AN ABSTRACT OF THE THESIS OF

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Title   SOME PROPERTIES OF A PARTICULATE AND A SOLUBLE NITRATE

         REDUCTASE FROM RHIZOBIUM JAPONICUM

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A particulate nitrate reductase complex from soybean-nodule bac-
teriods not only will catalyze the reduction of nitrate to nitrite by use of

DPNH or succinate as electron donors, but also will catalyze the trans-

fer of electrons from succinate to oxygen. This process is inhibited by

nitrate, suggesting that both oxygen and nitrate compete for electrons

from a common source.

In addition, the particulate complex possesses the capacity to

transfer electrons from succinate to oxyhemoglobin isolated from nodules.

Efforts to demonstrate a succinate-dependent reduction of nodule hemi-

globin to hemoglobin in the presence of the enzyme complex were nega-

tive. The role of nodule hemoglobin in nodule respiration and nitrogen

fixation was discussed, and it is proposed that sufficient evidence

exists to suggest that hemoglobin and oxyhemoglobin play a role in

oxygen uptake by whole nodules.

It was postulated that the nitrate reductase portion of the nitrate reducing particle may substitute for cytochrome _a_ as a terminal electron transfer protein. From the available evidence, an electron transport scheme was formulated describing a more or less parallel function for the nitrate reductase _per se_ and cytochrome _a_.

A method was devised for the preparation of a completely soluble nitrate reductase extract of _Rhizobium_ cells derived from pure cultures or from soybean nodules. This method involves reducing the bacterial suspension in a sealed Tygon tube containing glass beads and grinding the enclosed material after each of a series of freezing and thawing operations.

The soluble enzyme preparation is extremely susceptible to oxidation. It was necessary to maintain completely reduced conditions throughout purification procedures and studies of the properties of the enzyme. Since the soluble nitrate reductase is extremely labile to oxidation, it was necessary to assay the enzyme activity under reduced conditions. The conventional anaerobic assay in Thunberg tubes is tedious and time consuming. A simple assay procedure was developed which could be used under atmospheric pressure. In the procedure, sufficient hydrosulfite is added to the reaction mixture to maintain the system in the reduced state for the duration of the incubation period. Benzyl viologen, which is reduced in the presence of hydrosulfite, is used as the electron donor. After incubation, the hydrosulfite is destroyed by shaking in air and the nitrite formed is measured by a colorimetric

procedure.

The soluble system has been purified about 11-fold by $(NH_4)_2SO_4$ fractionation and chromatography on a calcium phosphate column. An attempt to purify a preparation without benzyl viologen was unsuccessful.

DPNH and succinate, which were effective electron donors in the particulate system, were completely inactive with the soluble preparation. A series of biological compounds were tested in an attempt to find a physiologically active electron donor; however, only reduced benzyl viologen or methyl viologen were found to be active.

The effect of pH and nitrate concentration on the rate of nitrate reduction by the soluble enzyme was determined. The K_m value for nitrate and the optimum pH are in relatively close agreement with those reported for the particulate nitrate reductase. Results of inhibition studies of the soluble nitrate reductase by sulfhydryl reagents and metal chelating agents are not consistent with the results obtained in similar studies with the particulate system. Variations obtained may be due at least partially to differences in steric properties of the two systems and to the necessary differences involved in assay procedures.

SOME PROPERTIES OF A PARTICULATE
AND A SOLUBLE NITRATE REDUCTASE
FROM RHIZOBIUM JAPONICUM

by

RICHIE HOWARD LOWE

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SOME PROPERTIES OF A PARTICULATE AND A SOLUBLE NITRATE REDUCTASE FROM RHIZOBIUM JAPONICUM

I. INTRODUCTION

The reduction of nitrate to nitrite is an oxidation-reduction reaction in which the nitrogen atom of nitrate is reduced from an oxidation number of five to three. Since soil nitrogen normally exists primarily as nitrate, the biological reduction of nitrate is of utmost importance. It is the initial step in the conversion of nitrate into ammonia and subsequently into protein and the other nitrogenous constituents of living organisms.

In 1954 Evans (16) reported the presence of a reduced diphospho-pyridine nucleotide (DPNH) specific nitrate reductase in Rhizobium isolated from soybean nodules. It was proposed that sufficient indirect evidence existed to consider the possibility of a role of nitrate reductase in nitrogen fixation. In subsequent studies Cheniae and Evans (13) demonstrated that the activities under various physiological conditions of the particulate nitrate reducing system isolated from soybean nodule bacteriods are in a number of ways closely correlated with rates of nitrogen fixation. Intensive studies (12) of the properties of the particulate nitrate reductase complex showed it to be similar in nature to the nitrate respiration system from Escherichia coli. These investigators concluded that the nitrate reducing system of nodules should be regarded as a system involved in nitrate respiration until evidence is presented to demonstrate directly its role, if any, in nitrogen fixation.

The purpose of the study presented here was to investigate further the nature of the terminal electron transfer catalyzed by the particulate nitrate reductase system in nodule rhizobia and to attempt to solubilize and study the properties of the nitrate reductase per se. Also, in view of the recent discoveries in the cobalt nutrition of legumes (14, 69), it was proposed to determine whether or not cobalt may be a limiting factor in the nutrition of cultures of Rhizobium japonicum grown for nitrate reductase studies. A cobalt requirement for Rhizobium species was found, but the quantities needed were so minute that reagent grade chemicals contained many times the amount required for optimum growth (9, p. 129). Since cobalt nutrition was not a factor influencing the growth of cultures for nitrate reductase preparations, the results of these studies are presented in Appendix I.

II. REVIEW OF LITERATURE

The early investigations of nitrate reduction have been reviewed

by Nightingale (61, p. 104-112, 62, p. 186-190), Burström (8, p. 1-5),

Street (76, p. 392-398), and Virtanen and Rautanen (84, p. 1121-1128).

It is apparent that many of the early studies of enzymatic nitrate reduc-

tion were not definitive due to lack of precautions necessary to prevent

microbial contamination during the long periods required for assays.

However, the detailed study of the nitrate reducing capacity of potato

extracts by Kastle and Elvove (34) in 1904 has been overlooked by the

above reviews. These workers used adequate precautions to prevent

microbial contamination and demonstrated that a heat labile component

was responsible for the catalytic conversion of nitrate to nitrite. In

addition to showing that nitrite production was proportional to concentra-

tion of the potato extract, time-course and substrate saturation studies

were presented which showed characteristics of enzymatic reactions.

The production of nitrite was inhibited by a number of metabolic poisons.

Unfortunately, these investigators lacked conclusive evidence that their

extracts were free of intact cells. Their statement indicating that filtra-

tion diminished the activity suggests that intact cells were present.

Green, Strickland, and Tarr (23, p. 1813-1819) were the first to un-

equivocally demonstrate nitrate reduction in cell-free extracts. These

investigators centrifuged disrupted cell suspensions of E. coli at high

speed and checked the resulting extract microscopically to be sure no

whole cells were present. Only reduced oxidation-reduction dyes were found to serve as electron donors for nitrate reduction by the extracts prepared by these investigators.

During the past decade numerous investigators have critically studied the nitrate reducing systems of a large number of organisms including higher plants, fungi, and bacteria. Nitrate reductases have been classified (21, 48, p. 17) as either "assimilatory types" or "dissimilatory types" depending on their physiological function. The nitrate respiration types of nitrate reductases are included in the dissimilatory category. In the first type (assimilatory), nitrate is converted into ammonia and incorporated into protein and other organic nitrogen components of the cell. This type is commonly found in higher plants, fungi, and some bacteria. In dissimilatory nitrate reduction the nitrate serves primarily as a terminal oxidant. Nitrate is converted into nitrite, nitrogen gas, and various oxides of nitrogen depending on the type of organism and the conditions where nitrate reduction takes place. The assimilatory enzymes are usually soluble, whereas the dissimilatory enzymes are particulate in nature.

Assimilatory Nitrate Reduction

The nitrate reductase from Neurospora was the first nitrate reducing system to be well characterized. Using a highly purified preparation Evans and Nason (18, 49) established that the nitrate reductase

from Neurospora is a metallo-flavoprotein requiring sulfhydryl groups for the transfer of electrons from reduced pyridine nucleotide to nitrate. The enzyme was found to be relatively specific for reduced triphosphopyridine nucleotide (TPNH) as electron donor. The flavin component could be removed and the enzyme inactivated by precipitation with ammonium sulfate. Activity was restored by addition of either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN); however, FMN was approximately 50 per cent as effective as FAD.

Subsequently, Nicholas and coworkers (53, 54, 58) demonstrated conclusively that Mo is a constituent of the Neurospora nitrate reductase and that the metal is directly involved in electron transfer. Molybdenum deficiency (58) resulted in as much as 95 per cent decrease in nitrate reductase. There were no decreases in activity as a result of deficiencies of Ca, Mg, Fe, Cu, Zn, or Mn. Nitrate reductase activity of deficient cultures could be specifically restored by adding Mo to the growth medium. When cell-free extracts were fractionated by several steps of $(NH_4)_2SO_4$ precipitation, the activities of the various fractions were closely correlated with their Mo contents (53). Nicholas and Nason (53) demonstrated that the enzyme is inactivated by dialysis against a purified buffer containing KCN, and that activity is specifically restored by Mo. Other metals, including Fe, Zn, Mn, Ni, Co, Hg, W, U, V and B, were without effect.

Nicholas and Nason (54) also demonstrated that FAD and Mo are

involved in electron transfer according to the following sequence:

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

Either reduced flavin or Mo reduced by dithionite will serve as an electron donor. Reduced flavin does not reduce nitrate when Mo is removed from the enzyme, but FAD is reduced by TPNH. Dithionite reduced Mo is an effective electron donor for nitrate reduction in the absence of flavin. Nicholas and Stevens (60) have reported that the Mo undergoes a change in oxidation state of Mo^{+5} to Mo^{+6} in the nitrate reductase reaction.

Phosphate ions are necessary for nitrate reduction by Neurospora extracts (38, 45, p. 572, 59). It has been reported by Nicholas and Scawin (59) that the phosphate requirement can be replaced by selenate, tellurate, or arsenate, and they propose the theory that phosphate is involved in binding molybdate to the apoenzyme. The observations by Kinsky and McElroy (38) that phosphate is required for the oxidation of enzyme bound Mo and not in the enzymatic oxidation of TPNH by FAD support this theory.

Kinsky and McElroy (38, p. 481-482) have reported that in Neurospora the ratio of cytochrome c reductase and nitrate reductase remained constant over a wide range of purification, and that both nitrate and cytochrome c reductase activities were induced in a parallel fashion. Considerable discussion has taken place concerning the suggestion that cytochrome c reductase and nitrate reductase are the same enzyme

(11, p. 5-6, 38, p. 481-482, 48, p. 19). However, observation of the data presented by Kinsky and McElroy show that the maximum ratio of nitrate reductase to cytochrome c reductase is more than twice the minimum ratio obtained. Although both enzymes are adaptively formed in the presence of nitrate in the growth medium, the pattern of adaptation is markedly different for the two enzyme systems. Cultures grown without nitrate possess a substantial amount of cytochrome c reductase activity, but such cultures show no nitrate reductase activity. The data discussed above appear to negate the proposal that the two activities reside on the same protein. It does seem likely, however, that the cytochrome c reductase and nitrate reductase may be linked to DPNH oxidation via a common intermediate, possibly a flavoprotein (11, p. 6, 47, p. 16).

In 1953 Evans and Nason (19) purified and partially characterized a pyridine nucleotide-nitrate reductase from soybean leaves. The presence of the enzyme also was demonstrated in several other plant species. The soybean nitrate reductase system appears to be very similar to that found in Neurospora except that it is non-specific for pyridine nucleotide electron donor. The Neurospora enzyme exhibits a marked specificity for TPNH. Evidence was presented that the enzyme is a metallo-flavoprotein containing an essential sulfhydryl group. Evans and Hall (17) demonstrated that during purification, radioactive Mo^{99} is accumulated in fractions with high nitrate reductase activity. After dialysis against reduced glutathione (GSH) and cyanide, the enzyme was

activated by Mo and to a lesser extent (15 per cent of original activity) by Fe. The effect of Fe may have resulted from Mo contamination of the Fe. Nicholas and Nason (55) have demonstrated that the sequence of electron transfer and mechanism of action of the nitrate reductase from soybean leaves are essentially the same as in Neurospora. A similar nitrate reductase has been studied in a variety of other higher plants (10, 24, 27, 63, 73, 75, 81, 82). The nitrate reductases isolated from germinating wheat (73) and from corn seedlings (24), however, appear to be specific for DPNH. In the cases where the metal constituent of nitrate reductase has been studied, it appears that Mo (10, 73, 82) is required for activation.

Many investigators have studied the effect of light on nitrate reduction by photosynthetic organisms (8, 19, p. 246-248, 24, 69, p. 186-199, 75, 84, p. 1127). Evans and Nason (19, p. 246-248) demonstrated the photo-chemical reduction of nitrate by the soybean nitrate reductase in the presence of triphosphopyridine nucleotide (TPN) and grana isolated from soybean leaves. There was no effect of light on purified nitrate reductase in the absence of grana. On the basis of their experiments it seems most likely that the photosynthetic process serves as a source of energy, primarily in the form of reduced pyridine nucleotide.

A nitrate reductase requiring either TPNH or DPNH for activity has been isolated from the yeast Hansenula anomala by Silver (71). The enzyme is a metallo-flavoprotein with FAD as the probable flavin

component. It was suggested that the metal constituent of the enzyme is Mo. Spectroscopic evidence indicated that no cytochromes were directly involved in the electron transfer sequence in the system.

Taniguchi and Ohmachi (79) have demonstrated the presence of a particulate nitrate reductase in Azotobacter vinelandii and have suggested that the enzyme is involved in nitrate assimilation. Either DPNH or TPNH served as an effective electron donor and succinate functioned to some extent. Activity was stimulated by both FAD and FMN. Cytochromes are not involved directly in the reduction of nitrate by this system, although the system does possess a DPNH oxidase. The following scheme of electron transfer was proposed (79):

$$
\text{DPNH} \xrightarrow{\quad} \begin{array}{c} \text{FAD} \\ \text{or} \\ \text{FMN} \end{array}
\begin{cases}
\xrightarrow{\quad} \text{Cytochrome system} \xrightarrow{\quad} \text{Cytochrome oxidase} \xrightarrow{\quad} O_2 \\
\xrightarrow{\quad} \text{Nitrate reductase (CO-insensitive heavy metal)} \xrightarrow{\quad} NO_3^-
\end{cases}
$$
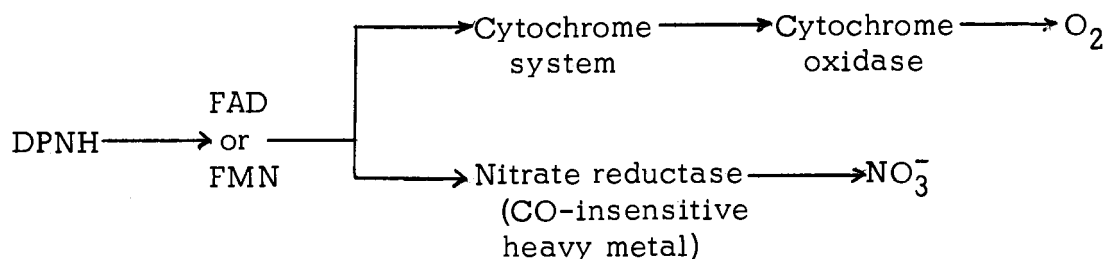
If one assumes that the metal is Mo, the system in Azotobacter is very similar to that in Neurospora.

Dissimilatory Nitrate Reduction

In 1949 Sato and Egami (67) provided evidence to support the following general scheme of electron transfer in nitrate reduction by crude extracts of E. coli:

Hydrogen— - $\tilde{}$ - — Intermediary hydrogen — — $\tilde{}$ — — NO_3^-
carrier

Dehydrogenase   Nitrate reductase
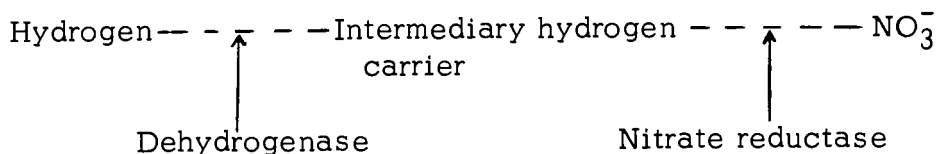
They showed that FAD could act as an intermediary hydrogen carrier,

and that the reduction of nitrate was inhibited by heavy metal complexing

agents. They also noted that under anaerobic conditions reduced cyto-

chrome b bands disappeared rapidly when nitrate was added to the incu-

bation mixture. From these results it was erroneously concluded that
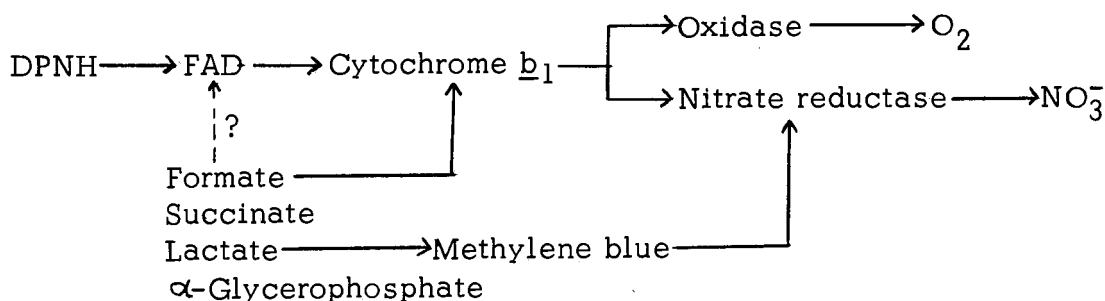
cytochrome b was identical with nitrate reductase.

