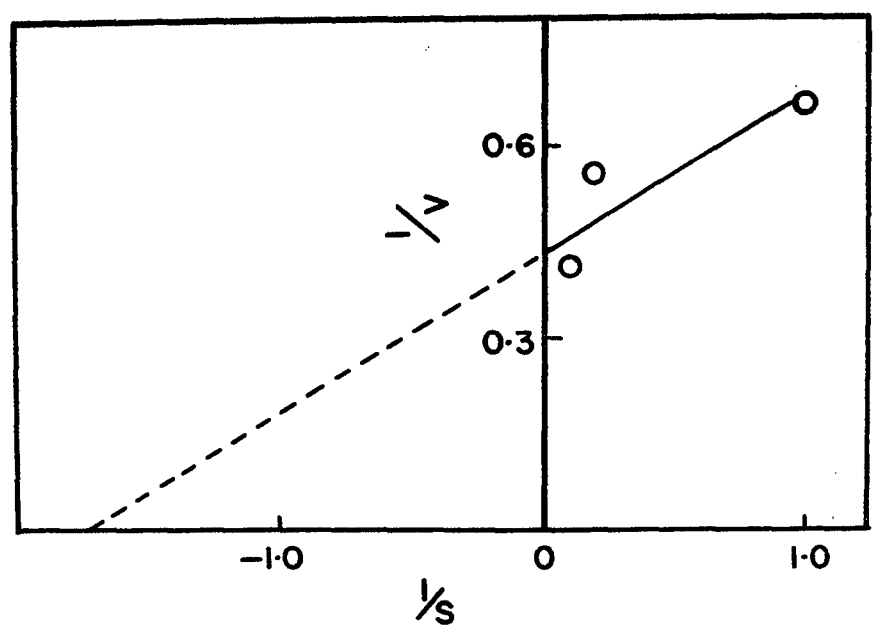

Figure 10. Lineweaver-Burk Plot of Nitrite Reduction. 5 mg clastic extract, 20 μmoles nitrite, 100 μmoles dithionite, 100 μmoles phosphate buffer pH 7, in 3 ml total. Warburg flask assay with He gas. 1/S = reciprocal μmoles benzyl viologen. 1/V = reciprocal μmoles ammonia formed in 30 min.

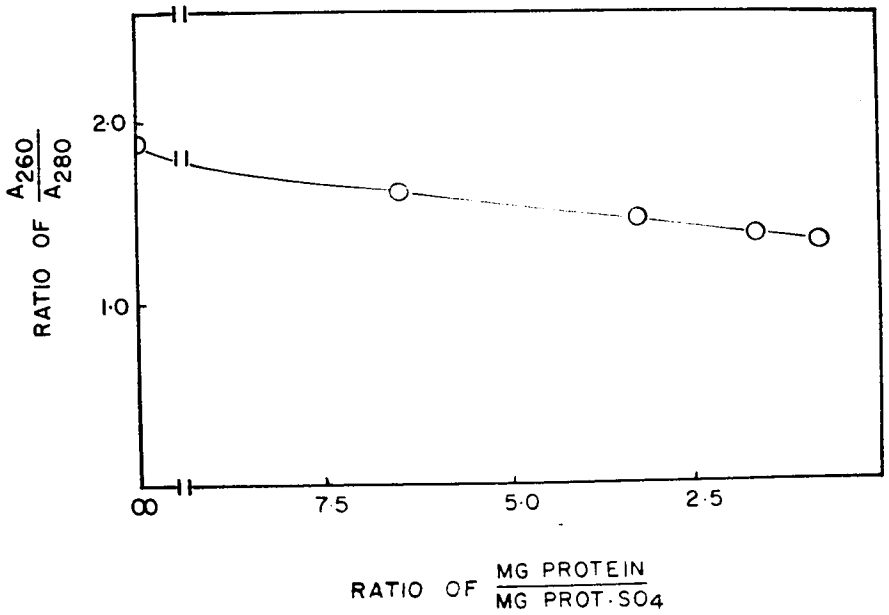

Figure 11. Effect of Protamine Sulfate Concentration on Nucleic Acid/Protein Concentration. 6.5 mg clastic extract samples treated with 10, 20, 40, and 80 mg protamine sulfate, respectively. Centrifuged at 30,000 X g for 10 min. Absorbency of supernatant at 260 mμ and 280 mμ determined on Beckman DB spectrophotometer.

