DIPHOSPHOPYRIDINE NUCLEOTIDE-NITRATE REDUCTASE

in Beta vulgaris L.

by

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ABSTRACT

A soluble DPNH-nitrate reductase (NRase) of the sugar

beet has been purified and characterized. The occurrence of NO_2^-

as an end product and the insensitivity of the enzyme to oxygen

indicate that the sugar beet NRase is of the nitrate assimilation

type.

The enzyme was not associated with any cell particle and

all NRase activity present in the homogenate of sugar beet leaves

was recovered in the 20,000 x g supernatant.

A sixtyfold purification was accomplished by ammonium

sulfate precipitation followed by adsorption on calcium phosphate

gel. At room temperatures and higher the enzyme was heat labile,

but was relatively stable at $-15^{o}C$. Dialysis at 4° C. did not

result in an appreciable loss of activity. The optimum pH was 7.0.

The NRase was sensitive to heavy metal inhibitors but it

was not possible to show that Mo was the specific prosthetic metal.

It was demonstrated, however, that chemically reduced Mo could

serve as an electron donor. Thus Mo may be a cofactor for the

enzyme. The reversal of p-chloromercuribenzoate inhibition by the

sulfhydryl reagents glutathione and cysteine, coupled with strong

inhibition by iodoacetate and cupric sulfate indicated the sulf-

hydryl nature of the enzyme.

The partially purified NRase was stimulated to a con-

siderably greater degree by FAD than by FMN. Rf values and co-

chromatography in different solvents showed that a substance

liberated from the enzyme preparation by acid and heat was not

riboflavin or FMN but very probably was FAD. It is suggested that, in common with other assimilatory NRases of higher plants, the flavin nucleotide prosthetic group of sugar beet NRase is FAD.

The presence of two NRases in the sugar beet was indicated by the fact that the crude "enzyme" was stimulated to the same extent by the addition of DPNH or TPNH, that the ratio of activities resulting from the addition of the two pyridine nucleotides changed with the degree of purity of the enzyme, and that the enzyme finally obtained by calcium phosphate gel adsorption and elution was DPNH-specific. That purification was not complete was shown by the presence of DPNH-quinone reductase and DPNH-cytochrome c reductase activity in the NRase preparation.

A low NRase activity and a high nitrate content were measured in sugar beet leaves during growth in darkness. The reverse occurred in light. It is suggested that the diurnal variation in NRase activity may be the result of the fall of leaf tissue pH during darkness and its rise to approximate the enzyme's optimum pH in light.

The possible participation of the NRase in a flavin nucleotide-catalyzed enzymatic photoreduction of nitrate was indicated by the coupling of photoreduction of FAD with the reduction of nitrate by NRase.

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I. DISCOVERY OF NITRATE REDUCTASE

By the processes of ammonification and nitrification the nitrogen of soil organic matter is converted to ammonia and finally to nitrate. Nitrate is the main source of nitrogen utilized by higher plants. The nitrogen of nitrate, with an oxidation number of +5, is in a highly oxidized state, but the nitrogen of the amino group and ammonia with an oxidation number of -3, is in a highly reduced state. The reduction of nitrogen is thus the first step in the synthesis of amino acids and other organic nitrogenous compounds when nitrate is the source of nitrogen. Although other nitrogen sources such as ammonia, nitrite and amino acids can be utilized by higher plants, nitrate usually supports better growth than these sources.

One of the earliest investigations, reported in 1903 by Pozjii Escot, demonstrated that the aqueous extracts of the stem of the burdock had the power to reduce nitrates to nitrites and ammonia. Kastle and Elvove (1904) also found that the reduction took place most rapidly at 40° to 50°C and that aldehyde was one of the most effective of various "accelerators" of the action.

Irving and Hankinson (1908) claimed to have found a nitrate reducing enzyme in Elodea, Potamogeton, and numerous members of Gramineae. They considered that the nitrate reduction required only the enzyme and a suitable carbohydrate.