Since the early work of Egami and Sato, the nitrate reductase of

E. coli has been investigated extensively by a host of workers. Sato

and Niwa (68) in 1949 showed that nitrate reductase and cytochrome b

are not identical, but that nitrate reductase follows cytochrome b in the

electron transport chain. This was based mainly on the fact that thio-

urea inhibited nitrate reduction but did not inhibit oxygen uptake. More

recently, Sato (66), working in Chance's laboratory, found evidence to

support this view. Using the double-beam spectrophotometer developed

by Chance, he demonstrated a steady state oxidation of cytochrome b
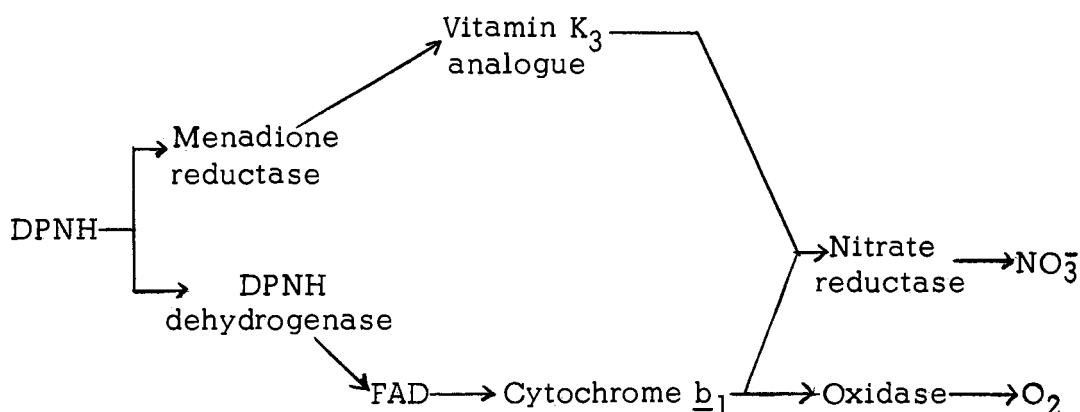
by nitrate.

Nicholas and Nason (56) isolated a diphosphopyridine nucleotide-

nitrate reductase from aerobically grown E. coli, and concluded that the

preparation was similar to the Neurospora enzyme. They demonstrated

that the enzyme is a metallo-flavoprotein and that its activity was

appreciably reduced in Mo deficient cultures. These workers did not determine whether the enzyme was particulate in nature. Taniguchi, Sato, and Egami (80, p. 96) have suggested that the preparation of Nicholas and Nason contained an active cytochrome component.

Taniguchi et al. (80) have discussed in considerable detail the properties of the particulate nitrate reductase preparation from E. coli. Formate, succinate, lactate, DPNH, α-glycerophosphate, and various oxidation-reduction dyes are effective electron donors for the particulate system. Nitrate reduction by this system is inhibited by 2-heptyl-4-hydroxyquinoline-N-oxide, a compound which has been considered to be a specific inhibitor for cytochromes b and b_1 (80, p. 90). The specificity of this compound however has been questioned by Kogut and Lightbrown (39). It has been suggested that the b type cytochrome involved in the E. coli nitrate reduction is cytochrome b_1 (80, p. 93). From the available evidence Taniguchi et al. (80, p. 94) drew the following scheme to visualize electron-transport pathways in the particulate preparation of E. coli.

```
                                    ┌──→Oxidase ──────→O₂
DPNH──→FAD ──→Cytochrome b₁─┤
             ↑              ↑       └──→Nitrate reductase──────→NO₃⁻
             ¦?             |
             |              |
          Formate ──────────┘
          Succinate                              ↑
          Lactate──────────→Methylene blue───────┘
          α-Glycerophosphate
```

Wainwright (85) found that nitrate reductase activity in extracts of

E. coli, strain 1431, was enhanced by vitamin K_3. Heredea and Medina

(26, 46) showed that an extract of E. coli inactivated by extraction with

light petroleum (b.p. 40-60°C) could be reactivated either by vitamin K_3

or by the dry residue obtained by vacuum evaporation of light-petroleum

extracts of previously boiled preparations. On the basis of these re-

sults, they suggest that the scheme proposed by Taniguchi et al. (80,

p. 90) should be modified to include an alternate pathway as follows:

DPNH — Menadione reductase → Vitamin K_3 analogue → Nitrate reductase → NO_3^-

DPNH dehydrogenase → FAD → Cytochrome $\underline{b}_1$ → Oxidase → O_2

Itagaki, Taniguchi, and coworkers (30, 31, 32, 77, 78) have

recently succeeded in solubilizing the particulate nitrate reductase from

E. coli. Results of experiments with the soluble preparation support the

proposed cytochrome nature of the enzyme system. Results obtained

from experiments with these preparations, however, indicate that the

vitamin K_3 replaceable lipid-soluble factor is involved in the cytochrome

pathway and is not necessarily an alternate pathway for nitrate reduc-

tion. This is based primarily on the fact that vitamin K_3 or the extracted

lipid-soluble factor is required for reduction of cytochrome b_1 by for-
mate. The solubilized enzyme prepared from E. coli particles appar-
ently contains either nitrate reductase; or cytochrome b_1 and nitrate re-
ductase; or formic dehydrogenase, cytochrome b_1, and nitrate reductase
depending on the techniques of preparation. The preparation containing
only nitrate reductase activity was purified to the extent that it gave a
single protein peak when it was centrifuged in the analytical centrifuge.
It contained approximately 1 mole of Mo and 40 moles of non-heme Fe
per mole of nitrate reductase (29, 31). These ions were not removed by
dialysis against distilled water. There was no detectable flavin in the
purified preparation. Reconstitution of the purified system has not yet
been reported.

In discussing the variety of proposed pathways for nitrate reduc-
tion in E. coli, Fewson and Nicholas (21) have pointed out that the use
of different hydrogen donors in assay systems, the lack of uniform con-
ditions for culturing the organism and the use of various strains of the
organism may contribute to the lack of agreement in results obtained.
In their opinion the nitrate reductase per se from all E. coli preparations
is very likely the same molybdo-protein.

Fewson and Nicholas (20) recently have accomplished over 100-
fold purification of a nitrate reductase from Pseudomonas aeruginosa.
The system appears to be a small particulate fragment containing DPNH
dehydrogenase and cytochromes b and c. The enzyme is DPNH

dependent; however, chemically reduced FAD, cytochrome $\underline{c}$, or Mo^{+5} also are effective electron donors. When DPNH was used as the electron donor, a flavin requirement could be demonstrated after the enzyme had been repeatedly precipitated with ammonium sulfate (pH 5.5). Either FAD or FMN was effective, but chromatographic evidence indicated that FAD was the sole flavin component of the purified system. Spectral evidence as well as removal of the cytochrome $\underline{c}$ component and restoration of activity by reconstitution of the system indicate that cytochrome $\underline{c}$ is involved in the electron transfer. When Mo^{+5} was the donor, no cytochrome requirement could be demonstrated. Reduced cytochrome $\underline{c}$ was oxidized either by Mo^{+6} or nitrate. Evidence against participation of cytochrome $\underline{b}$ includes: (a) no inhibition by either Antimycin A or 2-heptyl-4-hydroxyquinoline-N-oxide and (b) lack of stimulation from addition of cytochrome $\underline{b}$ after removal of most of the cytochrome $\underline{b}$ from the system. Electron spin resonance signals support the conclusion that FAD and Mo are involved in the enzymatic transfer of electrons to nitrate. Nitrate reduction by the crude extract was inhibited by oxygen, but this inhibition could be partially reversed by carbon monoxide. These results suggested that oxygen inhibited the nitrate reductase by diversion of electrons to oxygen $\underline{via}$ cytochrome oxidase thus bypassing the nitrate reductase. Fewson and Nicholas (20, p. 348) proposed the following scheme of electron transfer for the $\underline{Pseudomonas}$ system.

$$\text{DPNH} \longrightarrow \text{FAD} \longrightarrow \text{Cytochrome } \underline{c} \longrightarrow \text{Mo}^{+5} \longrightarrow \text{NO}_3^-$$

$$\downarrow$$

$$\text{Cytochrome}$$
$$\text{oxidase}$$

$$\downarrow$$

$$\text{O}_2$$
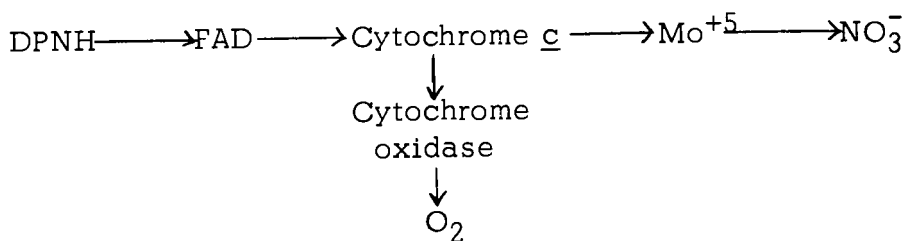
Sadana and McElroy (65) have studied the properties of a highly purified nitrate reductase from Achromobacter fischeri. Their preparation contained a cytochrome having a spectrum similar to that of mammalian cytochrome $\underline{c}$. Reduced mammalian cytochrome $\underline{c}$, however, would not function as an electron donor for nitrate reduction. DPNH and TPNH were effective electron donors in the crude preparation, but would not function as electron donors for the purified preparation unless a DPNH oxidase was added. Flavins were required by the DPNH-oxidase system, but no flavin requirement could be demonstrated when benzyl viologen was used as electron donor. The following scheme of electron transfer was proposed:

$$\begin{array}{l} \text{TPNH} \\ \text{or} \\ \text{DPNH} \end{array} \longrightarrow \begin{array}{l} \text{FAD} \\ \text{or} \\ \text{FMN} \end{array} \longrightarrow \text{Fe}^{+++} \longrightarrow \begin{array}{l} \text{Bacterial} \\ \text{cytochrome} \end{array} \longrightarrow \text{O}_2$$

$$\downarrow$$

$$\begin{array}{l} \text{Reduced} \\ \text{benzyl viologen} \end{array} \longrightarrow \text{Nitrate reductase}$$

$$\downarrow$$

$$\text{NO}_3^-$$
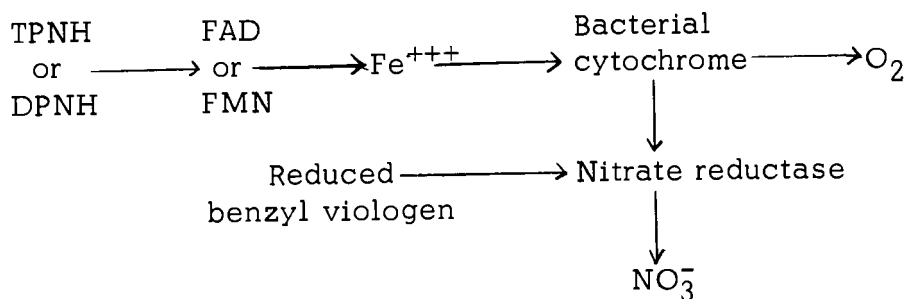
Sadana and McElroy (65, p. 32) suggested that bound iron participates in electron transfer. This conclusion was based on the formation of either a pink ferrous-o-phenanthroline or a ferrous-dipyridyl complex when o-phenanthroline or dipyridyl was added to the reaction mixture.

The suggestion that Mo is not involved in nitrate reduction by A. fischeri has been questioned by Nicholas (50, p. 68).

The nitrate respiration of Pseudomonas fluorescens (42), Mycobacterium tuberculosis (4), certain Micrococci (22, 28), and Aerobacter aerogenes (64) has been investigated to some extent; however, the results are less conclusive than those already considered. The system from A. aerogenes is a unique one in that it appears to carry out nitrate respiration without the participation of cytochromes or reduced pyridine nucleotides. Molecular hydrogen apparently serves as the electron source through a hydrogenase.

Nason (48, p. 24-25) has reviewed in detail the adaptive nature of nitrate reductases. With very few exceptions nitrate is required in the growth medium for induction of the enzyme.

Nitrate Reductase from Soybean Nodules

Cheniae and Evans (12, 13) have studied a nitrate reductase from soybean nodule bacteria. The enzyme is particulate in nature, and in addition to various oxidation-reduction dyes, it utilizes either DPNH or succinate as an electron donor. Inhibition by Antimycin A and spectral evidence indicate that a cytochrome is involved in electron transfer. The spectral evidence is rather inconclusive, however, due to the pronounced light scattering of the particulate fraction. When DPNH serves as a donor, a lipid fraction which is replaceable by either vitamin K_3 or

dried residue of an acetone extract is required for activity. Failure to show a flavin requirement was assumed to be associated with difficulties in dealing with the particulate system.

The activity of the nodule nitrate reductase is greatly reduced by deficiencies of either Fe or Mo (13). Cheniae (11, p. 83) has proposed a scheme of electron transport which is similar to that proposed for E. coli (see page 10 of this thesis).

There are two especially interesting aspects of the nodule nitrate reductase (13). In contrast to other nitrate reducing systems, it does not appear to be adaptive to nitrate. When soybean plants are grown in a culture solution lacking combined nitrogen, the nodule nitrate reductase is quite active. There is little or no detectable nitrate, however, in nodules of plants grown under these conditions. In fact, addition of nitrate to the culture solution used for soybean plants results in a considerable decrease in nitrate reducing activity. The second point is that in a number of ways the nitrate reducing activity of the enzyme complex is closely correlated with rates of nitrogen fixation. There is a close relation between the effectiveness of nitrogen fixation by various strains of R. japonicum and the nitrate reductase activities of nodules. Also there is a close correlation between the nitrate reductase activities and the hemoglobin contents of nodules collected at intervals during the growth period. It has been shown that nitrogen fixation is closely correlated with the hemoglobin content of nodules (83). There is no direct

evidence, however, that the nodule nitrate reductase participates in nitrogen fixation and the physiological role of the hemoglobin pigment remains obscure.

Relation of Co Metabolism to Nitrate Reduction

After the initial discovery of a cobalt requirement for Rhizobium species by Lowe and Evans (43, 44), Nicholas, Maruyana, and Fisher (52) demonstrated that the capacity to reduce nitrate by Co deficient R. japonicum was markedly decreased. Since radioactive Co did not accumulate in purified fractions of the enzyme, it was concluded that the effect of Co on the enzyme was very probably indirect. Subsequently Nicholas, Kobayashi, and Wilson (51) presented correlative data indicating that Co is involved in the synthesis of nitrate reductase by A. vinelandii. When Co^{58}-labeled ribosomes from A. vinelandii were resolved on a 15-60 per cent sucrose density gradient column, the bulk of the protein was in the top half of the gradient, but the enzyme was associated with two main peaks in the lower half of the column. These results led these workers to the conclusion that the synthesis of nitrate reductase is specifically influenced by Co and, therefore, the synthesis of this enzyme is different from the synthesis of protein in general. In the writer's opinion, this suggestion seems highly unlikely. Perhaps the results of these workers can best be reconciled by assuming that the activity of nitrate reductase is short-lived after the enzyme is

dissociated from the ribosomal particle and that cobalt is involved either directly or indirectly in protein synthesis in general. The writer has established that soluble nitrate reductase from R. japonicum is extremely labile whereas the particulate form is relatively stable.

As a result of the work of Lowe and Evans (43) which has been confirmed by Nicholas et al. (52) it is clear that Rhizobium japonicum requires cobalt for normal growth. The quantities present in nutrient media prepared from reagent grade chemicals (9, p. 129) results in a concentration that is many-fold that required for normal growth. Results supporting this view are included in Appendix I.

Properties and Possible Roles of Nodule Hemoglobin

Keilin and Wang (37) have shown that hemoglobin from soybean nodules has a high affinity for oxygen and Bergensen and Wilson (7) have demonstrated that oxygenation of nodule hemoglobin occurs when the surrounding atmosphere contains as little as 8 ppm of oxygen. Spectroscopic observations by Keilin and Smith (36) and Appleby (2) show that normal intact nodules contain a mixture of hemoglobin and oxyhemoglobin. No hemiglobin was identified. Bergersen and co-workers (5, 6) have presented evidence that groups of bacteriods in nodules are surrounded by a solution of hemoglobin within host tissue membranes. If this view is correct, it would seem logical to conclude that oxygen utilized by the bacteriods would first have to come in

contact with the nodule hemiglobin solution before reaching the bacteriods. In this respect, Kubo (40) has shown that nodule "hemoglobins"* stimulate the uptake of oxygen by bacteriods from soybean nodules. Smith (72) is of the opinion that the stimulatory effect is indirect because oxygen uptake by nodules was not inhibited by levels of carbon monoxide sufficient to tie up the hemoglobin. The oxygen tensions which existed under the conditions of Smith's experiments, however, were not similar to those expected under normal conditions of nodule growth.

Although it is apparent that hemoglobin from legume nodules possesses the capacity for oxygenation, the possibility of a physiologically important oxidation-reduction reaction involving hemoglobin and hemiglobin remains controversial. Hamilton, Shug, and Wilson (25) have presented spectral evidence indicating that extracts prepared from soybean nodules will catalyze the oxidation of hemoglobin to hemiglobin by use of nitrogen as oxidant. Bergersen and Wilson (7), taking precautions to insure complete removal of atmospheric oxygen from the gases used, later confirmed their work. These interpretations appear to be reasonably well based, but the possibility should not be overlooked that the spectral changes observed are the result of formation of a nitrogen-hemoglobin complex similar to the oxyhemoglobin complex.

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*The term "hemoglobins" includes hemoglobin, oxyhemoglobin, and hemiglobin.

Bergersen and Wilson (7) also present data which they interpret as evidence that bacteriods from soybean nodules exhibit the capacity to reduce hemiglobin to hemoglobin. This interpretation is definitely subject to criticism (see the "Discussion" section of this thesis).

III. MATERIALS AND METHODS

Preparation of Particulate Enzyme Extract

The particulate enzyme system used in the experiment described in figure 5 was prepared by the methods described previously (12, p. 141-142). Briefly the methods consisted of: (a) crushing washed soybean nodules and isolating bacteriods by centrifugation procedures; (b) grinding washed bacteriods with alumina using a mortar and pestle, and (c) separation of the particulate enzyme from other portions of the disrupted cell suspension by centrifugation. All operations unless otherwise indicated were carried out at 0-4°C. For the preparation of the particulate enzyme system utilized in other experiments, bacteriods from the nodules of soybean plants, Glycine max Merr. (var. Lee), grown in the field or greenhouse were obtained by centrifugation procedures (12, p. 141-142). The cell paste was suspended in a solution of (1 weight of wet cells in 5 weights of solution) 0.1 M phosphate buffer at pH 7.5 containing 0.001 M sodium ethylene diaminetetraacetate (EDTA). The suspension was placed in a Tygon tube (0.5 inch internal diameter and 2.5 feet in length). For each g of wet cells 2 g of glass beads (averaging 0.3 mm in diameter) were placed in the tube and the ends of the tube were closed with a clamp excluding all air space. The suspension of cells in the tube was rapidly frozen by use of solid carbon dioxide and then the tube was pulled back and forth over a steel rod

mounted on bearings until the contents of the tube had thawed. The entire operation was repeated twice. After grinding, the beads were removed from the mixture by squeezing the suspension through cheese cloth and the cell debris was removed by centrifugation for 10 minutes at 20,000 x g. The particulate enzyme complex remaining in the supernatant fluid was collected by centrifugation for 30 minutes at 80,000 x g. The particles were suspended in a quantity of phosphate buffer at pH 7.0 (0.1 M and containing 0.001 M EDTA) equivalent to 2.5 times the wet weight of the original cell paste. All evidence obtained to date indicates that the properties of the particulate enzyme complex prepared by the two procedures are indistinguishable.