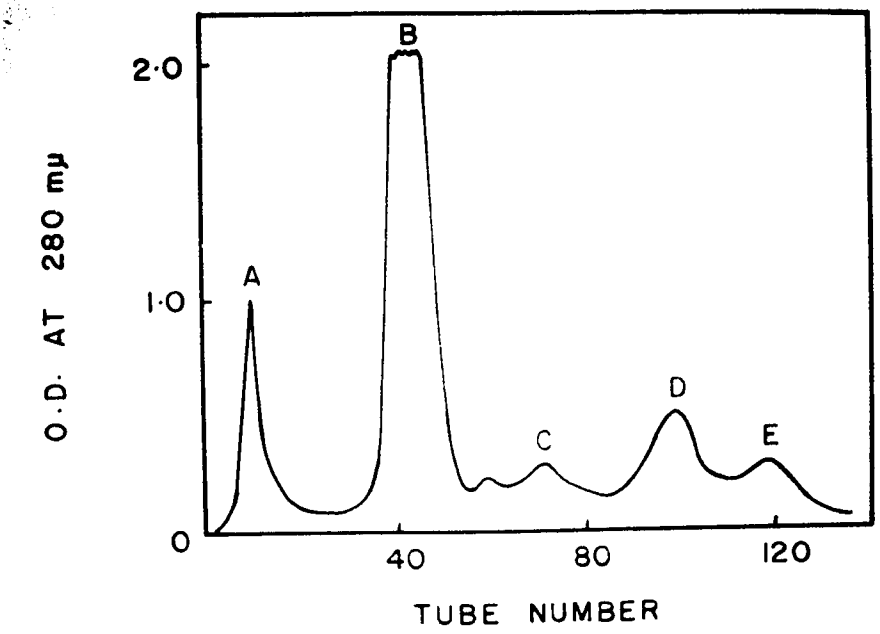

Figure 12. Separation of Nitrite Reductase on DEAE Cellulose. 142 mg protein placed on 2 X 15 cm column equilibrated with 0.01 M Tris-HCl pH 8.0. Eluants: tubes 1-22 - 150 ml 0.01 M Tris-HCl pH 7.3, tubes 23-52 - 200 ml 0.01 M Tris-HCl pH 7.3 + 0.1 M NaCl, tubes 53-140 - linear gradient of 300 ml 0.1 M NaCl to 300 ml 0.5 M NaCl, in 0.01 M Tris-HCl pH 7.3. Optical density at 280 mμ determined with a Beckman DB spectrophotometer.