Eckerson (1924) for the first time suggested that higher

plants contain an enzyme "reducase" responsible for the reduction of nitrate. The "reducase" activity was calculated from the rate of production of nitrite from nitrate. At the same time Anderson (1924) detected nitrite in the shoots of 25 of the 105 species examined. The nitrate-reducing substance, present in the foliar part of such plants as sunflower, artichoke, pea, radish, tomato, clover and wheat, was found to be thermolabile and oxidizable.

Wu and Loo reported in 1950 that an enzyme "reductase" had been detected in the roots and leaves of 11 higher plants, but not in the stem. This enzyme had an optimum pH of 5.2 and an optimum temperature of $52^{\circ}C$. The reduction of nitrate to nitrite in vitro was a first order reaction. Reductase from soybean sprouts appeared to use lactate as an electron donor.

Delwiche (1952) found that cell-free extracts of roots and leaves of various plants could convert N^{15}-labeled nitrate and nitrite to ammonia. Optimum activity was obtained with a dialyzed preparation. Citrate or other Kreb's intermediates were able to stimulate the reaction. The system was inhibited by Cu^{++}, $AsO_4^{\doteq}$, and iodoacetamide.

Nason and Evans (1953) and Evans and Nason (1953) successfully purified nitrate reductase (NRase) from Neurospora and soybean leaves, respectively. It proved to be a metalloflavoprotein containing FAD, molybdenum and active sulfhydryl groups. $DPNH_2$ or $TPNH_2$ served as hydrogen donor.

NRase was later shown to occur in many plants:

Higher Plants

Rice	Tang and Wu (1957)
Cauliflower	Candela, Fisher and Hewitt (1957)
Wheat leaf	Anacker and Stoy (1958)
Wheat embryo	Rijven (1958); Spender (1959)
Tomato roots	Vardyanathan and Street (1959)
Corn seedling	Hageman and Flesher (1960)
Tobacco	Fukuzumi and Tamaki (1962)
Vegetable marrow	Cresswell, Hageman and Hewitt (1962)
Tomato leaf	Sanderson and Cocking (1964)

Green Algae

Ankistrodesmus	Czyan (1963)
Anabaena	Hattori (1962)
Chlorella	Syrett and Morris (1963)
	Shafter, Baker and Thompson (1961)

Fungi

Neurospora	Nicholas and Scawin (1956)
	Kinsky and McElroy (1958)
Hansenula	Silver (1956)

Bacteria

Achromobacter fischeri	Sadana and McElroy (1957)

Azotobacter
  vinlandii   Taniguichi and Ohmachi (1960)

                     Becking (1962)

Escherichia
  coli   Nicholas and Nason (1955b)

                     Taniguchi, Sato and Egami (1956)

                     Farkas-Himsley and Artman (1957)

                     Medina and Heredia (1958)

                     Taniguchi and Itagaki (1959)

Rhizobium
  japonica   Evans (1954); Cheniae and Evans (1955,

                     1956, 1957, 1959, 1960); Lowe and

                     Evans (1964)

Rhodospirillum
  rubrun   Kato (1963)

Pseudomonas
  aeruginosa   Fewson and Nicholas (1961)

Pseudomonas
  denitrificans   Vernon (1956)

II. CLASSIFICATION OF NITRATE REDUCTASE

From the literature it is apparent that a variety of types

of nitrate reduction occurs in the organisms which have been studied.

The dissimilarity of electron transport sequences and of the end

product of reduction indicate that the NRase which catalyzes nitrate

reduction occurs in several different forms.

Several classifications have been proposed for the nitrate

reduction types. Verhoeven (1956) suggested three types of nitrate

reduction for microorganism: assimilation, incidental dissimilation

and true dissimilation. Sato (1956) classified nitrate reducing micro-

organisms on the basis of the behaviour of their cytochromes toward

nitrate. Egami et al.(1957) described three types of nitrate

reduction in germinating seedlings of Viginia sesquipedalis, e.g.

nitrate assimilation, nitrate respiration and nitrate fermentation.