For the preparation of nodule hemoglobin and oxyhemoglobin, a modification of the procedure of Sternberg and Virtanen (74) was used. Fresh nodules from soybean plants grown either in the field or the greenhouse were washed with cold water and macerated with a mortar and pestle in an equal amount of saturated $(NH_4)_2SO_4$ solution (weight per volume). The mass of the nodule tissue was removed by squeezing the material through several layers of cheesecloth and then the suspension was centrifuged at 22,000 x g for 20 minutes to remove the bacterioids. The supernatant fluid was collected, and sufficient solid $(NH_4)_2SO_4$ was added to bring the specific gravity to 1.2. After standing for 20 minutes, the precipitate containing the "hemoglobins" was collected by centrifugation (22,000 x g for 10 minutes) and then it was dissolved in 0.2 M

phosphate buffer pH 7.0 containing 0.001 M EDTA (0.5 ml buffer for each g of fresh nodules).

The solution of "hemoglobins" was placed in a Thunberg tube and after evacuation about 0.2 mg of solid $Na_2S_2O_4$ per ml of solution was tipped into the solution from the side arm. Oxygen was admitted into the tube and the contents were shaken gently. Spectra of this solution were recorded, and, as indicated in figures 1 and 2, absorption bands characteristic of oxyhemoglobin were obtained. Hemoglobin was prepared from oxyhemoglobin by adding about 2.0 mg of $Na_2S_2O_4$ to a Thunberg tube containing 10 ml of oxyhemoglobin (0.1 to 0.2 mg of heme per ml). Spectra of a typical hemoglobin preparation are presented in figures 1 and 2.

Hemiglobin was prepared by maceration, with a cold mortar and pestle, one weight of washed nodules from soybean plants with two weights of 0.1 M potassium phosphate buffer at pH 7.5 containing 0.001 M EDTA. The macerated nodules were shaken for ten minutes at $25^{\circ}C$. in order to allow the phenoloxidase to act (37). After this, the mass of nodular tissue was removed by squeezing through cheesecloth and the fluid suspension was centrifuged for 20 minutes at 22,000 x g. The supernatant liquid was shaken again for ten minutes at $25^{\circ}C$. An absorption spectrum of the preparation revealed characteristic bands of hemiglobin (figure 3). Spectral determinations were carried out with a Cary Model 11 recording spectrophotometer.
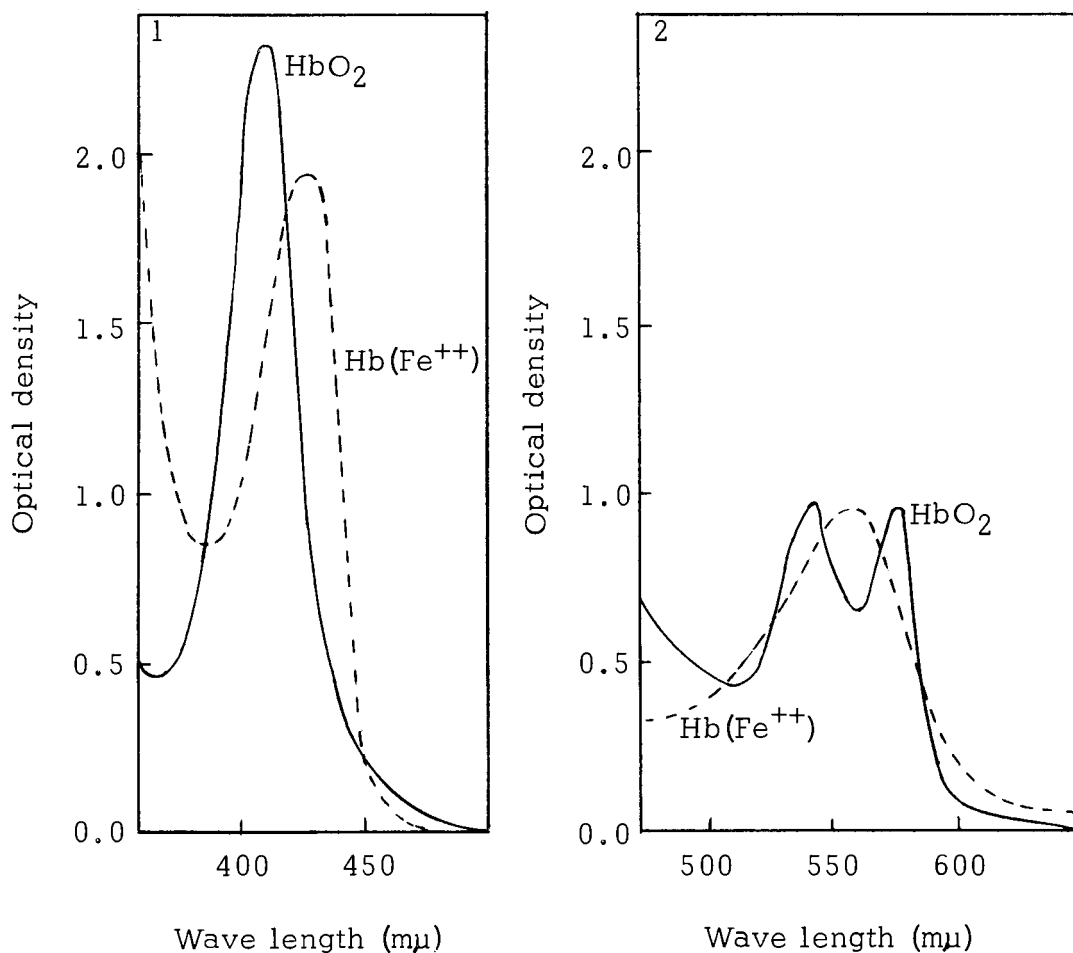
Fig. 1. Spectra of nodule oxyhemoglobin (HbO$_2$) and hemoglobin (Hb [Fe^{++}]) in the range of 360 to 500 mμ. The concentration of heme in the cuvette was 8 μg per ml.

Fig. 2. Spectra of nodule oxyhemoglobin and hemoglobin in the range of 470 to 650 mμ. The concentration of heme in the cuvette was 32 μg per ml.
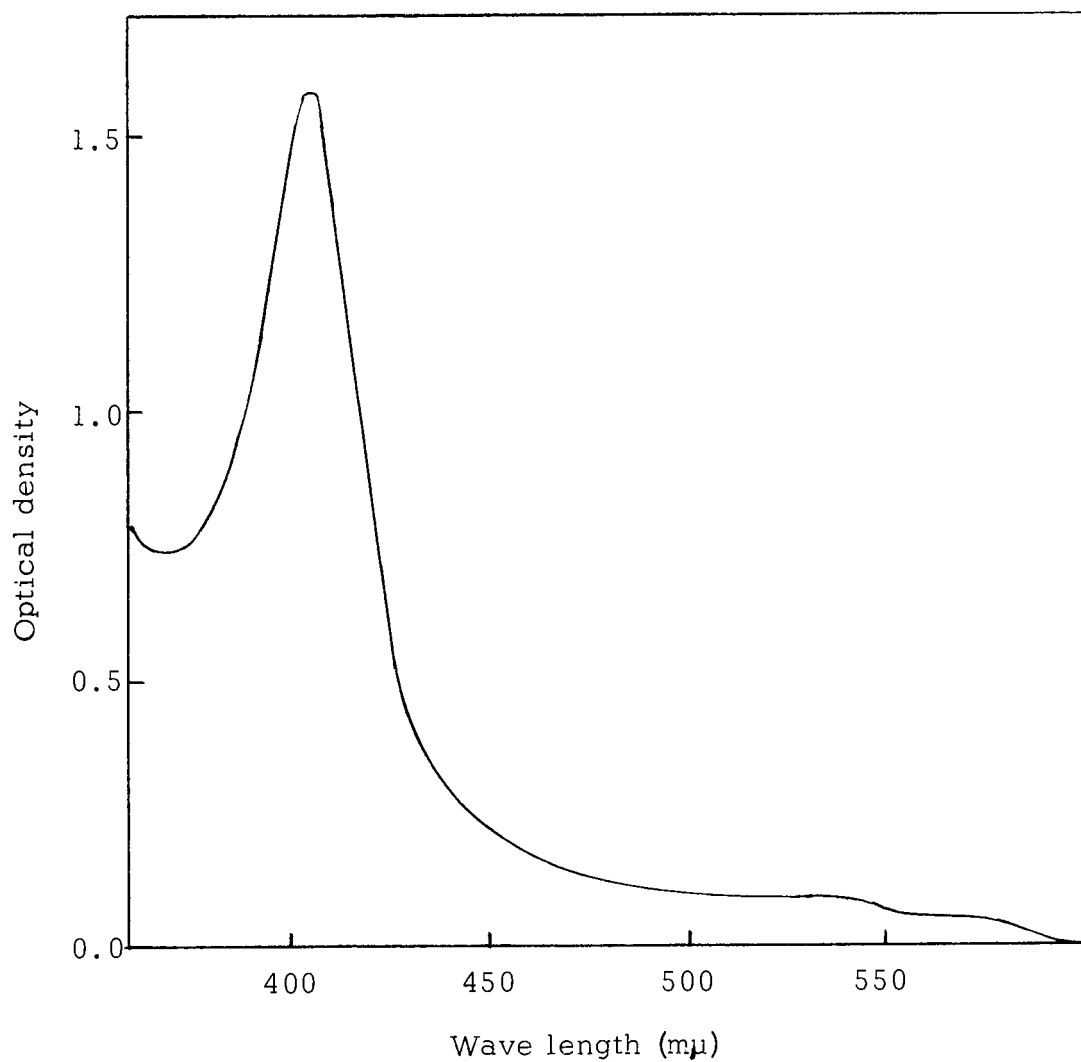
Fig. 3. Spectrum of nodule hemiglobin in the range of 360 to 600 mµ.
The concentration of heme in the cuvette was 5 µg per ml.

Quantitative Determinations

Heme contents of the various preparations were determined by the method of Keilin and Hartree (35, p. 89). Oxygen uptake was measured manometrically by use of a Warburg apparatus.

The total nitrogen contents of whole cells and of particulate preparations were determined by a micro-Kjeldahl procedure (33, p. 576). The protein content of cell-free preparations in soluble studies was determined by a turbidimetric procedure (41, p. 447). For this procedure, an aliquot of the extract was made to 1.0 ml with H_2O and mixed with 4.0 ml of 0.75 per cent $K_4Fe(CN)_6$ and one drop of glacial acetic acid was added. After about 10 minutes the turbidity was measured spectrophotometrically at a wavelength of 600 mμ. In cases where the protein was especially dilute, 1.5 ml of 1.0 per cent $K_4Fe(CN)_6$ was added instead of 4.0 ml of 0.75 per cent. Thus, the final concentration of $K_4Fe(CN)_6$ was the same in both cases.

Nitrate Reductase Assays

The procedure for determination of nitrate reductase activity of particulate preparations was the same as that used by Cheniae (12, p. 142-143). Since the electron donors that were active with the particulate preparation were completely ineffective with the soluble enzyme, it was necessary to develop a special procedure for the assay of the

soluble enzyme. The details of this procedure are described in the section of this thesis entitled, "Results Obtained with Soluble System."

The procedure for the determination of nitrite was the same for both the particulate and soluble nitrate reductase assay procedures. In this method, nitrite was determined by the diazo-coupling reaction. Unless otherwise stated, nitrite was determined by measuring the amount of nitrite formed in a 0.5 ml reaction mixture after a ten minute incubation period. At the end of the incubation period, 0.5 ml each of the diazo-coupling reagents (1 per cent sulfamilamide in 3 N HCl and 0.01 per cent N-(-1-naphthyl)-ethylenediamine hydrochloride) were added and the volume made to 3 ml with H_2O. The optical density was determined after 10 minutes using a Beckman B or DU spectrophotometer at a wave length of 540 mμ.

Sources of Rhizobium for Soluble Nitrate Reductase Studies

R. japonicum cells for the initial studies of the soluble nitrate reductase were isolated by the methods of Cheniae (12, p. 141-142) from soybean nodules grown in the field near Raleigh, North Carolina. The activity of nitrate reductase in bacteriods isolated from nodules grown in the field near Corvallis, Oregon, was extremely weak. In most of the experiments carried out at Oregon State University, therefore, pure cultures of R. japonicum served as a source of the enzyme. An effective N_2-fixing strain of R. japonicum used in the experiments was obtained

from Dr. L. W. Erdman of the U. S. Department of Agriculture and was maintained on agar slants. The nutrient medium used for mass culture of the <u>Rhizobium</u> contained the following per 1: K_2HPO_4, 0.5 g; KH_2PO_4, 1.0 g; KNO_3, 0.7 g; $MgSO_4 \cdot 7 H_2O$, 0.2 g; $CaSO_4 \cdot 2 H_2O$, 0.1 g; $FeCl_3$, 4.0 mg; L-arabinose, 1.0 g; and glycerol, 4 ml. Glycerol was used as a carbon source in preference to mannitol or sucrose because the organism produced much less slime under these conditions. This greatly simplified the collection of the <u>Rhizobium</u> cells by centrifugation.

The organism was successively transferred from the slants to 50 ml Bellco culture flasks containing 20 ml of sterile medium and then to 1,000 ml Bellco culture flasks containing 500 ml of sterile medium. The cultures were grown on a shaker at 30^oC. The contents of the 1,000 ml flasks were emptied into 12 l glass bottles containing 10 l of sterile medium to which had been added 10 ppm Dow Corning Antifoam C before sterilization. The cultures were incubated at 34^oC in a water bath and aerated slowly by passing air through a tube containing sterile cotton and then dispersing it into the inoculated nutrient medium. After about 40 hours the bacteria were collected by centrifugation in a Serval continuous flow system and washed twice with 0.1 M potassium phosphate buffer (0.001 M with respect to EDTA) at pH 7.0.

Culture of R. japonicum Under Controlled Oxygen Pressures

A series of cultures of R. japonicum were grown under varying oxy-
gen pressures to determine the effect of oxygen on nitrate reductase acti-
vity. Thirty milliliters of the medium described above was sterilized in
each of a series of 50 ml Erlenmeyer flasks fitted with a rubber stopper
through which was inserted an aeration tube containing cotton. One
liter suction flasks were evacuated and filled with either a mixture of air
and nitrogen or a mixture of air and oxygen to give the desired oxygen
pressure. The air in the culture flasks and aeration tubes was consi-
dered when calculating amounts of gases to be added to the suction
flasks. After inoculation, the flasks were connected and placed on a
rotary shaker at 31°C. Figure 4 shows a diagram of the culture system.

Source of Chemicals

Coenzymes and special compounds used were obtained from the
following sources: DPNH, TPNH, FAD and mammalian cytochrome $\underline{c}$
from Sigma Chemical Company of St. Louis, Missouri; FMN, GSH, and
cysteine from Nutritional Biochemicals Corporation of Cleveland, Ohio;
benzyl viologen and methyl viologen from Mann Research Laboratories of
New York, N. Y.; and Sephadex G-75 from Pharmacia, Uppsala, Sweden.
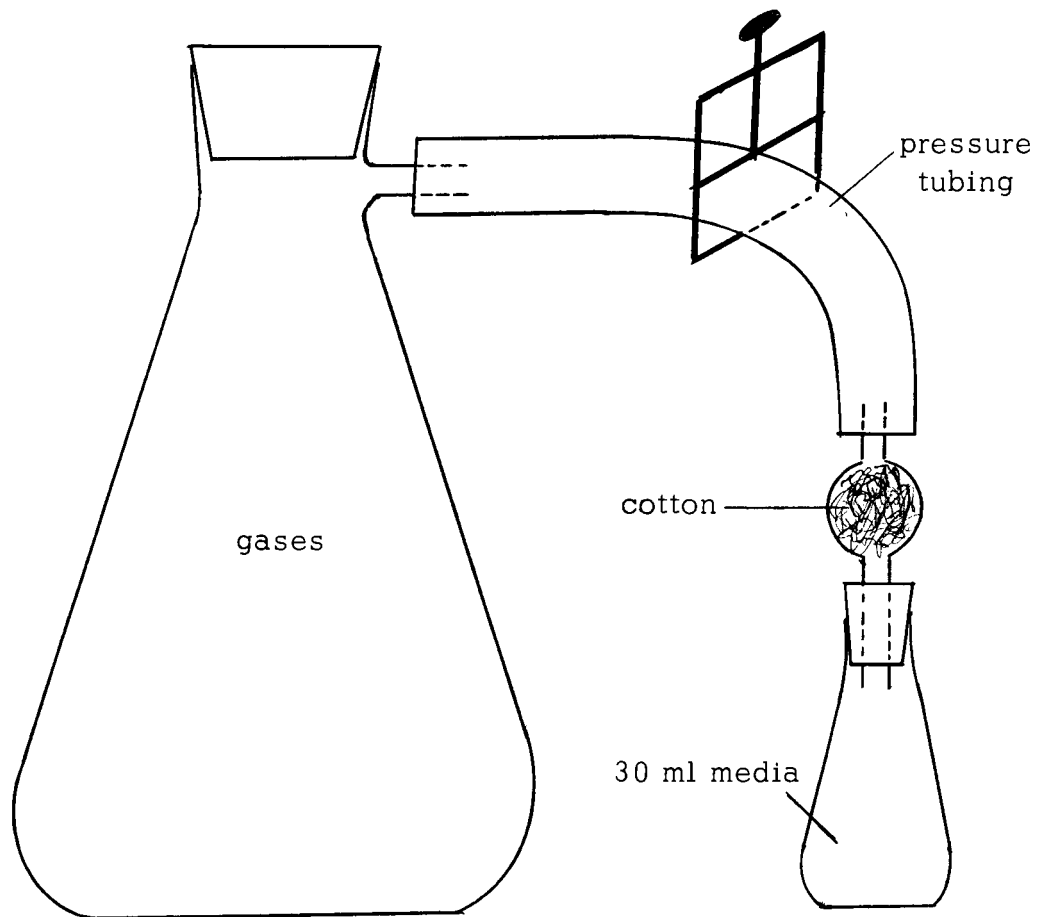Coenzyme Q (CoQ_{10}) was a gift from Merck Sharp and Dohme of West

Fig. 4. Culture apparatus for growing _Rhizobium_ under controlled oxygen pressures.

Point, Pa., and ferredoxin was kindly supplied by Dr.L. E. Mortenson

of Purdue University.