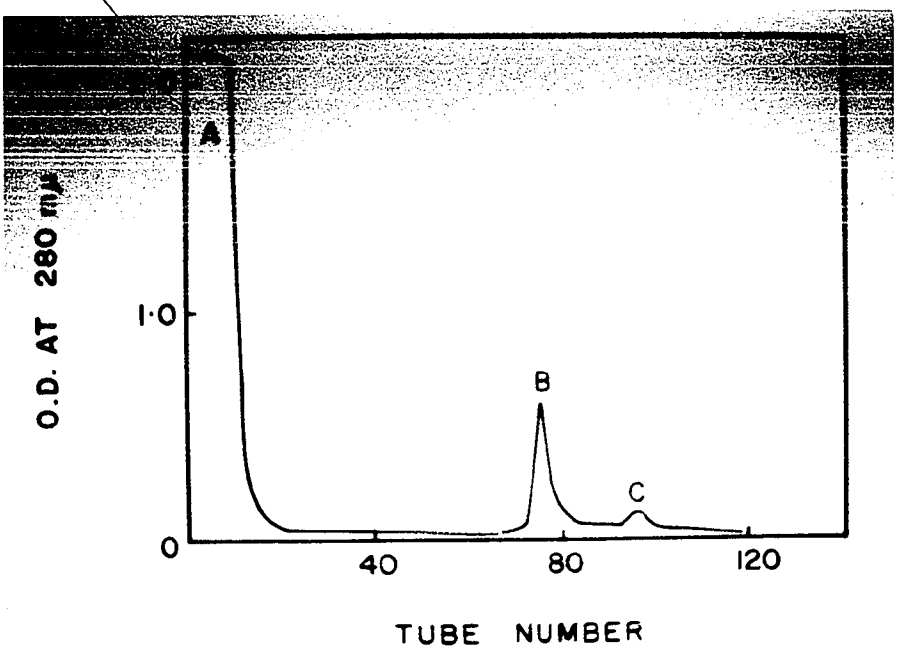

Figure 13. Separation of Nitrite Reductase on CM Sephadex. 400 mg protein placed on 1.6 X 12 cm column equilibrated with 0.01 M phosphate pH 6.0. Eluants: tubes 1-72 - linear gradient of 200 ml 0.01 M phosphate pH 6.0 to 200 ml 0.01 M phosphate pH 7.0, tubes 73-92 - 150 ml 0.1 M NaCl in 0.01 M phosphate pH 7.0. Optical density at 280 mμ determined with a Beckman DB spectrophotometer.

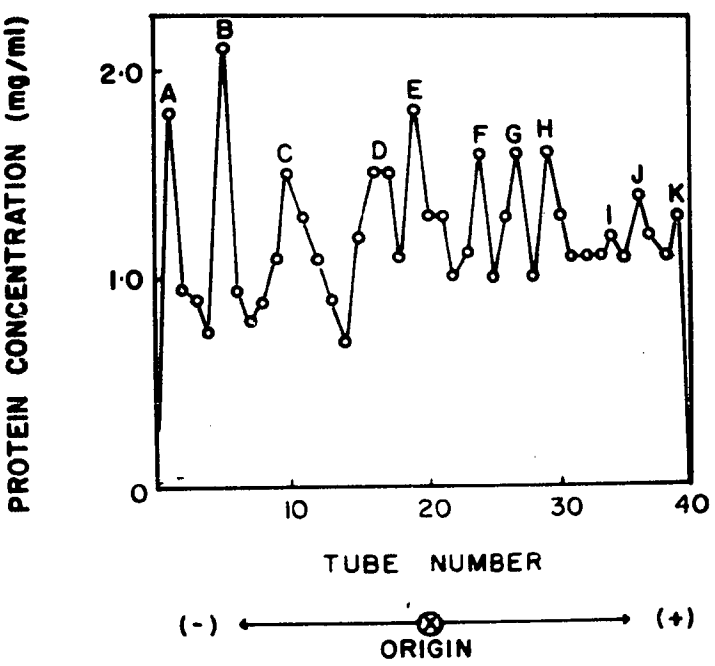

Figure 14. Starch Block Electrophoresis of Clastic Extract. 45 mg protein placed at midpoint of 40 X 13 X 0.5 cm starch bed prepared as under Methods section. Run 12 h at 20 ma constant current (450-600 v). Each tube contained eluted protein from 1 cm cross-section of bed. Protein concentration determined by Lowry method.