The classification of nitrate reduction by Nason (1962) is

widely used at the present. He divided nitrate reduction into two

classes: 1) nitrate assimilation, or assimilatory nitrate reduction,

in which nitrate is reduced to ammonia or the amino level with the

products being used for the biosynthesis of nitrogen-containing cell

constituents, for example, proteins and nucleic acids; 2) nitrate

respiration, or dissimilatory nitrate reduction, in which nitrate is

used as the terminal electron acceptor in place of oxygen, usually

under anaerobic conditions. Nason's two types of NRase are dis-

cussed briefly.

1. Assimilatory NRase

Assimilatory NRase catalyzes the first step in the sequence of reduction by which nitrate is converted to the ammonia or amino acid level for the ultimate synthesis of protein, nucleic acids and other nitrogen-containing cell constituents. The enzyme obtained by Evans and Nason (1953) and Nason and Evans (1953) from Neurospora and soybean leaves is the typical assimilatory enzyme.

With purified NRase from Neurospora, Nicholas and Nason (1954) have shown that, during the enzymatic transfer of electrons from TPNH to nitrate, both FAD (or FMN) and molybdenum function as electron carriers. They proposed the following reduction sequence:

$$TPNH \longrightarrow FAD \ (or \ FMN) \longrightarrow Mo \longrightarrow NO_3^-$$
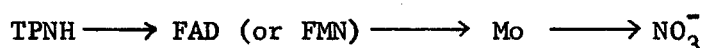
An assimilatory type of pyridine nucleotide-NRase similar to that described for Neurospora and soybean leaves, has been reported to occur in tissues of higher plants e.g. wheat leaves (Anacker and Stoy, 1958), wheat embryo (Spencer, 1959) and tomato leaves (Sanderson and Cocking, 1964), etc.

Nicholas and Nason (1955b) reported the presence of an assimilatory NRase in E. coli. The enzyme was DPNH-linked and required FAD and probably molybdenum as cofactors.

Silver (1957) purified and characterized a pyridine nucleotide-NRase in the extracts of the yeast, Hansenula anomala,

grown on a nitrate-containing medium. The enzyme was similar

to that found in _Neurospora_ and soybean leaves. The possibility

that cytochromes play a direct part in nitrate reduction by _Hansenula_

has been eliminated by direct spectrophotometric observations of

living cells.

Taniguchi and Ohmachi (1960) described an inducible

pyridine nucleotide NRase of sulfhydryl CO-insensitive metalloenzyme

nature in the large particles from nitrate-grown Azotobacter cells.

The activity was stimulated 1.5 to 2.0-fold by the addition of FAD

or FMN. Except for its particulate nature, the enzyme strongly

resembled that of _Neurospora_ and higher plants and it was apparently

of the assimilatory type. The following electron-transfer sequence

was proposed:

$$\text{Cytochrome} \longrightarrow \text{Cytochrome oxidase} \longrightarrow O_2$$

$$\text{system}$$

$$\uparrow$$

$$\text{DPNH} \longrightarrow \underset{\text{(or FMN)}}{\text{FAD}} \searrow \underset{\underset{\text{metal)}}{\text{(CO-sensitive heavy}}}{\text{Nitrate reductase}} \nearrow NO_3^-$$