IV. RESULTS OBTAINED WITH PARTICULATE SYSTEM

Oxygen Uptake

Since there is no evidence that nitrate is involved in the nitrogen

fixing process, it has been postulated (13) that the enzyme complex may

be non-specific for oxidant and that some unidentified substance with

the appropriate oxidation-reduction potential may serve as the natural

oxidant under physiological conditions. It has been observed (12, p.

143) that the rate of nitrate reduction by the particulate preparation was

more rapid in assays conducted under anaerobic than under aerobic con-

ditions. In view of these results, a manometric experiment was de-

signed to determine whether or not the particulate preparation would

utilize atmospheric oxygen as an electron acceptor. Oxygen utiliza-

tion was determined using a Warburg apparatus. The components of the

reaction mixtures are described in figure 5. From the data obtained, it

is apparent that the particles catalyzed rapid oxygen uptake that was de-

pendent upon addition of succinate to the assay medium. These results

also show that oxygen uptake is inhibited by nitrate and that the de-

gree of inhibition is related to the concentration of nitrate in the reac-

tion mixtures. From the data in table 1 it can be seen that the de-

creased oxygen uptake resulting from the addition of nitrate was accom-

panied by an increase in nitrite production.

In view of the observations of Kubo (40) that nodule "hemoglobins"
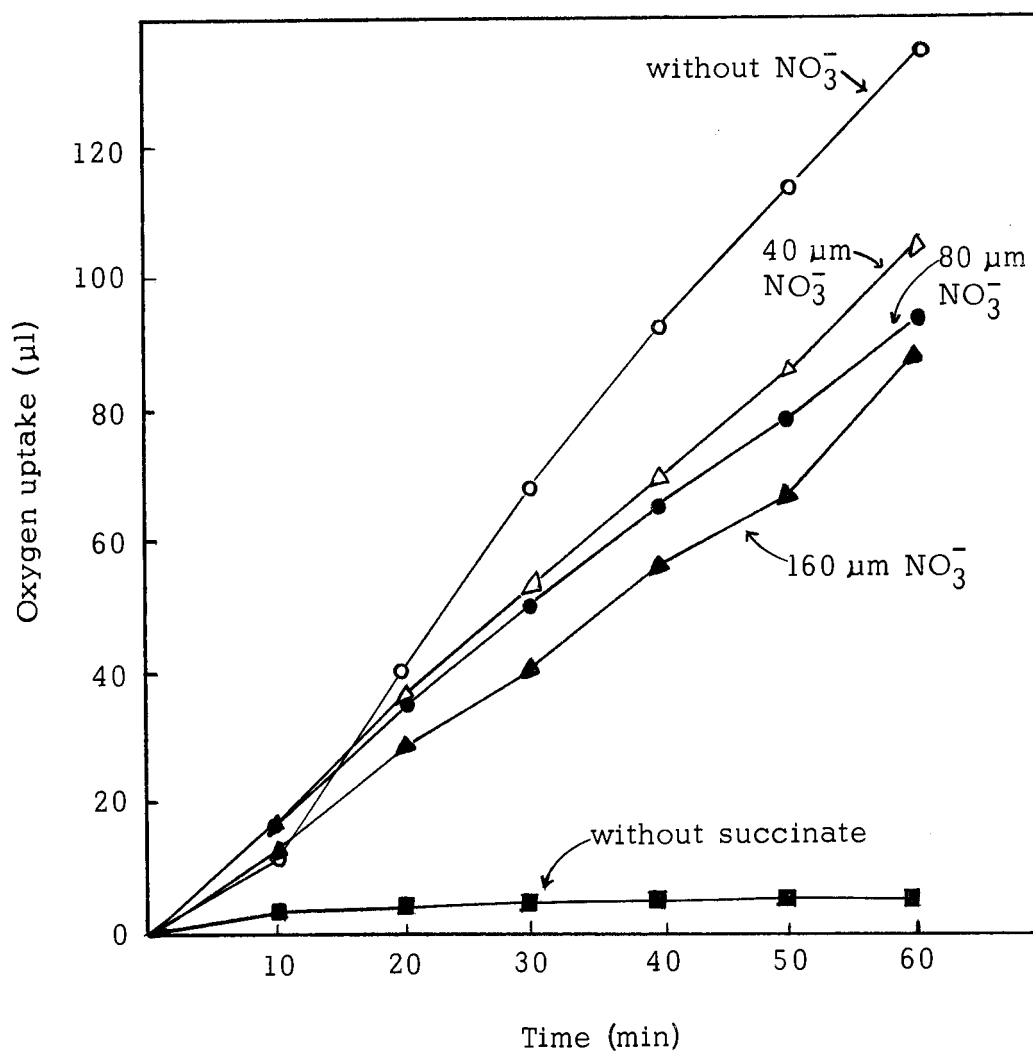
Fig. 5. Oxygen uptake by bacteriod particulate enzyme system and its inhibition by nitrate. The complete reaction mixture consisted of enzyme (1.2 mg protein), succinate (60 μmoles), phosphate buffer (120 μmoles), and the amounts of nitrate indicated. The final volume of each reaction mixture was 3.6 ml. The center well in each flask contained 0.2 ml 20 per cent KOH. The temperature was 30°C.

Table 1. Effect of Nitrate on Oxygen Uptake and Nitrite Formation in Presence of Particulate Enzyme*

Addition	Oxygen uptake** μmoles	Nitrite produced** μmoles
None	4.1	0.02
0.09 μmoles NaNO$_3$	3.8	0.28
0.9 " "	3.6	1.03
3.0 " "	3.2	2.77
9.0 " "	3.5	3.02
30.0 " "	3.1	3.07
90.0 " "	2.4	3.18

*Components of the reaction flasks were: enzyme (2.4 mg protein), succinate (60 μmoles), phosphate buffer (150 μmoles), and the nitrate additions indicated. The final volume of each reaction mixture was 3.0 ml. The center well in each flask contained 0.2 ml 20 per cent KOH, the gas phase was air, and the temperature was 30°C.

**Measurements were made after incubating for 60 minutes. Nitrite was determined by the procedure described under "Materials and Methods."

stimulated the uptake of oxygen by bacteriods from soybean nodules, an experiment was designed to test the effect of nodule "hemoglobins" on the rate of oxygen uptake by the particles from nodule Rhizobium. An experiment was conducted comparable with that described in figure 5 with the exception that nitrate additions were omitted and nodule "hemoglobins" at concentrations of 1.9, 19.0, and 32.0 μg heme per ml were included in the reaction flasks. Two experiments of this type revealed no consistent effect of the "hemoglobins" on the rate of succinate oxidation. Other experiments have revealed that hemoglobin is rapidly converted to hemiglobin in the presence of the particulate system and oxygen.

Attempts to Reduce Hemiglobin

Bergersen and Wilson (7) claim that bacteriods from soybean nodules exhibit the capacity to reduce hemiglobin to hemoglobin. In view of this report, an experiment was designed to determine whether or not the particulate enzyme complex, possessing the nitrate reducing capacity, possessed the capacity to reduce hemiglobin to hemoglobin. A complete reaction mixture (final volume of 3 ml) containing 300 μ moles buffer, 1 ml of particulate suspension (2 mg protein/ml) and 1 ml of hemiglobin solution (45 μg heme/ml) was placed in the main compartment of a cuvette fitted with a Thunberg attachment. The side arm of the cuvette contained 10 mg of solid sodium succinate. A reference cuvette was

prepared identical with the sample cuvette except it contained no succinate in the side arm. The cuvettes were closed, evacuated, and flushed with oxygen-free helium (scrubbed through two towers of $CrCl_2$). This procedure was repeated three times. A difference spectrum was recorded and then the contents of the sample cuvette were mixed with the succinate in the side arm. Difference spectra were recorded again at 10, 20, 30, and 60 minutes after adding the succinate but nothing indicated the reduction of the hemiglobin to hemoglobin. The trace obtained was no different from the base line which was recorded before the addition of succinate. The absolute spectrum after the experiment showed the same peak as that of hemiglobin (figure 3). This experiment was repeated several times using variations in the experimental techniques but in all cases results were negative.

Deoxygenation of Oxyhemoglobin

In another series of experiments the capacity of the particulate preparation to catalyze the transfer of electrons from succinate to oxyhemoglobin was studied. In a typical experiment (see figure 6 for results and description) reaction mixtures were prepared in each of two cuvettes fitted with ground glass stoppers. Both cuvettes contained buffer, oxyhemoglobin, and the particulate enzyme, and were filled in air. The base line, (trace a, figure 6) which falls close to the zero optical density scale at all wave lengths, is the difference spectrum prior to adding succinate. Solid sodium succinate (10 mg) was then mixed with
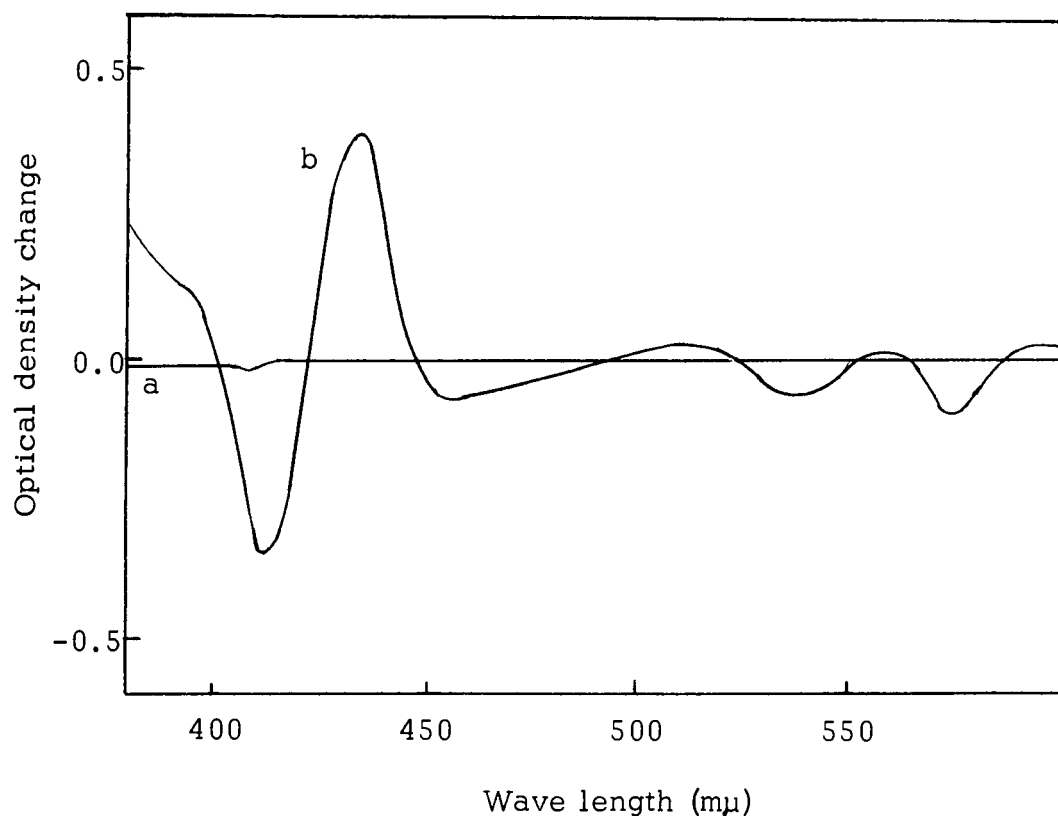
Fig. 6. A difference spectrum resulting from the conversion of oxyhemo-
globin to hemoglobin under aerobic conditions. Trace "a" is a
recording of the difference spectrum of two cuvettes each con-
taining oxyhemoglobin (12.5 μg heme/ml), particulate enzyme
(0.4 mg protein/ml), and phosphate buffer (100 μmoles/ml).
Trace "b" was recorded 40 minutes after mixing succinate
(0.33 mg solid sodium succinate/ml) into the sample cuvette.
(See text for further experimental details.)

the contents of one of the cuvettes and both cuvettes were stoppered to exclude all air space. The ground glass stoppers were coated with a thin film of vacuum grease. Difference spectra were recorded at 10 minute intervals. The difference spectrum in figure 6 was recorded 40 minutes after initiating the reaction. Recordings prior to this one indicated a lag in the reaction during the first 20 minutes. Decreases in absorbance observed at 575 and 540 mμ and the decrease at about 412 and increase near 430 (trace b) are conclusive evidence that oxyhemoglobin was converted to hemoglobin (see figures 2 and 3 for direct spectra of these two forms of the pigment).

An identical experiment was conducted with the exception that the cuvettes were filled under helium. The cuvettes and a beaker containing the reaction mixture were placed inside a plastic bag. The bag was flushed several times with helium, and the reaction mixture was shaken intermittently during the flushing procedure. The solution was transferred inside the bag to the cuvettes and the cuvettes were stoppered, removed from the bag, and difference spectra were recorded (trace a, figure 7). The cuvettes were returned to the bag and then flushed several times with helium. To one cuvette 10 mg of solid sodium succinate were added. The cuvette was stoppered and then both cuvettes were removed from the bag for difference spectra (trace b, figure 7). The base line falling near the zero optical density scale (trace a) is the difference spectrum prior to adding succinate to the mixture. The difference
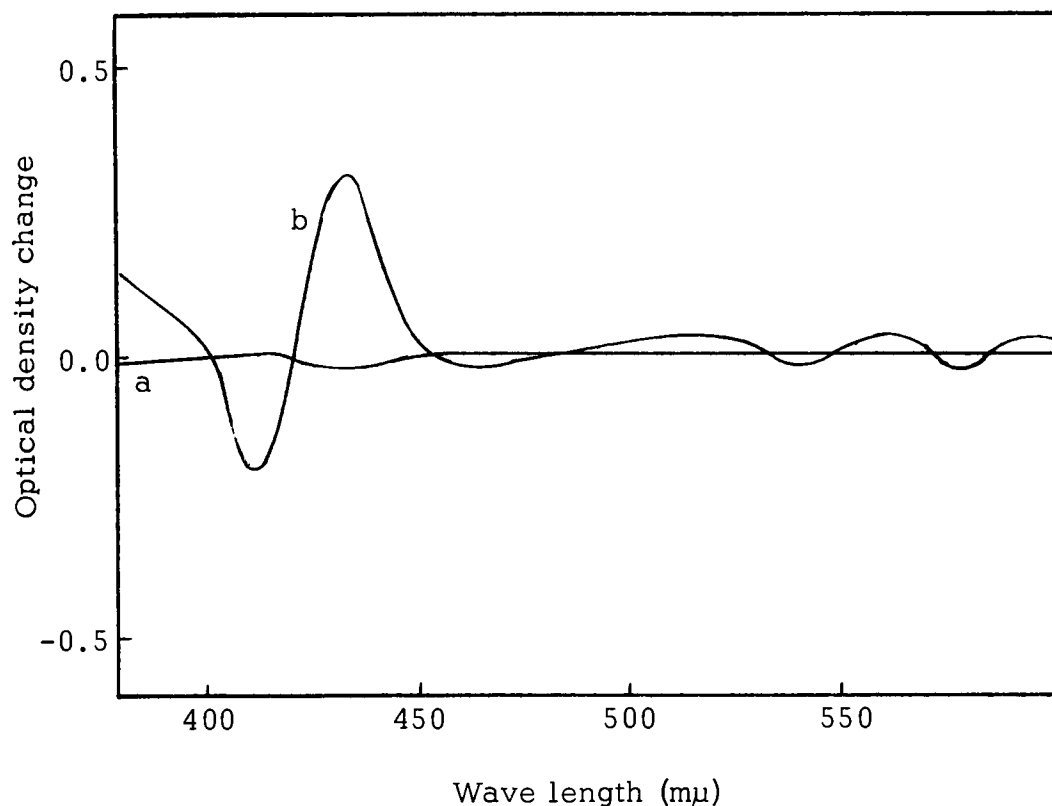
Fig. 7. A difference spectrum resulting from the conversion of oxy-
hemoglobin to hemoglobin in an atmosphere of He. The experi-
ment was identical with that described in figure 6 except that
both cuvettes were flushed with He (see text) and the differ-
ence spectrum (trace b) was recorded 7 minutes after mixing
succinate with the mixture. The slight curvature of the differ-
ence spectrum prior to adding succinate (trace a) is a result of
a slight difference in concentration of the oxyhemoglobin in the
two cuvettes.

spectrum showing considerable changes in optical density near 412 and 430 mμ (trace b) was recorded 7 minutes after the reaction was initiated. In both experiments (figures 6 and 7) the changes in the spectra after adding succinate are those expected when oxyhemoglobin is converted to hemoglobin. In the experiment reported in figure 7 there was no lag in the reaction.

That the time course of the conversion of oxyhemoglobin to hemoglobin is succinate-dependent is illustrated by the data plotted in figure 8. The reaction mixture containing succinate was identical with the complete reaction mixture used in the experiment reported in figure 7. The reference cuvette contained buffer, particulate enzyme, and oxyhemoglobin; therefore, the only difference between the two cuvettes was the succinate. In this case, however, the deoxygenation of oxyhemoglobin was followed by the optical density decrease at 575 mμ. As shown by the curve the reaction rate is approximately linear during the first 20 minutes of the reaction. In all experiments where reaction mixtures were flushed with purified helium, the conversion of oxyhemoglobin to hemoglobin by the particulate preparation could be confirmed by direct spectra as well as difference spectra.