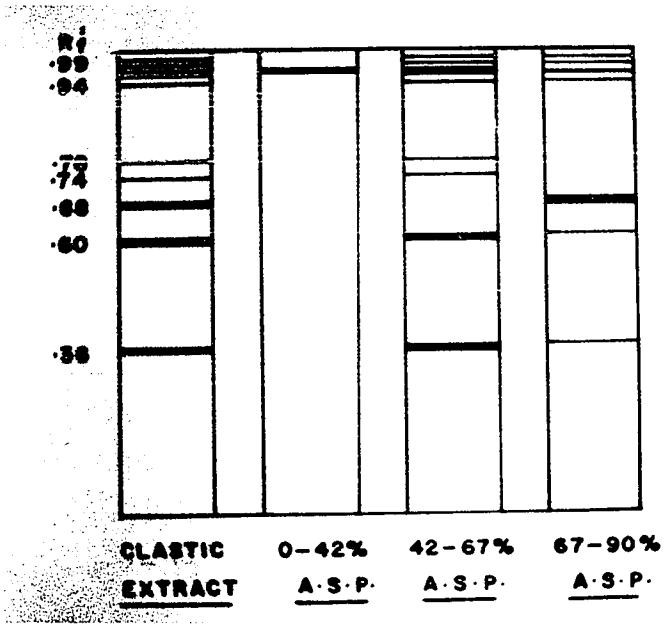

Figure 15. Starch Gel Electrophoresis of $(NH_4)_2SO_4$ Fractions. Ca. 2-5 mg protein run at 40 ma constant current (500-900 v) to 12 cm path length in 3 h. Gel prepared as under Methods section. Protein stained with amido black, developed with 50 MeOH: 50 H_2O: 10 HOAc.

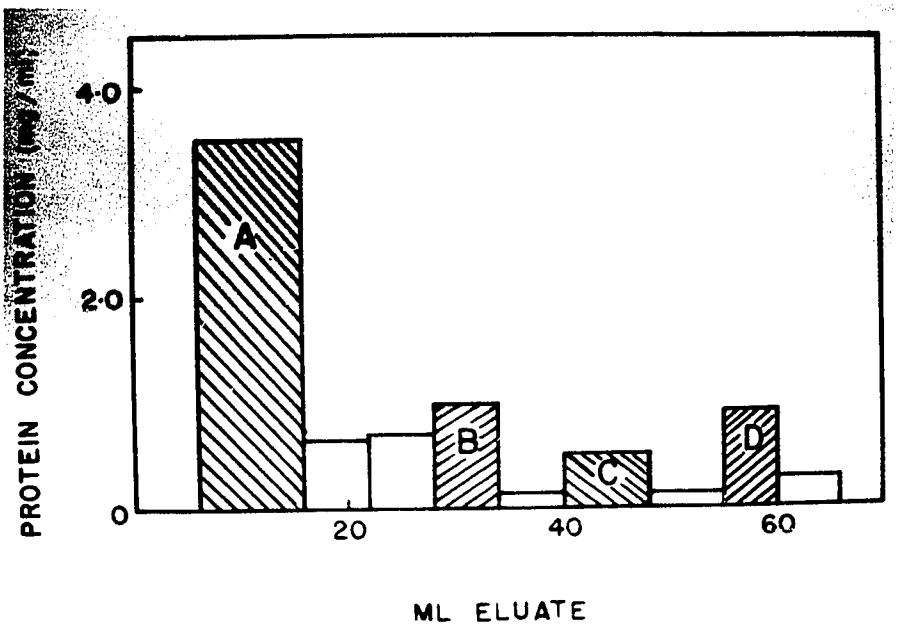

Figure 16. Separation of Nitrite Reductase on Sephadex G-200. 65 mg clastic extract placed on 1 X 17 cm column equilibrated with 0.1 M Tris-HCl buffer pH 7.3 + 10^{-3} M cysteine + 10^{-4} M reduced benzyl viologen. Protein concentration determined by Lowry method.