$$\uparrow$$

$$\text{Reduced nile blue}$$
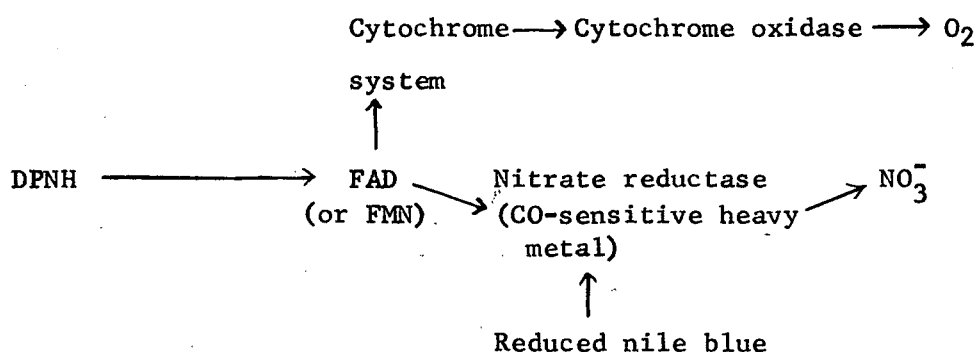
2. Respiratory NRase

According to Nason's classification, respiratory

NRase catalyzes the first step in nitrate respiration by which

nitrate is used as the terminal electron acceptor in place of

oxygen by microorganisms under anaerobic or partially anaerobic

conditions. The end products of nitrate respiration may include

nitrite, nitric oxide, nitrous oxide, molecular or other oxidation

states of nitrogen. Most of the reduction products are not

further utilized and for the most part are excreted into the growth

medium.

The distinguishing characteristics of respiratory NRase

is the participation of cytochrome in its electron transport

sequence. Taniguchi et al. (1956) formulated the following scheme

to visualize electron transport pathways in the particulate prepara-

tion of E. coli.

$$DPNH \longrightarrow FAD \longrightarrow Cytochrome\ b_1 \begin{cases} \longrightarrow Oxidase \longrightarrow O_2 \\ \\ \longrightarrow \underset{reductase}{Nitrate} \longrightarrow NO_3^- \end{cases}$$

formate ·

reduced by
methylene blue
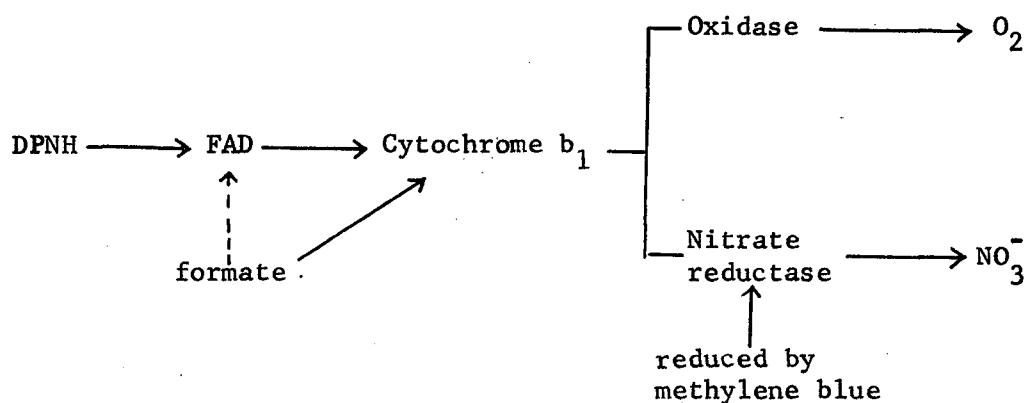
The participation of cytochrome b_1 in the nitrate reducing

mechanism in E. coli was based on the spectroscopic observation

that the addition of nitrate to a cell-free extract of this organism

(in the absence of oxygen) caused the characteristic bands of the

reduced cytochrome to fade, and that this anaerobic oxidation of cytochrome b_1 was prevented by cyanide. They were able to obtain more direct evidence for this with the aid of a specific inhibitor of cytochrome b and b_1, 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO).

When formate was used as the hydrogen donor, the reduction of nitrate was strong inhibited by this HOQNO. In this type of electron transfer system, NRase acts as a terminal enzyme, mediating the direct transfer of electrons to nitrate.