Oxyhemoglobin from soybean nodules will dissociate non-enzymatically into hemoglobin and oxygen if the solutions of the pigment are placed in an evacuated vessel and incubated at $37^{\circ}C$ (37), but this reaction is much slower than the enzymic dissociation. When an
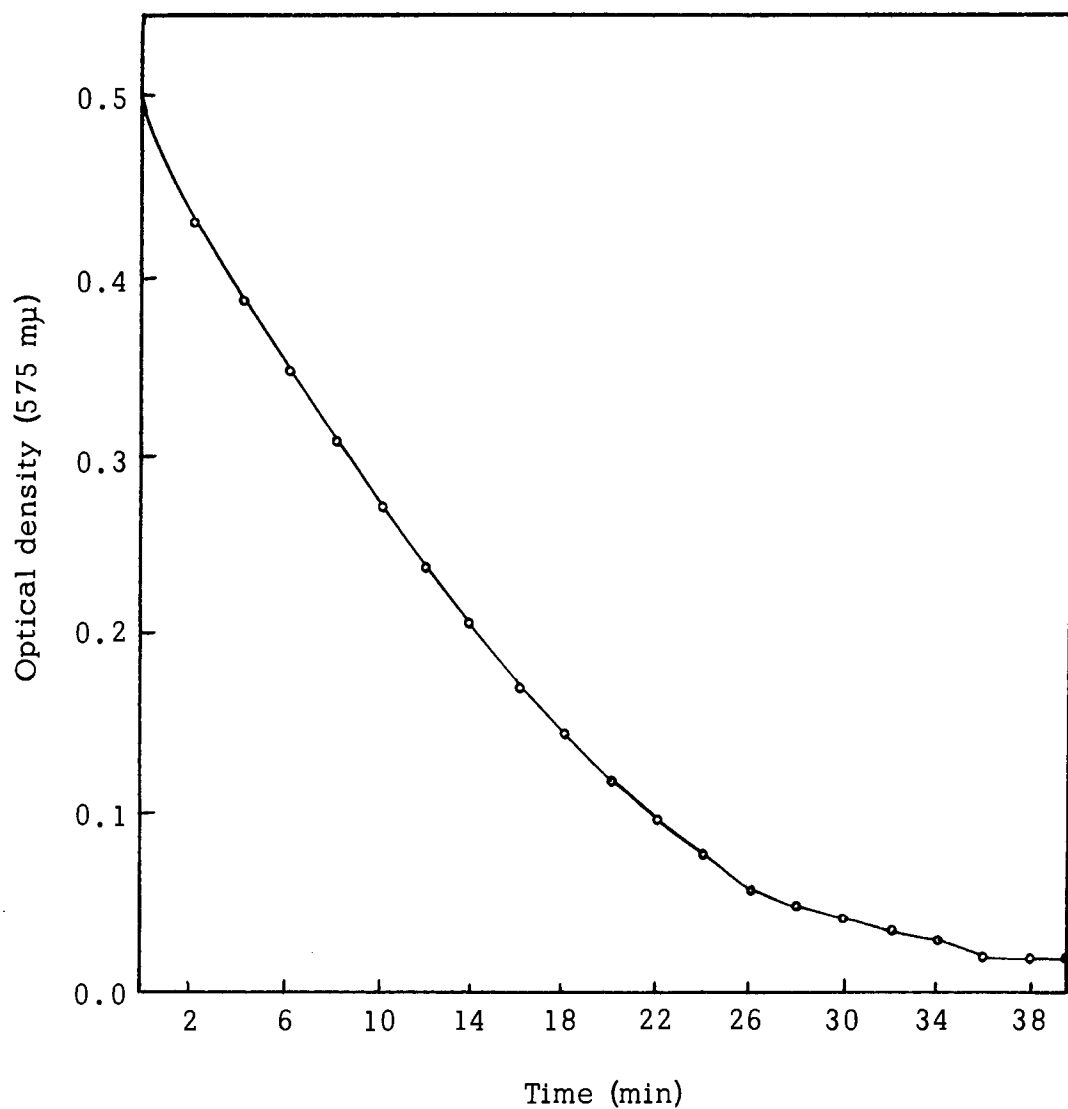
Fig. 8. Conversion of oxyhemoglobin to hemoglobin in an atmosphere
of He indicated by the decrease in optical density at 575 mµ.
The reaction mixture was the same as the complete reaction
mixture described in figure 6. The reference cuvette contained
all the reactants except succinate.

oxyhemoglobin solution (at a concentration used in experiments de-
scribed above) was placed in a Thunberg cuvette and flushed at $0^{\circ}C$
with purified He and then incubated at $25^{\circ}C$, only 5 to 10% of the oxy-
hemoglobin dissociated within 1 hour. In contrast, active preparations
of the particulate complex supplied with succinate caused an almost
complete deoxygenation of the oxyhemoglobin within 20 minutes. To
date no evidence has been obtained indicating whether the nitrate reduc-
tase component of the particulate complex is involved in the final trans-
fer step to oxyhemoglobin. The particulate system, however, is essen-
tial for rapid oxidation of succinate by oxyhemoglobin.

When reactions were carried out under aerobic conditions, a series
of spectra recorded during the course of the reaction revealed that oxy-
hemoglobin was converted to hemoglobin and then the hemoglobin was
oxidized to hemiglobin. Apparently the phenoloxidase activity (37) was
responsible for this oxidation.

V. RESULTS OBTAINED WITH SOLUBLE SYSTEM

Development of a Procedure for Preparation of the Soluble Enzyme

Previously all attempts to prepare a soluble extract of the nitrate reductase from R. japonicum had been completely unsuccessful. Treatment with any of the usual materials used to solubilize particulate enzymes, including cholate, deoxycholate, and isobutanol, resulted in complete and irreversible loss of activity. It had been observed that activity of the particulate enzyme was enhanced by addition of reducing reagents such as GSH and cysteine. Also Taniguchi and Itagaki (78) have shown that E. coli grown under anaerobic conditions possesses a much higher nitrate reductase activity than cells grown under aerobic conditions. In consideration of the possibility that the soluble enzyme may be labile to oxidation, a method was devised for the disruption of the cells anaerobically, under reduced conditions. This method involves a procedure where the bacterial suspension is reduced with hydrosulfite in the presence of benzyl viologen, and then the suspension is ground in a sealed Tygon tube containing glass beads. This method results in a highly active nitrate reductase preparation which is not sedimented after centrifugation for 2 hours at 140,000 x g.

In the preparation of the extract, Rhizobium japonicum cells from field grown nodules or pure cultures grown in an artificial medium were obtained by centrifugation procedures described in the section on

"Materials and Methods." The wet cell paste was suspended in 0.1 M K_2HPO_4 buffer (1 weight of cells in 2.5 weights of buffer). To the suspension was added the following per 100 ml of buffer: 10 mg benzyl viologen, about 40 mg of 1 to 1 $Na_2S_2O_4$-$NaHCO_3$ mixture, 1 g of Steapsin and sufficient GSH and EDTA to make the suspension 0.001 M with each of these compounds. This mixture was placed in a Tygon tube (internal diameter of 0.5 inch and 3 feet long) containing 1 g glass beads (diameter 0.3 mm) for each g of cells in the suspension. The ends of the tube were closed with metal clamps excluding as much air space as possible. The suspension of cells was rapidly frozen by placing the tube in crushed dry ice. The frozen tube then was pulled back and forth over a steel rod mounted on bearings until the suspension thawed. The entire procedure was repeated twice. After completion of the grinding procedure, the mixture was incubated at $25^{\circ}C$ for 30 minutes to allow further action of the lipase. The beads were removed by squeezing the mixture through cheese cloth and the cell debris was removed by centrifuging for 10 minutes at 20,000 x g or for 5 minutes at 35,000 x g. The turbid suspension obtained was then centrifuged at 80,000 x g for 30 minutes and the resulting supernatant used for fractionation and characterization studies. Throughout the procedure the $Na_2S_2O_4$-$NaHCO_3$ mixture was added as needed in order to maintain a blue color in the preparation.

Development of a Standard Assay Procedure for the Soluble Enzyme

Since benzyl viologen was a constituent of the preparation mixture, it was convenient to use it as an electron donor in the standard assay procedure. The conventional methods of assay utilizing oxidation-reduction dyes involve anaerobic incubation in Thunberg tubes. Such assays are tedious and time consuming. Especially troublesome are problems of evaporation of mixtures under reduced pressures. It was found that a suitable assay could be carried out under atmospheric pressure provided that sufficient hydrosulfite was added. The components of the assay system are listed in table 2. After a 10 minute incubation at $30^{\circ}C$, the reaction is stopped by vigorously shaking the assay mixture until all blue color has disappeared. The nitrite produced is measured as described in the section on "Materials and Methods." It is especially important to be sure that all the $Na_2S_2O_4$ has been oxidized prior to addition of the diazo-dye reagents because nitrite is rapidly reduced by the $Na_2S_2O_4$ under strongly acid conditions. That the assay is reasonably reliable is illustrated by the proportionality curve in figure 9. From these data it is apparent that nitrite production is proportional to volume of enzyme over a wide range of enzyme concentrations. In the remainder of this thesis, this assay will be referred to as "the standard assay procedure."

Table 2. Components of Soluble Nitrate Reductase Assay Mixture

Component	Quantity*
Phosphate buffer pH 7.0	30 µmoles
$NaNO_3$	10 µmoles
Benzyl viologen	0.02 mg
$Na_2S_2O_4$-$NaHCO_3$ (1 to 1)	0.80 mg
Enzyme extract	0.1 ml**

*The final volume of the reaction mixture was 0.5 ml.

**The enzyme solution was diluted so that the rate of nitrite formation in the reaction mixture ranged between 0 and 60 mµmoles per ten minutes.

Purification

The soluble nitrate reductase has been purified about 11-fold by use of $(NH_4)_2SO_4$ precipitation and chromatography on a calcium phosphate column. An $(NH_4)_2SO_4$ solution, saturated at about 3°C was cooled to its freezing point by placing the container in dry ice immediately before use. A sufficient quantity of the pre-cooled $(NH_4)_2SO_4$ to bring the solution to 25 per cent saturation at 3°C was slowly added to the crude extract (designated as Fraction I in table 3). During the addition, the solution was stirred with a magnetic stirrer at a rate sufficient to give fairly rapid mixing of the $(NH_4)_2SO_4$ and enzyme solution. Vigorous stirring was avoided because this resulted in excessive
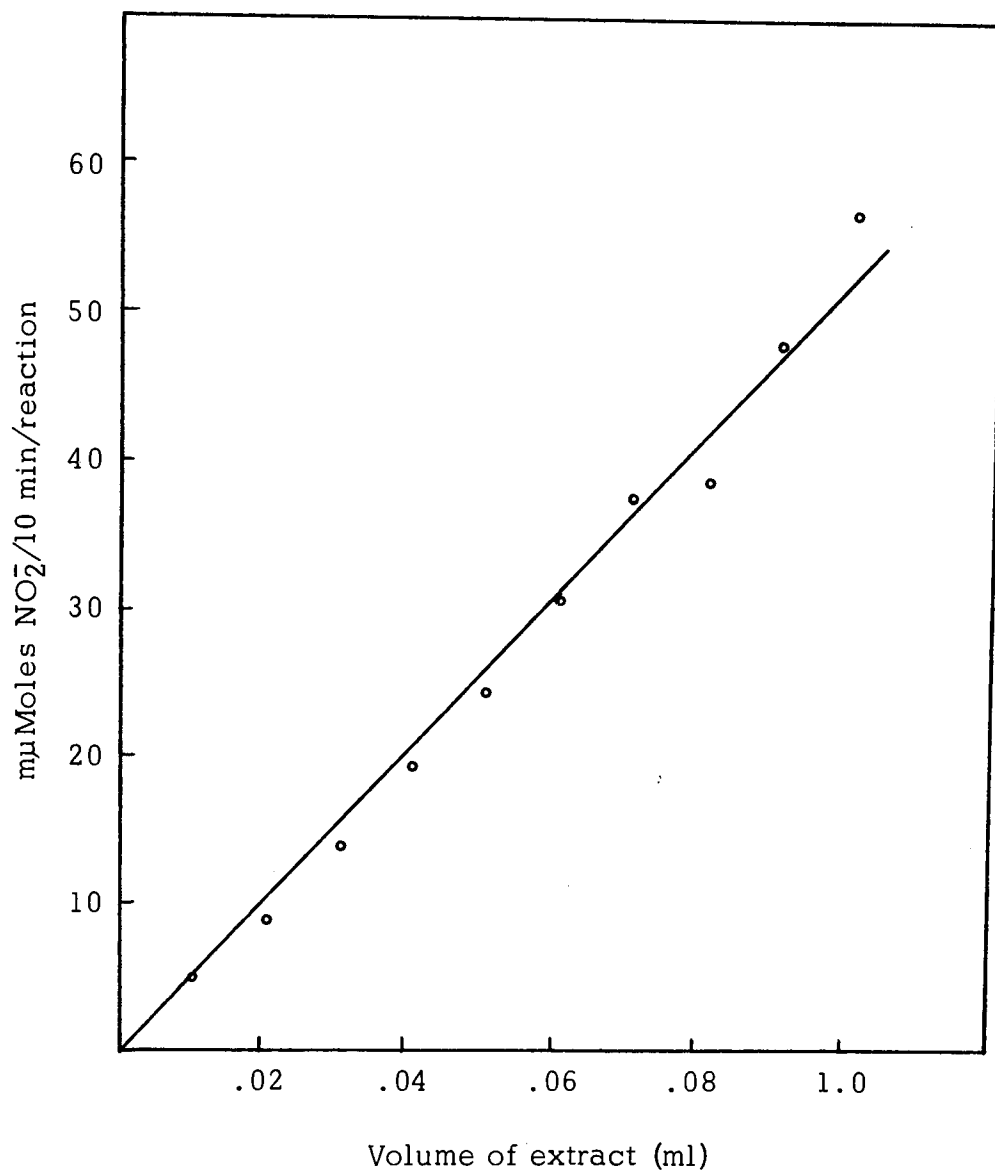
Fig. 9. The effect of increasing amounts of enzyme extract on nitrite
formation. The components of the reaction mixture are the
same as described in table 2 except that the volume of the
enzyme (Fraction I table 3) is varied as shown. The final
volume of each reaction mixture was 0.5 ml.

oxidation and inactivation of the enzyme. The suspension was allowed

to stand for 10 minutes and then it was centrifuged at 10,000 x g for 10

minutes. The resulting supernatant was brought to 45 per cent satura-

tion with $(NH_4)_2SO_4$ and allowed to stand 10 minutes. The precipitate

was collected by centrifugation and dissolved in 0.01 M potassium

phosphate buffer at pH 7.0. This enzyme solution (Fraction II in table

3) was placed on a bed of calcium phosphate prepared by the method of

Anacker and Stoy (1). The glass column used to retain the calcium

phosphate was 30 mm in diameter and 50 mm in height. A fritted glass

plate in the bottom of the column retained the adsorbent. A filter paper

disc was placed on the fritted glass and sufficient calcium phosphate,

suspended in 0.01 M potassium phosphate at pH 7.0, was added to ob-

tain a bed of calcium phosphate 16 mm high. Prior to packing the

column, about 0.004 mg of benzyl viologen and 0.5 mg of a 1 to 1 mix-

ture of $NaHCO_3$ and $Na_2S_2O_4$ were mixed with each ml of the calcium

phosphate suspension. A second filter paper disc was carefully placed

on the top of the calcium phosphate and then the column was packed

under a pressure of three pounds per square inch.

The enzyme and buffers used for elution were added to the column

from a separatory funnel placed 35 cm above the column. A flow rate

of about 1 ml per minute was maintained by adjusting the stopcock of

the separatory funnel. A typical elution pattern is illustrated in figure

10. Inactive protein was first separated from the column by washing
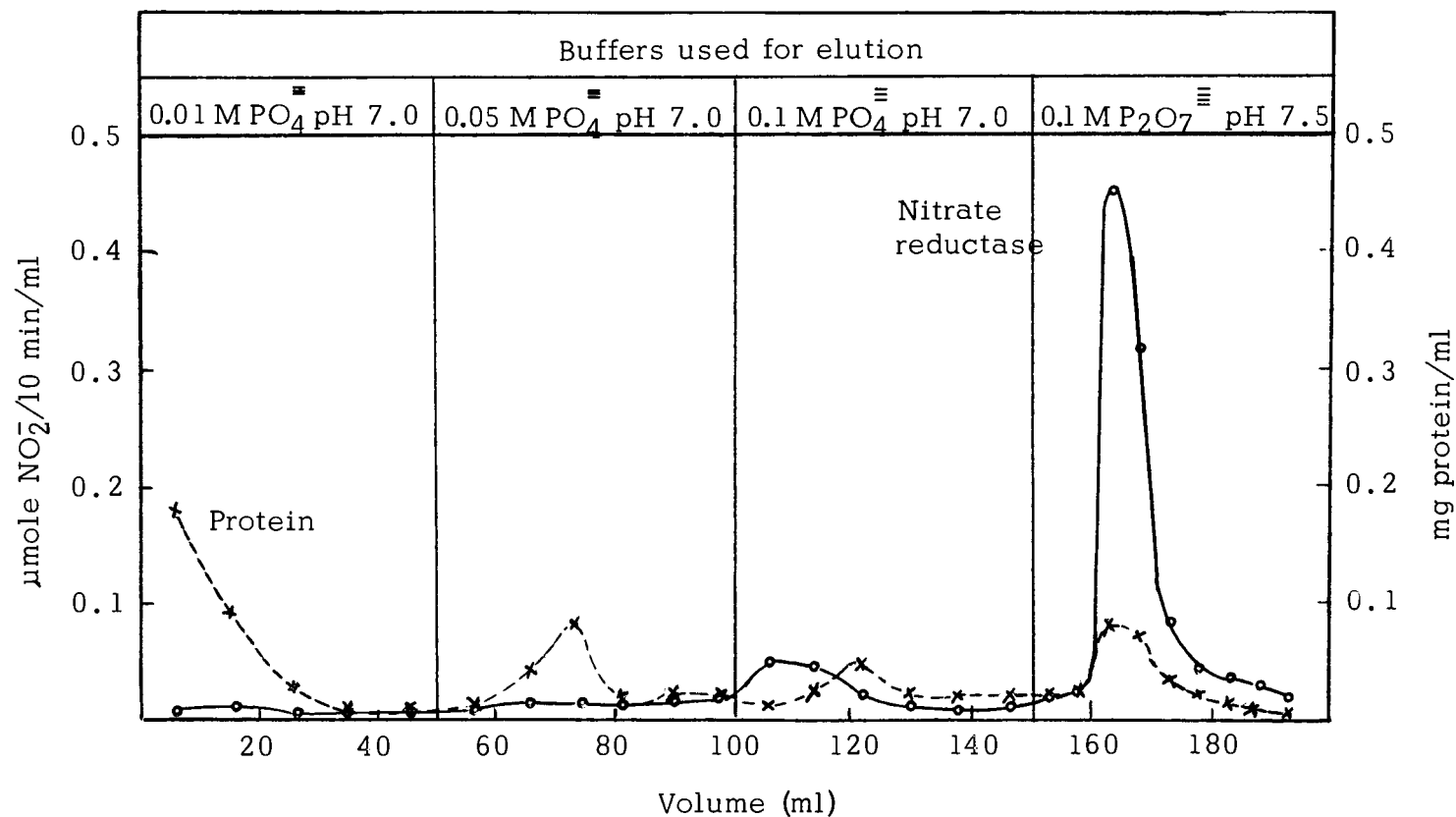
Fig. 10. Elution pattern of nitrate reductase from a calcium phosphate
column. The standard assay procedure was used to determine
nitrate reductase activity.

with increasing concentrations of potassium phosphate buffer at pH 7.0. The nitrate reductase fraction was then eluted with 0.1 M sodium pyrophosphate buffer at pH 7.5. The elution buffers contained 0.004 mg of benzyl viologen and 0.5 mg of the $Na_2S_2O_4$-$NaHCO_3$ mixture. Summary data for a typical purification are presented in table 3.