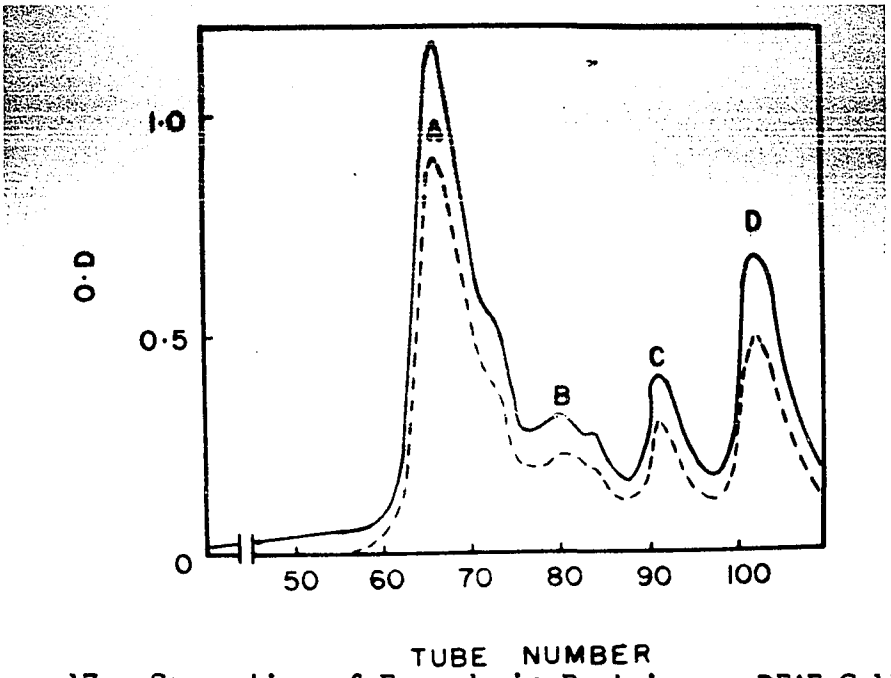

Figure 17. Separation of Ferredoxin Proteins on DEAE Cellulose. 25 mg crude ferredoxin placed on 2.2 X 13 cm column equilibrated with 0.01 M Tris-HCl pH 7.3. Eluants: tubes 1-84 - linear gradient of 500 ml 0.1 M Tris-HCl pH 7.3 to 500 ml 0.2 M NaCl in 0.1 M Tris-HCl pH 7.3, tubes 84-112 - linear gradient of 500 ml 0.2 M NaCl to 500 ml 0.4 M NaCl, in 0.1 M Tris-HCl pH 7.3. Optical density at 280 mμ ( ) and 390 mμ (- - - - - -) determined with a Beckman DB spectrophotometer.

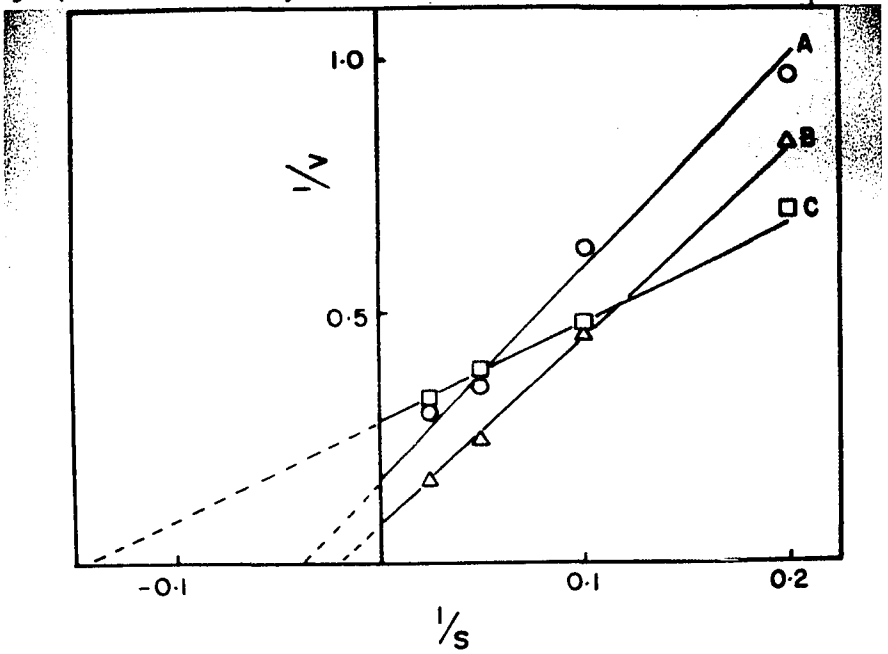

Figure 18. Lineweaver-Burk Plot of Nitrite Reduction in Several Clostridial Strains. 15 mg clastic extract, 10 μmoles benzyl viologen, 50 μmoles dithionite, 100 μmoles phosphate buffer pH 7, in 3 ml. Warburg flask assays with He gas. 1/S = reciprocal μmoles nitrite. 1/V = reciprocal μmoles ammonia formed in 30 min. Curve A = C. pasteurianum, Curve B = C. felsineum, and Curve C = C. butyricum.