The finding of an unidentified soluble factor necessary to the DPNH- or formate-NRase system led Iida and Taniguchi (1959) to modify the sequence proposed by Taniguchi et al. (1956). This unidentified factor could not be replaced by FAD, menadione or ferrous ion. The marked inhibition of the terminal NRase by several metal-chelating agents suggested the presence of a metal component in the terminal step.
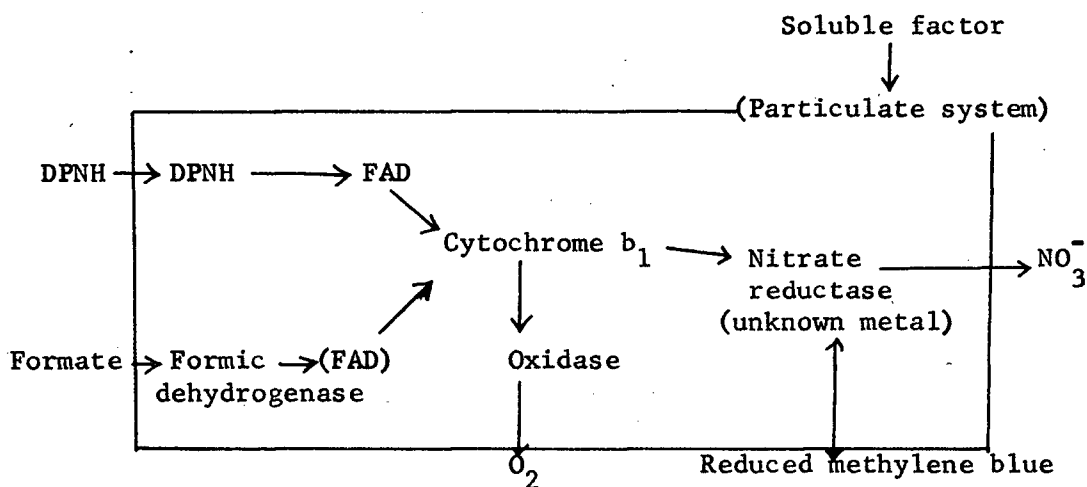
Itagaki and Taniguchi (1959) extracted a soluble

DPNH-NRase from $\underline{E}$. $\underline{coli}$ grown in synthetic medium under aerobic

conditions. In contrast to the particulate preparation this soluble

extract was unable to use formate as an electron donor. It was

sensitive to HOQNO and stimulated by the addition of FAD to the

dialyzed enzyme.

The ferrous ion could act as an electron donor for nitrate

via cytochrome b_1. An unknown heavy metal participation in the

terminal step was proposed because of the inhibtion by cyanide

and azide.

$$\text{DPNH} \xrightarrow{\substack{\text{FAD} \\ \text{or} \\ \text{FMN}}} \text{manadione, } Fe^{2+} \rightarrow \text{Cytochrome } b_1 \xrightarrow[\text{(unknown heavy metal)}]{\text{NRase}} NO_3^-$$
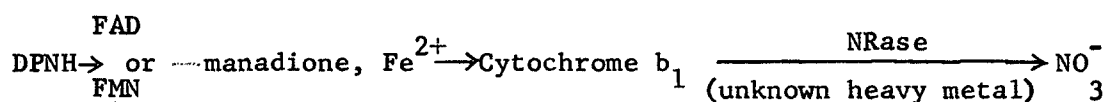
reduced dyes

Medina and Heredia (1960) extracted a particulate $\underline{E}$. $\underline{coli}$

NRase which required DPNH or TPNH as electron donor and vitamin K_3

as electron carrier. They suggested two mechanisms by which

nitrate can be reduced to nitrite.