An attempt to fractionate a soluble extract which was prepared without benzyl viologen resulted in complete loss of activity during the ammonium sulfate precipitation, even though reduced conditions were maintained. Apparently the enzyme is not maintained in the reduced form unless the benzyl viologen is present. It was desirable to obtain a purified enzyme without the use of reducing agents and benzyl viologen because these compounds interfered in certain preincubation experiments and in experiments where various electron donors were tested. As indicated in subsequent sections of this thesis, it was necessary to use the Fraction I preparation (table 3) for these studies.

Table 3. Summary of Purification of Soluble Nitrate Reductase

Fraction	Vol. (ml)	Specific Activity*	Recovery (%)	Purification
I. Crude	16	0.46	100	---
II. 25-45% $(NH_4)_2SO_4$ ppt.	10	1.58	67	3.5
III. 10-50 ml $P_2O_7^{\equiv}$ eluate**	40	4.40	36	9.6
IV. 15-25 ml $P_2O_7^{\equiv}$ eluate**	10	5.06	27	11.1

*The specific activity is listed in μmoles nitrite formed in 10 min per mg protein. Nitrate reductase activity was determined by the standard assay procedure.

**See figure 10 for a diagram of the calcium phosphate column chromatography of the enzyme.

Properties of Soluble Enzyme

Stability of the Enzyme. The soluble enzyme is extremely labile to oxidation by atmospheric oxygen. Any manipulation of the enzyme solution resulting in excessive exposure to air results in rapid loss of activity. The effect of leaving out various components of the grinding mixture (table 4) illustrates the susceptibility of the soluble enzyme to oxidation. Although the extract was prepared in a closed system, omitting the reducing reagent and benzyl viologen from the grinding mixture resulted in approximately 18 per cent of the activity obtained when all the components of the grinding medium were included. In a

Table 4. Effect of Various Components Used in the Grinding Medium on Activity of Soluble Nitrate Reductase

Components in medium	Nitrate reductase activity (μmole NO_2^-/10 min/ml)*
Complete including EDTA, GSH, $Na_2S_2O_4$ and benzyl viologen**	0.89
Complete, except EDTA and GSH omitted	0.69
Complete, except $Na_2S_2O_4$ omitted	0.26
Complete, except $Na_2S_2O_4$ and benzyl viologen omitted	0.16

*Standard assay procedure described in table 2 was used, which included 0.1 ml of a 1 to 5 dilution of the crude soluble extract.

**The complete grinding medium is described in the section entitled, "Development of a Procedure for Preparation of the Soluble Enzyme."

separate experiment similar to that for which the results are presented

in table 4, the omission of Steapsin from the grinding medium resulted

in a decrease in activity of the extract from 0.44 μmoles nitrite formed

per 10 minutes per mg of protein to 0.22 μmoles nitrite formed per 10

minutes per mg of protein.

In an experiment where an extract of the soluble nitrate reductase

was shaken until all the blue color had disappeared, allowed to remain

oxidized for 5 minutes at 3°C, and then sufficient reducing reagent

added to restore the blue color, 59 per cent of the original activity

was lost. Overnight storage of the enzyme under nitrogen and under reduced conditions at approximately $2^{\circ}C$ resulted in almost complete loss of activity. There is very little loss of activity of preparations stored under nitrogen at $-15^{\circ}C$ for periods as great as six months. The enzyme is relatively stable to dialysis for a period of two to four hours if reduced conditions are maintained.

Nitrate reductase activity of Rhizobium cells is markedly affected by the oxygen pressure of the culture medium. The data in table 5 show that nitrate reductase activity is not detectable in cells grown at high oxygen pressures. It should be noted that the O_2 pressures listed in table 5 are the initial pressures after inoculation. These pressures would be reduced as the cells began to grow and respire. Perhaps cells grown at the lowest oxygen pressure were supplied with insufficient oxygen for normal metabolism.

Specificity for Electron Donors. The electron transfer system present in the particulate enzyme is not functional in the soluble extract. DPNH and succinate, which were effective electron donors in the particulate system, were completely inactive with the soluble preparation (table 6). Either methyl viologen or benzyl viologen served as effective electron donors; however, the latter dye is slightly more active than the former. A series of biological compounds were used in an attempt to find a physiologically active electron donor for the soluble preparation.

Table 5. Effect of Oxygen Tension During Growth on the Nitrate Reductase Activity of R. japonicum

Oxygen pressure (atm.)	Growth (O.D.)*	Nitrate reductase activity (μmoles NO$_2$/10 min/30 ml culture)**
0.05	0.72	0.00
0.10	0.96	2.90
0.15	0.75	2.45
0.21	1.00	2.54
0.30	0.92	0.00
0.50	1.09	0.00

*Growth of the cultures was determined by measuring the optical density at 600 mμ in 0.5 inch cuvette using a Bausch and Lomb Spectronic 20 colorimeter.

**Cells from each 30 ml culture were harvested by centrifugation after about 66 hours of growth, washed with 20 ml 0.1 M potassium phosphate buffer at pH 7.0 and suspended in 5 ml of 0.1 M potassium phosphate buffer at pH 7.0. An aliquot of 0.1 ml from each 5 ml suspension was added to each assay mixture containing 10 μmoles sodium succinate at pH 7.0, 10 μmoles of NaNO$_3$, and 20 μmoles potassium phosphate buffer at pH 7.0. The final volume was 0.5 ml. Nitrite produced was estimated by the method used for the standard nitrate reductase assay.

Table 6. The Effectiveness of Various Electron Donors in Nitrate Reduction by the Soluble Nitrate Reductase

Experiment	Electron donor	Amount of donor (μmoles)	Nitrate reductase activity (μmoles NO_2^-/10 min/mg protein)*
I	Benzyl viologen	0.055	0.12
	DPNH	0.21	0.00
	TPNH	0.21	0.00
	Sodium succinate	10.00	0.00
	DPNH + CoQ$_{10}$	0.21 of DPNH and 0.016 of CoQ$_{10}$	0.00
	DPNH + FAD	0.21 of DPNH and 0.012 of FAD	0.00
II	Benzyl viologen	0.055	0.25
	Cytochrome $\underline{c}$	0.008	0.00
	FAD	0.006	0.00
	FMN	0.100	0.00
	Methyl viologen	0.070	0.22
III	Benzyl viologen	0.055	0.63
	Ferredoxin	0.008	0.00
	Sodium molybdate	0.100	0.00

*In addition to the compounds listed, the reaction mixtures contained 30 μmoles phosphate buffer pH 7.0, 10 μmoles $NaNO_3$, 0.8 mg of $Na_2S_2O_4$-$NaHCO_3$ (1 to 1) and 0.1 ml of a diluted and modified Fraction I enzyme preparation. The final volume was 0.5 ml. Nitrite formation was measured by the method used in the standard nitrate reductase assay.

Since reduced benzyl viologen in the enzyme preparation interfered with tests of other electron donors, the extract was prepared with benzyl viologen omitted from the grinding mixture and 0.1 M potassium phosphate buffer pH 7.0 was substituted for 0.1 M K_2HPO_4.

As shown by the data in table 6, none of the compounds tested except the oxidation-reduction dyes, benzyl viologen and methyl viologen, were effective under the conditions of the assay.

It has been reported that ferredoxin will serve as an electron donor for nitrate reduction (47). To determine whether or not nitrite may have been formed and rapidly reduced in the assay containing ferredoxin, 0.1 μmole nitrite was added to reaction mixtures containing this electron donor. There was a slight reduction of nitrite by ferredoxin but the magnitude of nitrite disappearance during the assay period was not sufficient to appreciably influence the nitrate reductase assay.

Effect of pH on Activity. The standard assay system contains a mixture of $Na_2S_2O_4$ and $NaHCO_3$ to maintain the reaction in a reduced condition. This mixture has considerable buffer capacity and therefore influences the pH of buffer solutions utilized for investigation of the effect of pH on enzyme activity. For the study of the effect of pH on activity, therefore, the $NaHCO_3$ was omitted from the standard reaction mixture and a minimum amount of $Na_2S_2O_4$ was utilized to maintain the benzyl viologen in the reduced form. The pH values of the reaction mixtures therefore were close to those of the various buffer solutions added (see figure 11 for further details). As indicated in figure 11, the pH of all complete mixtures were determined to further increase the accuracy of the study.
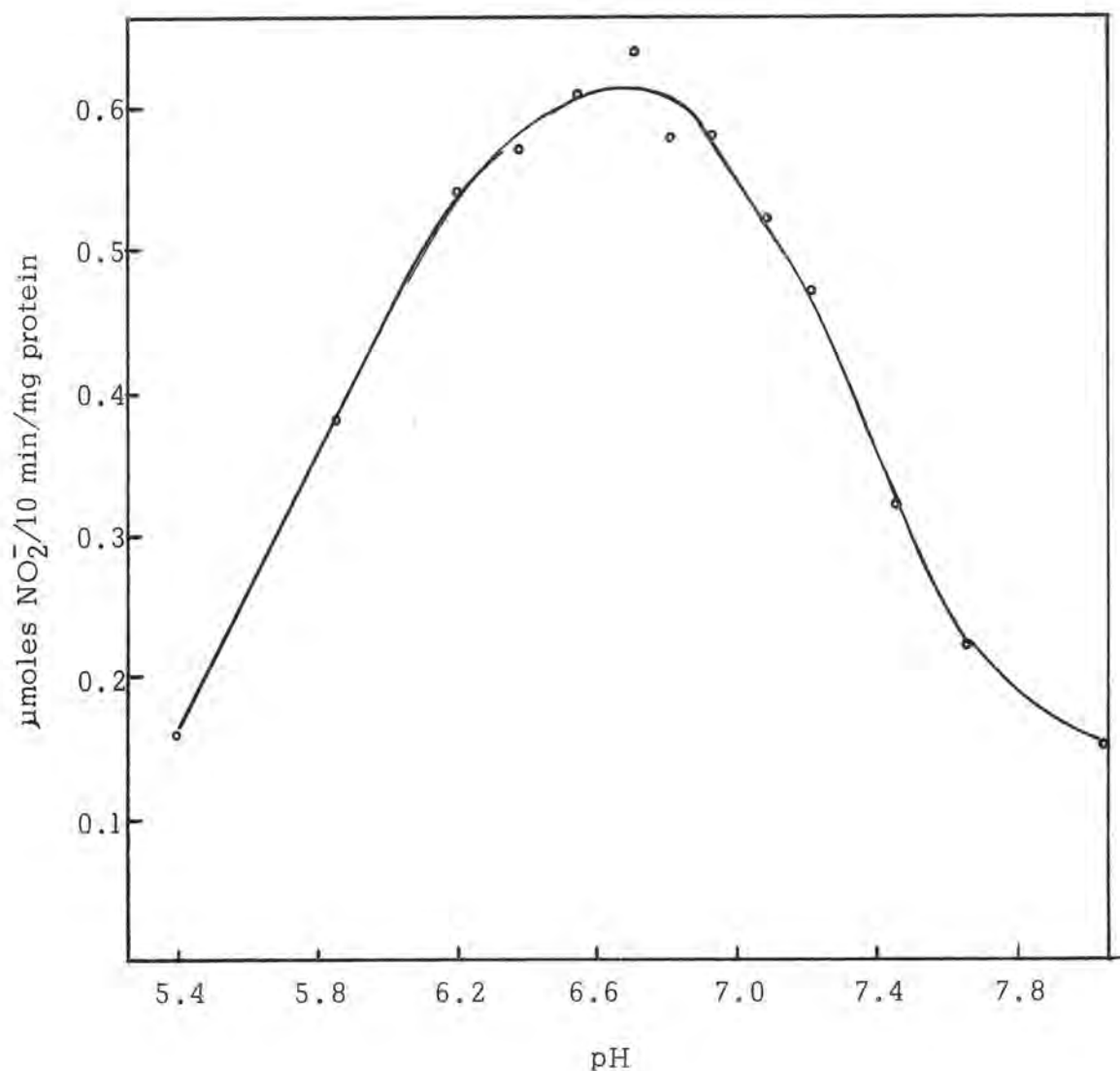
Fig. 11. Effect of pH on nitrate reductase activity of the soluble pre-
paration. The reaction mixture contained; 300 μmoles potas-
sium phosphate buffer (in a range of pH from that of KH_2PO_4
to that of K_2HPO_4), 100 μmoles $NaNO_3$, 0.2 mg benzyl violo-
gen, 2.0 mg $Na_2S_2O_4$, and 0.2 ml Fraction I extract in 0.1 M
potassium phosphate buffer pH 7.0. The final volume was
5 ml. At the end of the 10 minute incubation period 0.5 ml
of each reaction mixture was removed and assayed for nitrite.
The pH of the remaining portions was measured.

The rate of nitrate formation was not greatly influenced by pH in the range between 6.2 and 7.0 (figure 11). There was a very marked decline in activity, however, either above pH 7.0 or below pH 6.2. The optimum pH as shown by the curve in figure 11 is in the range between 6.7 and 6.8. The effect of buffers other than phosphate was not investigated.

Effect of Concentration of Substrate. The dependence of the rate of the reaction on the concentration of nitrate in the assay mixture is illustrated in figure 12. The data are presented in the form of a reciprocal substrate concentration vs. reciprocal velocity curve (Lineweaver-Burk plot (15, p. 21) from which a Michaelis constant (K_m) of 7.3×10^{-5} moles per l was calculated.

Inhibition by Sulfhydryl Reagents and Metal Chelating Agents. In preparation of the enzyme extract used for inhibition studies, benzyl viologen, GSH, EDTA, and lipase were omitted from the grinding mixture. Also, 0.1 M potassium phosphate at pH 7.0 was used instead of 0.1 M K_2HPO_4. This deviation from the usual procedure was necessary because it was desired to compare results with those published by Cheniae and Evans (11, p. 60-63, 12, p. 147-148). The GSH in the enzyme preparation influenced the inhibition by sulfhydryl reagents. Of the sulfhydryl binding reagents tested (table 7) only p-chloromercuri-benzoate (PCMB) resulted in appreciable inhibition of nitrate reduction.
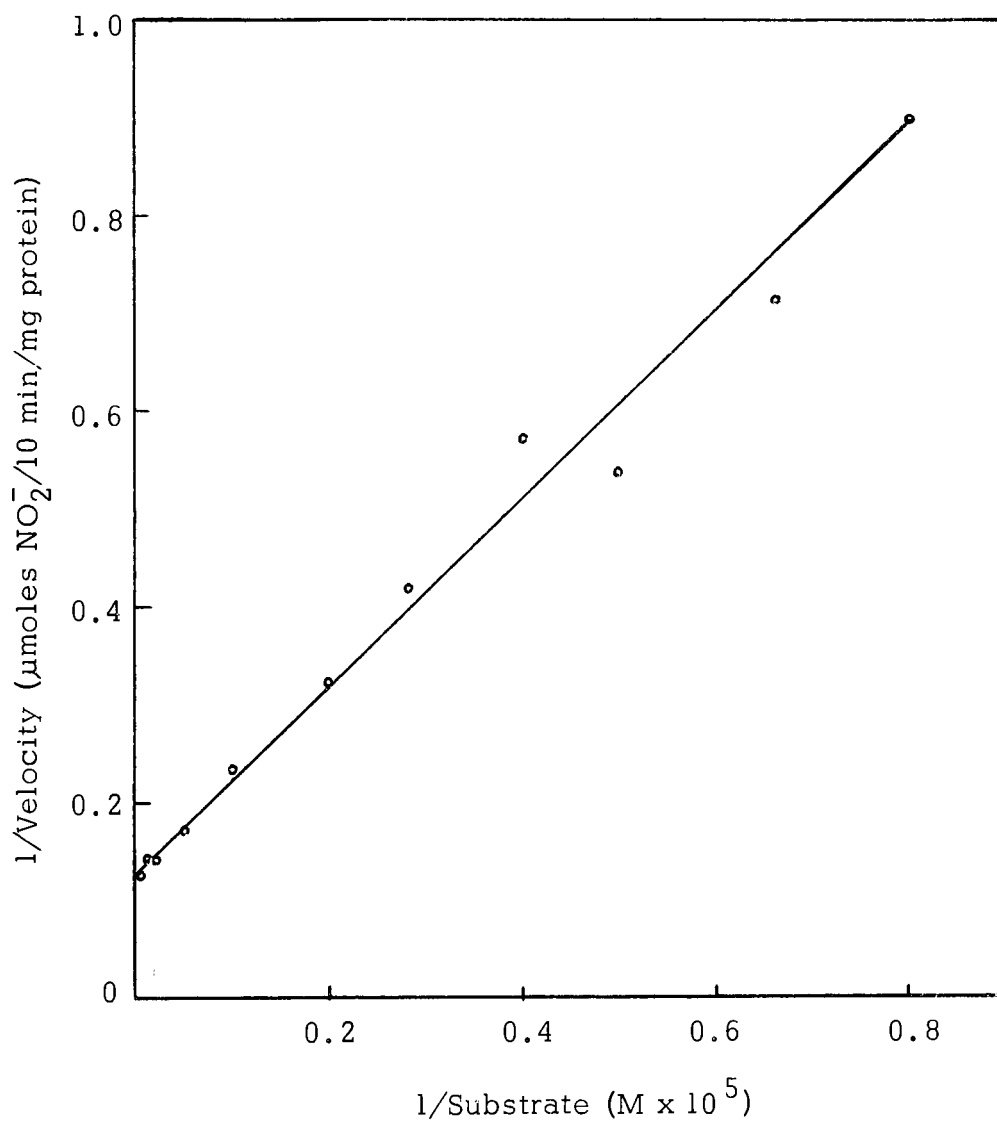
Fig. 12. Reciprocal plot of the effect of nitrate concentration on the nitrate reductase activity of the soluble nitrate reductase. The standard assay procedure was used with variations in nitrate concentration as indicated. The enzyme extract consisted of 0.1 ml of Fraction I.

The inhibition caused by PCMB was to a large degree reversed by the addition of either GSH or cysteine. There was slight inhibition by arsenite and iodoacetate; however, this was not reversed by GSH or cysteine.

Very low levels of metal chelating agents generally resulted in a stimulation of the soluble nitrate reductase activity (table 8). With exception of sodium citrate, there was some inhibition by most compounds used at high levels; however, only thiourea and sodium diethyl-dithiocarbamate resulted in a particularly high degree of inhibition. Dialysis against 0.001 M KCN for four hours resulted in complete loss of activity. After passing the extract through a column of Sephadex G-75 to remove KCN, addition of Mn^{++}, Co^{++}, $MoO_4^{=}$, Cu^{++}, Fe^{++}, and Zn^{++} (0.001 M final concentration) either singly or in combination failed to restore activity.

Table 7. Inhibition of Soluble Nitrate Reductase by Sulfhydryl Reagents and Its Reversal by Sulfhydryl Compounds

Addition	Final concentration (molar)	Inhibition (%)*		
		No sulfhydryl reagent	GSH $(10^{-4}M)$	Cysteine $(10^{-4}M)$
PCMB	2×10^{-5}	3	7	3
PCMB	5×10^{-5}	40	13	20
PCMB	1×10^{-4}	65	21	14
Sodium arsenite	4×10^{-3}	1	0	7
Sodium arsenite	2×10^{-2}	11	9	15
Sodium iodoacetate	2×10^{-2}	8	5	8

*The standard assay procedure was used with the exception that sulfhydryl reagents and either cysteine or GSH were added to reaction mixtures as indicated. All components of reaction mixtures except benzyl viologen were preincubated for 10 minutes at $0^{o}C$ prior to initiation of the reactions by the addition of benzyl viologen. For the preparation of the enzyme extract for inhibition studies, benzyl viologen, EDTA, GSH, and lipase were omitted from the grinding medium; also 0.1 M potassium phosphate buffer at pH 7.0 was used instead of K_2HPO_4. Each reaction mixture contained 0.1 ml of a 1 to 5 dilution of the extract. The enzyme used was the Fraction I preparation (table 3) prepared by the modified procedure indicated above.

Table 8. Inhibition of Soluble Nitrate Reductase by Metal Chelating Agents

Addition	Final concentration (molar)	Relative activity* (% of control)
o-Phenanthroline	1×10^{-3}	107
	2×10^{-3}	100
Sodium azide	2×10^{-5}	118
	2×10^{-4}	94
EDTA	2×10^{-2}	88
	4×10^{-2}	78
Thiourea	1×10^{-2}	68
	2×10^{-3}	54
2, 2-Dipyridyl	1×10^{-3}	101
	2×10^{-3}	88
Sodium citrate	1×10^{-2}	101
	2×10^{-2}	118
Sodium diethyl dithiocarbamate	1×10^{-2}	45
	2×10^{-2}	29
Potassium cyanide	1×10^{-3}	103
	5×10^{-3}	72
Potassium cyanate	2×10^{-4}	130
	1×10^{-3}	142

*The standard assay procedure was used with the exception that the inhibitors, at the concentrations indicated, were added and all components of reaction mixtures except benzyl viologen were preincubated for 10 minutes at $0^{\circ}C$ prior to the initiation of the reaction by addition of benzyl viologen. The enzyme extract used was the same as that utilized in the experiment reported in table 7.

VI. DISCUSSION

Function and Properties of the Particulate Nitrate Reductase

Previous investigations (13) on the possible physiological role of the nodule-nitrate reductase complex have provided considerable evidence that the enzyme activity, as measured by the rate of nitrate reduction, is correlated positively with the capacity, or with factors associated with the capacity, of leguminous plants to fix atmospheric nitrogen. The evidence for a relation between the nodule nitrate reductase and the capacity to fix nitrogen includes the following observations (13). (a) The activity of the enzyme in nodules of soybean plants inoculated with different strains of R. japonicum is positively correlated with the nitrogen fixing efficiencies of the various strains. (b) The nodule nitrate reductase activity is correlated with the hemoglobin content of legumes during the growth season. (c) The specific activity of the nodule enzyme is greatest when legumes are grown in the absence of combined nitrogen, and the addition of either nitrate or ammonium nitrogen to cultures results in reduced activity. This is in contrast to observations on most other organisms which require nitrate as an inducing agent for nitrate reductase formation. Since there is no evidence that nitrate is involved in the nitrogen fixing process, it has been postulated (13) that the enzyme complex containing the nitrate reductase may be non-specific for oxidant and that some unidentified substance

with the appropriate oxidation-reduction potential may serve as the

natural oxidant under physiological conditions. This postulation is

supported by the fact that xanthine oxidase, a molybdo-flavoprotein,

not only will catalyze the oxidation of hypoxanthine to xanthine by

molecular oxygen but also will catalyze the oxidation of hypoxanthine

to xanthine by nitrate provided that the assay system is devoid of oxy-

gen. It seems entirely reasonable to postulate that the nodule nitrate

reductase is referred to as a "nitrate reductase" because no other

oxidizing agent had been identified that would substitute for nitrate.

The fact that the addition of nitrate to cultures of soybean plants results

in an inhibition of the formation of the enzyme suggests that nitrate is

not the natural substrate (13).

It is apparent from the results presented here that the particulate

enzyme system from nodules of soybeans or pure cultures of R. japoni-

cum not only catalyzes a succinate-dependent nitrate reduction but

also catalyzes the transfer of electrons from succinate to oxygen. The

inhibition of oxygen uptake by nitrate (figure 5) strongly suggests that

oxygen and nitrate are competing for electrons from a common source.

Conclusive evidence that the terminal activation enzyme for each of the

two acceptors is the same has not been demonstrated. It is entirely

possible that the competition for electrons takes place at some link in

the electron transport chain prior to the terminal activation enzyme.

Kubo (40) has shown that nodule "hemoglobins" stimulate the uptake of oxygen by bacteriods from soybean nodules. Attempts to demonstrate a similar stimulation by the particulate nitrate reductase resulted in no consistent effect of the "hemoglobins" on the rate of succinate oxidation. Subsequent experiments revealed that hemoglobin is rapidly converted to hemiglobin in the presence of the particulate enzyme system and oxygen. If one postulates an involvement of hemoglobin and oxyhemoglobin in the oxygen uptake process, it is apparent that the oxidation of hemoglobin to hemiglobin might interfere with the normal role of the pigment under the experimental conditions. Since the particulate enzyme preparation used in the manometric experiments lacked the capacity to reduce hemiglobin to hemoglobin, the irreversible oxidation would rapidly remove all hemoglobin or oxyhemoglobin from the experimental system. In this regard, hemoglobin and oxyhemoglobin appear to be the major form in normal nodules (2, 36). The possibility remains, therefore, that hemoglobin and oxyhemoglobin may participate in the metabolism of intact nodules.

Bergersen and Wilson (7) claim that extracts prepared from soybean nodules will catalyze the oxidation of hemoglobin to hemiglobin by nitrogen gas. In view of the physiological experiments reported (13) and the experimental evidence of Bergersen and Wilson (7) concerning the role of nodule hemoglobin in the nitrogen fixing process, the possibility has been considered that some form of the nodule "hemoglobins" may

serve as an electron acceptor for the nitrate reductase complex. Bergersen and Wilson (7) also claim that bacteriods from soybean nodules exhibit the capacity to reduce the hemiglobin to hemoglobin. In view of these reports, a series of experiments were conducted to determine whether the particulate nitrate reductase would catalyze the reduction of hemiglobin to hemoglobin. As stated in the section entitled "Results Obtained with Particulate System," these experiments consistently provided negative results. These negative findings suggested a more thorough examination of the spectra presented by Bergersen and Wilson (7). Their results show that a large portion if not all the absorption bands in the difference spectrum resulting from the addition of bacteriods to their hemiglobin preparation may be accounted for by changes in the spectrum associated with the conversion of oxyhemoglobin to hemoglobin. Certainly the absorption minima which were obtained at 540 to 575 mμ are the result of the disappearance of oxyhemoglobin and not hemiglobin. It is concluded that the experimental results obtained in this research are in agreement with those reported by Bergersen and Wilson (7); however, the interpretations differ.

Results presented here (figures 6 and 7) show that the particulate system from soybean nodules possesses the capacity to transfer electrons from succinate to oxyhemoglobin. Keilin and Wang (37) have shown that hemoglobin from soybean nodules has a high affinity for oxygen, and Bergersen and Wilson (7) have demonstrated that

oxygenation of nodule hemoglobin occurs when the surrounding atmosphere contains as little as eight parts per million of oxygen. Bergersen and coworkers (5, 6) have presented evidence that groups of bacteriods in nodules are surrounded by a solution of hemoglobin within host tissue membranes. If this view is correct it would seem logical to conclude that oxygen utilized by bacteriods would first have to come in contact with the nodule hemoglobin solution before reaching the bacteriods.

Spectroscopic observations by Keilin and Smith (36) and Appleby (2) show that normal intact nodules contain a mixture of oxyhemoglobin and hemoglobin and no hemiglobin. Observations by Appleby (2) indicate that the major portion of the pigment is in the hemoglobin form and therefore it would be available for combining with any oxygen that is present. In view of these considerations, and of the demonstrated capacity of the particulate complex to transfer electrons from succinate to oxyhemoglobin, the possibility of the hemoglobin playing a metabolic role in oxygen uptake by whole nodules still remains. In regard to the proposed role of hemoglobin in the nitrogen fixing process as proposed by Bergersen and Wilson (7), one might postulate that the particulate enzyme may play a role in the conversion of oxyhemoglobin to hemoglobin, thus making the pigment available for electron transport to nitrogen. Alternately, the particulate complex may catalyze the deoxygenation of oxyhemoglobin at the expense of electrons from succinate, thus providing increasing anaerobic conditions for the nitrogen

fixing reaction.

In an investigation of the cytochrome components of Rhizobium grown under various conditions, Appleby and Bergersen (3) reported that they were unable to detect cytochrome a in bacteriods isolated from effective soybean nodules. Appreciable concentrations of cytochrome a, however, were found in cells from cultures grown on an artificial medium and in bacteriods isolated from ineffective nodules. From the results obtained in this research and from those of Cheniae and Evans (13), it seems apparent that the activity of the particulate nitrate reductase is highest under the conditions where the cytochrome a of the Rhizobium cells is either absent or too low a concentration to be detected. It seems logical to postulate, therefore, that the nitrate reductase per se is substituting for cytochrome a as a terminal electron transfer protein. Thus, the results presented earlier (11, 12) and those reported here support the formulation of an electron transport scheme for the particulate system as follows:
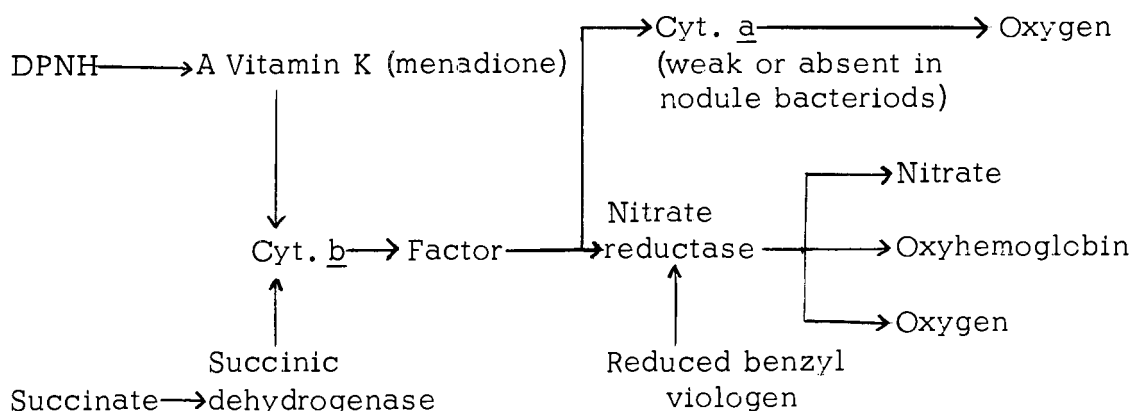
Physiologically the nitrate reductase of nodule bacteriods would then parallel cytochrome a in function.

Significance of the Soluble Nitrate Reductase

A great deal of effort was expended in the development of a procedure for solubilization and purification of nitrate reductase from Rhizobium cells. The particulate nitrate reductase complex in the cells of Rhizobium japonicum was discovered in 1954 (16) and extensive investigations of the properties and physiological role of the system have been conducted since then. Prior to the efforts of the writer, all attempts to obtain a completely soluble enzyme resulted in failure. As previously mentioned, it has been postulated that the nitrate reductase component of the particulate nitrate reductase complex may be non-specific for oxidant and thus possess the capacity of transferring electrons to substances other than nitrate. It was demonstrated that the particulate complex, indeed, possessed the capacity to catalyze the deoxygenation of oxyhemoglobin from nodules and also the capacity to catalyze succinate-dependent oxygen uptake in manometric experiments. It seemed absolutely essential, therefore, to attempt to separate the nitrate reductase component from the remainder of the particulate complex and then determined whether the soluble system would catalyze the reduction of oxygen or the deoxygenation of oxyhemoglobin.

The extreme lability of the soluble nitrate reductase has greatly impeded studies of the properties and function of the enzyme. The highly reduced conditions necessary to retain the activity of the enzyme have made it impossible to determine whether or not it will catalyze the transfer of electrons to oxyhemoglobin. Oxyhemoglobin is chemically deoxygenated in the presence of $Na_2S_2O_4$ or appreciable concentrations of other reducing agents. Also it was not possible to determine whether the soluble nitrate reductase would catalyze the transfer of electrons to molecular oxygen because oxygen resulted in an irreversible inactivation of the enzyme.

The fact that the soluble preparations required $Na_2S_2O_4$ and benzyl viologen for stability has complicated other experimentation. When preparations contained these two components, it was not possible to test other electron donors because the reduced benzyl viologen served as an excellent donor and thus competed with other donors. It was necessary, therefore, to use preparations containing $Na_2S_2O_4$ but lacking benzyl viologen for the experiments where several possible electron donors were tested (table 6). Since a purified preparation could not be obtained without the use of the benzyl viologen, it was necessary to use the soluble extract without purification in these studies. In other experiments the presence of $Na_2S_2O_4$ and benzyl viologen also interfered with the design of experiments. For example, it was desired to preincubate the enzyme and inhibitors in absence of

the electron donor and then add the donor to determine the degree of inhibition. The omission of another component of the reaction during preincubation with inhibitors would have resulted in an experimental procedure that was not comparable with that published (11, p. 60-67, 12, p. 145-148) in previous studies on the particulate nitrate reductase preparation.

Obviously, it is necessary to consider whether the soluble nitrate reductase is the same enzyme as that associated with the particulate complex. The K_m value for nitrate for the soluble nitrate reductase was calculated to be 7.3×10^{-5} moles per liter. Cheniae and Evans (12, p. 144), using succinate as an electron donor for the particulate complex, reported that the K_m for nitrate was 7.8×10^{-5} M. In view of the fact that the preparation used by Cheniae and Evans was particulate in nature and the one used in this investigation was relatively crude, the two values are in close agreement. The pH optimum for the soluble enzyme was shown to be between 6.7 and 6.8 which is in the same range as that reported (12, p. 143) for the particulate nitrate reductase complex. This evidence is consistent with the conclusion that the soluble nitrate reductase and the nitrate reductase portion of the particulate complex are the same enzyme.

If the soluble nitrate reductase is the terminal electron transfer constituent of the particulate system, the question arises as to what component of the particle normally serves as the physiological electron

donor. The evidence presented here does not provide sufficient basis for any conclusions in this respect. It seems feasible, however, to draw an analogy with somewhat similar systems in other organisms. Results of recent experiments with the nitrate reductases of E. coli (31) and P. aeruginosa (20) provide good evidence that cytochromes are the physiological electron donors for these systems. It seems reasonable to postulate, therefore, that a specific native cytochrome may be the physiological electron donor for the soluble nitrate reductase from R. japonicum. If this postulation proves to be correct, the results obtained by Cheniae and Evans (12, p. 148-152) suggest that a cytochrome of the b type is involved.

The soluble nitrate reductase exhibits a low sensitivity to the sulfhydryl binding agent, PCMB. When benzyl viologen was used as an electron donor for nitrate reduction by the particulate system (11, p. 58), no inhibition by PCMB was observed. Perhaps the particle provides steric protection for the sensitive sulfhydryl site or sites and these are exposed by the procedure used for solubilization.

The pattern of inhibition by metal chelating agents is markedly different for the soluble and particulate nitrate reductase even though benzyl viologen was used as the electron donor in both cases. In general the particulate system exhibited a greater specificity toward metal chelating agents than the soluble system (11, p. 60-63). Benzyl viologen and hydrosulfite may in some way partially protect the soluble

enzyme from the complexing agent. Another possibility that may account

for the differential inhibition of the two forms of nitrate reductase is

that the sensitive component in the particulate system is some inter-

mediate between benzyl viologen and the nitrate reductase. If this

postulation is correct, the benzyl viologen would not transfer electrons

directly to the nitrate reductase but would donate electrons at some

other point in the electron transport pathway.

VII. SUMMARY

An investigation has been conducted to further characterize a nitrate reductase obtained from bacteriods of soybean nodules or from pure cultures of <u>Rhizobium japonicum</u>. This investigation was initiated because the activity of the particulate nitrate reductase from nodules has been found to be closely correlated with the capacity of leguminous plants to fix atmospheric nitrogen. The enzyme, therefore, is not necessarily associated with nitrate metabolism. Previously it has been postulated that the particulate nitrate reductase may be non-specific for oxidant and that some unidentified substance with the appropriate oxidation-reduction potential may serve as the natural oxidant under physiological conditions.

The investigation has been divided into two separate phases. The first is an investigation of the possible physiological electron acceptors for the particulate system. The second involves the development of a method for solubilizing the nitrate reductase for the purpose of studying the properties of the nitrate reductase <u>per se</u>. The following are the major findings with the particulate system.

1. The particulate system not only will catalyze the reduction of nitrate to nitrite by use of DPNH or succinate as electron donors, but also will catalyze the transfer of electrons from succinate to oxygen.

2. Oxygen uptake by the particulate system is inhibited by nitrate suggesting that both oxygen and nitrate compete for electrons from a common source.

3. The particulate complex possesses the capacity to transfer electrons from succinate to a preparation of oxyhemoglobin from soybean nodules.

4. Efforts to demonstrate a succinate-dependent reduction of hemi-globin to hemoglobin by the particulate complex were negative.

The major findings in regard to the soluble nitrate reductase are summarized below.

1. A method was devised for the preparation of a completely soluble nitrate reductase extract of Rhizobium cells derived from either pure cultures of the organism or from nodule bac-teriods.

2. The soluble nitrate reductase is extremely susceptible to oxi-dation and therefore previous investigators who considered the enzyme to be insoluble have lost soluble enzyme during prepar-atory procedures.

3. An assay was developed using hydrosulfite to maintain reduced conditions and benzyl viologen as the electron donor. This procedure eliminates the necessity of using the more tedious anaerobic assay in Thunberg tubes.

4. DPNH and succinate, which were effective electron donors in the particulate system, were completely inactive with the soluble preparation. Reduced benzyl viologen or reduced methyl viologen were the only compounds found to be active with the soluble nitrate reductase.

5. The pH optimum for the soluble enzyme ranged between 6.7 and 6.8 when phosphate buffer was utilized in the assay system.

6. The Michaelis constant (K_m) for nitrate was calculated to be 7.3×10^{-5} moles per liter. This value may be compared with a value of 7.8×10^{-5} moles of nitrate per liter which has been reported for the particulate nitrate reductase from soybean nodule bacteriods.