Vitamin K_3
analogue
Menadione
reductase
DPNH
DPNH
dehydrogenase
FAD → cytochrome b_1 → Oxidase → O_2
→ NRase → NO_3^-
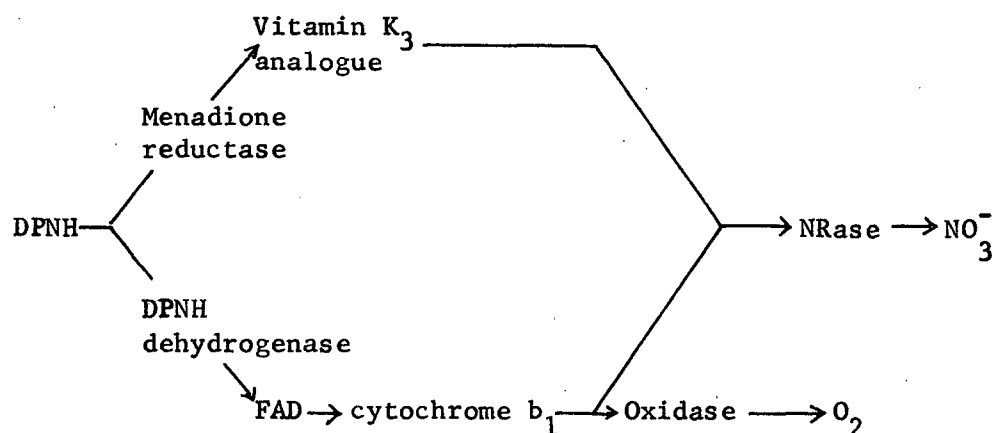
Under aerobic as well as anaerobic conditions, vitamin
K_3 or its analogue can act as the electron carrier without the
participation of flavins. Under anaerobic conditions nitrate
reduction takes place through an electron-transport chain invol-
ving FAD and DPNH-oxidase chain.

Evans (1954) and Cheniae and Evans (1956, 1957, 1959,
1960) reported that a particulate DPNH-NRase had been extracted
from Rhizobium japonium of soybean nodules. Their results indi-
cated that this enzyme catalyzes the reduction of nitrate to
nitrite with either DPNH or succinate as electron donor. When DPNH
is used as a source of electrons for the system, the evidence
indicates involvement of a vitamin K, or a related quinone, a cyto-
chrome, an Antimycin A-sensitive site, two cyanide-sensitive sites,
and NRase. When succinate is used as the electron donor for the
system it is suggested that succinic dehydrogenase, a cytochrome,
an Antimycin A-sensitive site, two cyanide sites, and NRase are
involved in the electron transport to nitrate. Enzyme activity is
probably correlated with the capacity to fix atmospheric nitrogen.
The particulate enzyme exhibited properties similar to those of the
nitrate respiration system from E. coli described by Taniguchi and
Itagaski (1960).

Lowe and Evans (1964) developed a method for the prepara-
tion of a soluble NRase extract of Rhizobium japonium cells. This
soluble enzyme was purified about elevenfold by ammonium sulfate

fractionation and chromatography on a calcium phosphate column.
DPNH and succinate were completely ineffective donors for nitrate
reduction with the soluble preparation. Reduced benzyl viologen
was employed as the electron donor. Judging from the similarity
of their substrate affinity and of pH optimum, the soluble NRase
and the NRase portion of the particulate complex from R. japonicum
cells are the same enzyme.

Sadana and McElroy (1957) extracted a nitrate reducing
system from the salt-water luminous bacterium, Achromobacter
fisheri, and suggested the following electron transport system:

$$DPNH \longrightarrow FAD \longrightarrow Fe^{3+} \longrightarrow Bacterial \longrightarrow O_2$$
$$(TPNH) \quad (FMN) \qquad\qquad cytochrome$$
$$\downarrow$$
$$Reduced\ benzyl\ viologen \longrightarrow Nitrate\ reductase \longrightarrow NO_3^-$$
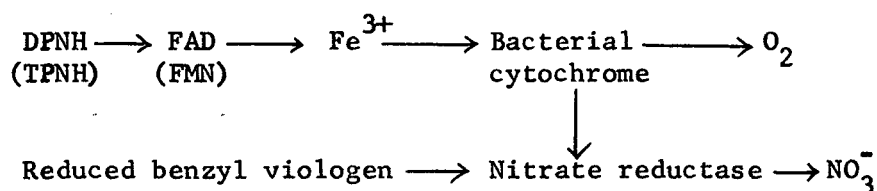
The nitrate-reducing system from this bacterium was
solubilized by osmotic rupture and separated into two soluble
fractions, a DPNH-cytochrome c reductase moiety with a require-
ment for FAD or FMN, and a terminal NRase. The enzyme could
catalyze the reduction of nitrate by using reduced cytochrome or
benzyl viologen. This enzyme is quite similar to the soluble
respiratory NRase from E. coli.

Fewson and Nicholas (1961) purified and characterized

a particulate respiratory NRase from the denitrifying bacterium, Pseudomonas aeruginosa. Cytochrome c and molybdenum were required as functional components. The following sequence of electron transport was suggested:

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

$$\downarrow$$

$$\text{Cytochrome oxidase} \longrightarrow \text{O}_2$$
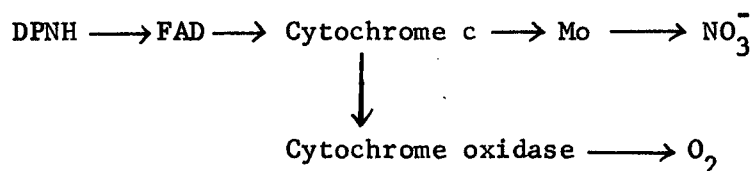
A summary comparison of the properties of assimilatory and respiratory NRase is given in Table I.

Table 1

Comparison of Assimilatory NRase with

Respiratory NRase

Properties	Assimilatory Nitrate Reductase	Respiratory Nitrate Reductase
Original Form	Soluble or particulate	Soluble or particulate
Sensitivity to oxygen	None	Inhibited
Enzyme-bound Components		
Flavin	FAD	FAD
Cytochrome	None	Present (Cytochrome b_1 in E. coli)
Molybdenum	Present	Present (1 atom/molecule in E. coli)
Other Component	Unknown	Iron (40 atoms/molecule in E. coli)
Typical Electron Transfer Sequence	$TPNH \rightarrow FAD \rightarrow Mo \rightarrow NO_3^-$	DPNH $\downarrow$ FAD, (Menadione) (Fe^{2+}) $\downarrow$ cytochrome b_1 $\downarrow$ Mo $\downarrow$ NO_3^-
Km for nitrate	1.4×10^{-3} M (Neurospora)	5.1×10^{-4} M (E. coli)
Physiological function	Nitrate assimilation	Nitrate respiration

III. ASSIMILATORY NITRATE REDUCTASE

As a background for the study of the assimilatory
NRase of the sugar beet, a literature review of the ex-
traction, purification and characterization of NRase of a
variety of other plants is presented in the following section.

1. Methods of Extraction

Nason and Evans (1953) isolated NRase from Neurospora
by the following procedure. After freezing at -15° C,
Neurospora mycelia were ground in a Ten Broeck homogenizer in
three times their weight of 0.1 M K_2HPO_4 buffer, pH 7.0, and
centrifuged at 20,000 x g for 10 minutes at 4° C. About 85%
of the total activity was obtained in the supernatant.

The enzyme from soybean leaves (Evans and Nason, 1953)
was extracted by grinding the leaves in a Waring Blendor in
the presence of phosphate buffer, pH 9.0, and alumina powder.
The mixture was further ground for three minutes in a Ten
Broeck homogenizer at 0 - 4° C, then centrifuged at 20,000
x g for 10 minutes at 4° C. About 10% of the enzyme was lost
by this procedure.

Nicholas and Nason (1955a) omitted the alumina powder
and added cysteine and EDTA, each at a final concentration of
10^{-4} M. This improved the stability and yield of the enzyme
from soybean leaves by protecting it against inactivation by

heavy metals or oxidation of sulfhydryl groups.