7. Para-chloromercuribenzoate at a concentration of 10^{-4} M resulted in 65 per cent inhibition of the enzyme. This inhibition was partially reversed by either glutathione or cysteine.

8. The soluble nitrate reductase also was inhibited by metal chelating agents as follows: 0.02 M sodium diethyl dithio-carbamate, 71 per cent; 0.002 M thiourea, 46 per cent; 0.005 M potassium cyanide, 28 per cent; and 0.04 M EDTA, 22 per cent. A series of other metal complexing agents were not particularly effective inhibitors of the enzyme.

BIBLIOGRAPHY

1. Anacker, Walter F. and Volkmar Stoy. Proteinchromatographie an Calciumphosphat. I. Reinigung von Nitrat-Reductase aus Weizen-blattern. Biochemische Zeitschrift 141-159. 1958.

2. Appleby, C. A. The oxygen equilibrium of leghemoglobin. Biochimica et Biophysica Acta 60:226-235. 1961.

3. Appleby, C. A. and F. J. Bergersen. Cytochromes of Rhizobium. Nature 182:1174. 1958.

4. Bastarrachea, Fernando and Dexter S. Goldman. Diphosphopyridine nucleotide-linked nitrate reductase from Mycobacterium tuberculosis. Biochimica et Biophysica Acta 50:174-176. 1961.

5. Bergersen, F. J. Biochemical pathways in legume root nodule nitrogen fixation. Bacteriological Reviews 24:246-249. 1960.

6. Bergersen, F. J. and M. J. Briggs. Studies on the bacterial component of soybean root nodules: Cytology and organization in the host tissue. Journal of General Microbiology 19:482-490. 1958.

7. Bergersen, F. J. and P. W. Wilson. Spectrophotometric studies of the effects of nitrogen on soybean nodule extracts. Proceedings of the National Academy of Science, U. S. 45:1641-1646. 1959.

8. Burström, Hans. Photosynthesis and assimilation of nitrate by wheat leaves. Uppsala Lantbrukshogskal Annaler 11:1-50. 1943.

9. Bolle-Jones, E. W. and V. R. Mallikarjuneswara. Cobalt: effects on the growth and composition of Hevea. Journal of Rubber Research Institute, Malaya 15:128-140. 1957.

10. Candela, M. I., E. G. Fischer and E. J. Hewitt. Molybdenum as a plant nutrient. X. Some factors affecting the activity of nitrate reductase in cauliflower plants grown with different nitrogen sources and molybdenum levels in sand culture. Plant Physiology 32:280-288. 1957.

11. Cheniae, George Maurice. The physiological role and mechanism of action of nitrate reductase from Rhizobium japonicum. Ph.D. thesis. Raleigh, North Carolina State College, 1959. 106 numb. leaves.

12. Cheniae, George and Harold J. Evans. Properties of a particulate nitrate reductase from the nodules of the soybean plant. Biochimica et Biophysica Acta 35:140-153. 1959.

13. Cheniae, George and Harold J. Evans. Physiological studies on nodule-nitrate reductase. Plant Physiology 35:454-462. 1960.

14. Delwiche, C. C., C. M. Johnson and H. M. Reisenauer. Influence of cobalt on nitrogen fixation by Medicago. Plant Physiology 36:73-78. 1961.

15. Dixon, Malcolm and Edwin C. Webb. Enzymes. New York, Academic Press. 1958. 782 p.

16. Evans, Harold J. Diphosphopyridine nucleotide-nitrate reductase from soybean nodules. Plant Physiology 29:298-301. 1954.

17. Evans, H. J. and N. S. Hall. Association of molybdenum with nitrate reductase from soybean leaves. Science 122:922-923. 1955.

18. Evans, Harold J. and Alvin Nason. The effect of reduced triphosphopyridine nucleotide on nitrate reduction by purified nitrate reductase. Archives of Biochemistry and Biophysics 39:234-235. 1952.

19. Evans, Harold J. and Alvin Nason. Pyridine nucleotide-nitrate reductase from extracts of higher plants. Plant Physiology 28:233-254. 1953.

20. Fewson, C. A. and D. J. D. Nicholas. Nitrate reductase from Pseudomonas aeruginosa. Biochimica et Biophysica Acta 49:335-349. 1961.

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

22. Fewson, C. A. and D. J D. Nicholas. Respiratory enzymes in Micrococcus denitrificans. Biochimica et Biophysica Acta 48:208-210. 1961.

23. Green, David Ezra, Leonard Hurbert Strickland and Hugh Lewis Aubrey Tarr. CCXL. Studies on reversible dehydrogenase systems. III. Carrier-linked reactions between isolated dehydrogenases. The Biochemical Journal 28:1812-1824. 1934.

24. Hageman, R. H. and Donna Flesher. Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. Plant Physiology 35:700-708. 1960.

25. Hamilton, P. B., A. L. Shug and P. W. Wilson. Spectroscopic examination of hydrogenase and nitrogenase in soybean nodules and _Azotobacter_. Proceedings of the National Academy of Science, U. S. 43:297-304. 1957.

26. Heredia, C. F. and A. Medina. Nitrate reductase and related enzymes in _Escherichia coli_. The Biochemical Journal 77:24-30. 1960.

27. Hewitt, D J. and M. M. R. K. Afridi. Adaptive synthesis of nitrate reductase in higher plants. Nature 183:57-58. 1959.

28. Hori, Katsuji. Electron transporting components participating in nitrate and oxygen respirations from a halotolerant _Micrococcus_. I. Purification and properties of cytochromes b_4 (I) and b_4 (II). Journal of Biochemistry (Tokyo) 50:440-449. 1961.

29. Iida, Chuzo and Kazuo Yamasaki. Spectrographic determination of molybdenum in the nitrate reductase from _Escherichia coli_. Biochimica et Biophysica Acta 44:352-353. 1960.

30. Iida, Katsuhira and Shigehiko Taniguchi. Studies on nitrate reductase system of _Escherichia coli_. I. Particulate electron transport system to nitrate and its solubilization. Journal of Biochemistry (Tokyo) 46:1041-1055. 1959.

31. Itagaki, Eiji, Takeshi Fujita and Ryo Sato. Cytochrome b_1-nitrate reductase interaction in solubilized system from _Escherichia coli_. Biochimica et Biophysica Acta 51:390-392. 1961.

32. Itagaki, Eiji and Shigehiko Taniguchi. Studies on nitrate reductase system of _Escherichia coli_. II. Soluble nitrate reductase system of aerobically grown cells in a synthetic medium. Journal of Biochemistry (Tokyo) 45:1419-1436. 1959.

33. Johnson, Marvin. Isolation and properties of a pure yeast polypeptidase. Journal of Biological Chemistry 137:575-592. 1941.

34. Kastle, J. H. and Elias Elvove. On the reduction of nitrates by certain plant extracts and metals, and the accelerating effect of certain substances on the progress of the reduction. American Chemical Journal 31:606-641. 1904.

35. Keilin, D. and E. F. Hartree. Purification of horse-radish perioxidase and comparison of its properties with those of catalase and methaemoglobin. The Biochemical Journal 49:88-104. 1951.

36. Keilin, D. and J. D. Smith. Haemoglobin and nitrogen fixation in the root nodules of leguminous plants. Nature 159:692-694. 1947.

37. Keilin, D. and Y. L. Wang. Haemoglobin in the root nodules of leguminous plants. Nature 155:227-229. 1945.

38. Kinsky, Stephen C. and W. D. McElroy. Neurospora nitrate reductase: The role of phosphate, flavin and cytochrome c reductase. Archives of Biochemistry and Biophysics 73:466-483. 1958.

39. Kogut, M. and J. W. Lightbrown. Selective inhibition by 2-heptyl-4-hydroxyquinoline-N-oxide of some peroxidative reactions catalyzed by certain haemoproteins and by a flavoprotein. The Biochemical Journal 76:8p-9p. 1960.

40. Kubo, Hideo. Uber das Hamoprotein aus den Wurzelknollchen von Leguminosen. Acta Phytochimica 11:195-200. 1939.

41. Layne, Ennis. Spectrophotometric and turbidimetric methods for measuring proteins. In: Sidney P. Colowick and Nathan O. Kaplan's Methods in Enzymology Vol. III, New York, Academic Press, 1956. p. 447-454.

42. Lenhoff, Howard M., D. J. D. Nicholas and Nathan O. Kaplan. Effects of oxygen, iron, and molybdenum on routes of electron transfer in Pseudomonas fluorescens. Journal of Biological Chemistry 220:983-995. 1956.

43. Lowe, R. H. and Harold J. Evans. Cobalt requirement for the growth of rhizobia. Journal of Bacteriology 83:210-211. 1962.

44. Lowe, Richie H., Harold J Evans and Shaukat Ahmed. The effect of cobalt on the growth of Rhizobium japonicum. Biochemical and Biophysical Research Communications 3:675-678. 1960.

45. McElroy, W. D. Comment. In: A Symposium on Inorganic Nitrogen Metabolism, Ed. by William D. McElroy and Bently Glass, 1956, Baltimore, Johns Hopkins Press. p. 572.

46. Medina, Antonia and Claudia F. de Heredia. Vitamin K-dependent nitrate reductase in Escherichia coli. Biochimica et Biophysica Acta 28:452-453. 1958.

47. Mortenson, L. E., R. C. Valentine and J. E. Carnahan. An electron transport factor from Clostridium pasteurianum. Biochemical and Biophysical Research Communications 7:448-452. 1962.

48. Nason, Alvin. Symposium on inorganic nitrogen compounds. II. Enzymatic pathways of nitrate, nitrite, and hydroxylamine metabolisms. Bacteriological Reviews 26:16-41. 1962.

49. Nason, Alvin and Harold J. Evans. Triphosphopyridine nucleotide-nitrate reductase in Neurospora. Journal of Biological Chemistry 202:655-673. 1953.

50. Nicholas, D. J D. Minor mineral nutrients. Annual Review of Plant Physiology 12:63-90. 1961.

51. Nicholas, D. J. D., M. Kobayashi and P. W. Wilson. Cobalt requirement for inorganic nitrogen metabolism in microorganisms. Proceedings of the National Academy of Science, U. S. 48:1537-1542. 1962.

52. Nicholas, D. J D., Yoshiharu Maruyama and D. J. Fisher. The effect of cobalt deficiency on the utilization of nitrate nitrogen in Rhizobium. Biochimica et Biophysica Acta 56:623-626. 1962.

53. Nicholas, D. J. D. and Alvin Nason. Molybdenum and nitrate reductase II. Molybdenum as a constituent of nitrate reductase. Journal of Biological Chemistry 207:353-360. 1954.

54. Nicholas, D. J. D. and Alvin Nason. Mechanism of action of nitrate reductase from Neurospora. Journal of Biological Chemistry 211:183-197. 1954.

55. Nicholas, D. J. D. and Alvin Nason. Role of molybdenum as a constituent of nitrate reductase from soybean leaves. Plant Physiology 30:135-138. 1955.

56. Nicholas, D. J D. and Alvin Nason. Diphosphopyridine nucleotide-nitrate reductase from Escherichia coli. Journal of Bacteriology 69:580-583. 1955.

57. Nicholas, D. J Donald, Alvin Nason and William D. McElroy. Effect of molybdenum deficiency on nitrate reductase in cell-free extracts of <u>Neurospora</u> and <u>Aspergillus</u>. Nature 172:34. 1953.

58. Nicholas, D. J. D., Alvin Nason and William D. McElroy. Molybdenum and nitrate reductase. I. Effect of molybdenum on the <u>Neurospora</u> enzyme. Journal of Biological Chemistry 207:341-351. 1954.

59. Nicholas, D. J. D. and J. H. Scawin. A phosphate requirement for nitrate reductase from <u>Neurospora crassa</u>. Nature 178:1474-1475. 1956.

60. Nicholas, D. J. D. and H. M. Stevens. Valency changes of molybdenum during the enzymatic reduction of nitrate in <u>Neurospora</u>. Nature 176:1066-1067. 1955.

61. Nightingale, Gordon T. The nitrogen nutrition of green plants. Botanical Review 3:85-174. 1937.

62. Nightingale, Gordon T. The nitrogen nutrition of green plants. II. Botanical Review 14:185-221. 1948.

63. Omachi, Kazuchiyo, Shigehiko Taniguchi and Fujio Egami. The soluble and cytochrome-lacking nitrate-reducing system in germinating cotyledons of bean seed embryos, <u>Vigna</u> <u>sesquipedalis</u>. Journal of Biochemistry (Tokyo) 46:911-915. 1959.

64. Pichinoty, F. and J. C. Senez. Extraction et propriétés de la nitrate-réductase d'<u>Aerobacter</u> <u>aerogenes</u>. Biochimica et Biophysica Acta 35:537-539. 1959.

65. Sadana, J. C. and W. D. McElroy. Nitrate reductase from <u>Achromobacter</u> <u>fischeri</u>. Purification and properties: Function of flavins and cytochrome. Archives Biochemistry and Biophysics 67:16-34. 1957.

66. Sato, Ryo. The cytochrome system and microbial reduction of nitrate. In: A Symposium on Inorganic Nitrogen Metabolism, ed. by William D. McElroy and Bently Glass. Baltimore, Johns Hopkins Press, 1956. p. 163-175.

67. Sato, Ryo and Fujio Egami. Studies on nitrate reductase. III. Bulletin of the Chemical Society of Japan 22:137-143. 1949.

68. Sato, Ryo and Makato Niwa. Studies on nitrate reductase. VII. Reinvestigation on the identity of the enzyme with cytochrome b. Bulletin of the Chemical Society of Japan 25:202-210. 1949.

69. Shaukat-Ahmed and Harold J. Evans. The essentiality of cobalt for soybean plants grown under symbiotic conditions. Proceedings of the National Academy of Science, U. S. 47:24-36. 1961.

70. Shaukat Ahmed and Harold J. Evans. Cobalt: a micronutrient element for the growth of soybean plants under symbiotic conditions. Soil Science 90:205-210. 1960.

71. Silver, Warren. Pyridine nucleotide-nitrate reductase from Hansenula anomala, a nitrate reducing yeast. Journal of Bacteriology 73:241-246. 1957.

72. Smith, J. D. Haemoglobin and the oxygen uptake of leguminous root nodules. The Biochemical Journal 44:591-598. 1948.

73. Spencer, D. A DPNH-specific nitrate reductase from germinating wheat. Australian Journal of Biological Science 12:181-191. 1959.

74. Sternberg, H. and Artturi I. Virtanen. Studies on the absorption spectrum of leghemoglobin, especially of leghemiglobin. Acta Chemica Scandinavica 6:1342-1352. 1952.

75. Stoy, V. Riboflavin-catalyzed enzymic photoreduction of nitrate. Biochimica et Biophysica Acta 21:395-396. 1956.

76. Street, H. E. Nitrogen metabolism of higher plants. Advances in Enzymology 9:391-454. 1949.

77. Taniguchi, Shigehiko and Eiji Itagaki. Solubilization and purification of particulate nitrate reductase of anaerobically grown Escherichia coli. Biochimica et Biophysica Acta 31:294-295. 1959.

78. Taniguchi, Shigehiko and Eiji Itagaki. Nitrate reductase of nitrate respiration type from E. coli I. Solubilization and purification from the particulate system with molecular characterization as a metalloprotein. Biochimica et Biophysica Acta 44:263-279. 1960.

79. Taniguchi, Shigehiko and Kazuchiyo Ohmachi. Particulate nitrate reductase of Azotobacter vinelandii. Journal of Biochemistry (Tokyo) 48:50-62. 1960.

80. Taniguchi, Shigehiko, Ryo Sato and Fujio Egami. The enzymatic mechanisms of nitrate metabolism in bacteria. In: A Symposium on Inorganic Nitrogen Metabolism, ed. by William D. McElroy and Bently Glass. Baltimore, Johns Hopkins Press, 1956. p. 87-108.

81. Tang, Pei-sung and Hsiang-yü Wu. Adaptive formation of nitrate reductase in rice seedlings. Nature 179:1355-1356. 1957.

82. Vaidyanathan, C. S. and H. E. Street. Nitrate reductase by aqueous extracts of excised tomato roots. Nature 184:531-533. 1959.

83. Virtanen, A. I., J. Erkama and H. Linkola. On the relation between nitrogen fixation and leghaemoglobin content of leguminous root nodules. II. Acta Chemica Scandinavica 1:861-870. 1947.

84. Virtanen, Artturi I. and Niilo Rautanen. Nitrogen assimilation. In: James B. Summer and Karl Myrbäck's The Enzymes, New York, Academic Press, 1952. p. 1089-1130.

85. Wainwright, S. D. Menadione derivatives and ferrous iron as cofactors of the nitrate reductase system of a coliform organism. Biochimica et Biophysica Acta 18:583-585. 1955.

APPENDIX

APPENDIX I

Effect of Cobalt and Other Elements on the Growth of <u>Rhizobium</u> <u>japonicum</u>*

Addition to basal medium		Growth***	
Cobalt (ppb)	Twelve other elements (ppb each)**	Turbidity (O.D.)	Nitrogen content (mg/flask)
--	--	0.32	0.16
--	0.1	0.35	0.14
0.1	0.1	1.33	1.08
0.1	--	1.35	1.05
0.0003	--	0.85	0.65
0.001	--	1.20	0.72
0.003	--	1.25	1.16
0.01	--	1.35	1.16
0.03	--	1.45	0.91
1	--	1.40	0.92
10	--	1.40	0.94
100	--	0.53	0.25
1000	--	0.35	0.15
10000	--	0.00	0.01

*The components of the basal nutrient medium and methods for their purification have been described in previous publications (43, 70). The R. japonicum strain described in the "Materials and Methods" section was used for this investigation. After growing the inoculum through

three cultures of the purified medium lacking Co, 0.1 ml of the deficient culture was transferred to a series of 50 ml flasks, each containing 20 ml of the purified medium with the treatments indicated. The cultures were incubated at 30°C on a reciprocating shaker for 6 days. Each culture was washed in 30 ml of H_2O by centrifugation and resuspended in 5 ml of H_2O. The nitrogen content of a 1 ml portion was determined by a micro-Kjeldahl procedure (33).

**To determine the specificity of the cobalt requirement, twelve elements which were considered likely to be removed from the medium by the purification procedures were included in a series of cultures. These elements were Ni, V, Ga, Al, Ag, U, Pd, Cr, Ru, Sn, Bi, and W.

***Values reported for cultures containing no cobalt (both with and without the other element) and the cultures containing 0.1 ppb cobalt and the other elements are means of triplicate determinations. The values reported for cultures containing 0.1 ppb cobalt without the other elements are means of duplicate determinations. The other cultures were not replicated.