Nicholas and Nason (1955b) isolated a soluble assimilatory NRase from $\underline{E}$. $\underline{coli}$. The frozen cells were homogenized in a cold mortar using an equal weight of alumina powder (Alcoa A-301). After grinding for 10 minutes, three times their weight of cold 0.1 M K_2HPO_4, pH 7.0, containing sodium versenate (10^{-4} M final concentration) was slowly added and grinding continued for another 5 minutes. After centrifugation for 15 minutes at 4° C., the supernatant solution contained 75% of the enzyme activity of the homogenate.

Silver (1956) extracted from the yeast $\underline{Hansenula}$ $\underline{anomala}$ a soluble enzyme which was concerned with the assimilation of nitrate. The cells were suspended in sufficient 0.2 M phosphate buffer, pH 7.5, to make a thick cream. A 50-ml aliquote of the cream and 5 g of 200-mesh powdered pyrex were placed in a 9-KC Raythreon sonic oscillator and the cells disrupted for 40 minutes in the cold. Unbroken cells, large debris, and glass were removed by low speed centrifugation and the supernatant was subjected to 107,000 x g in a preparative Spinco centrifuge for 50 minutes. The supernatant contained 95 to 100% of the enzymatically active material of the whole homogenate.

Candela et al. (1957) extracted NRase from cauliflower by grinding fresh tissue for 15 minutes with neutral acid-washed silica sand and three times its weight of extractant at 0^oC in a chilled mortar. The extracting reagent was 0.1 M phosphate buffer, pH 7.0, containing 10^{-4} M cysteine and ethylene diamine tetracetate (EDTA). The homogenate was filtered through muslin and the filtrate was centrifuged at 20,000 x g at 0° C.

Spencer (1959) separated wheat embryos from the endosperm after 48 hours germination on filter paper moistened with 10 mM potassium nitrate, and ground them in a cold mortar at 0 - 2° C with ten times their weight of 0.1 M K_2HPO_4/KH_2PO_4, pH 7.5, containing 10^{-3} M glutathione. The grindate was then centrifuged at 5000 x g for 20 minutes.

Hageman and Flesher (1960) extracted NRase from maize seedlings. The seedlings were cut into small pieces and ground in a Omnimixer at maximum speed for 2 minutes. The grinding medium was 0.1 M Tris-(hydroxymethyl)-amino-methane (Tris), 0.01 M cysteine and 3 x 10^{-4} M EDTA at a pH of 7.3 to 7.8. Four ml of cold (2° C) grinding medium was added for each gram of tissue. The homogenate was pressed through cheesecloth and centrifuged for 15 minutes at 20,000 x g. The supernatant was decanted through glass wool and assayed.

Sanderson and Cocking (1964) ground tomato leaves in a cold mortar containing acid-washed sand. The grinding medium was 0.1 M Tris-HCl buffer containing 10^{-3} M cysteine at pH 7.5. The macerate was pressed through cheesecloth and the filtrate was centrifuged at 1750 x g for 20 minutes.

Table II summarizes the methods of extraction employed by numerous investigators.

Table II

Summary of Methods of Extracting Assimilatory NRase

Source of Enzyme	Extraction Medium	Ml medium/ g tissue	Method of Extraction	Reference
Soybean leaves (1st tri- foliate)	0.1 M K_2HPO_4 (pH 9.0)	3:1	Add 2 wts. alumina, Waring Blendor 2 min. at 0-4° C, Ten Broeck homogen- ation, centrifuged 20,000 x g. 10 min. at 4°C. 90% acti- vity obtained.	Evans and Nason (1953)
Neurospora crassa	0.1 M K_2HPO_4 (pH 7.5)	3:1	Ten Broeck homoge- nation at 0-4°C, centrifuged at 20,000 x g. 85% activity obtained.	Nason and Evans (1953)
Soybean leaves	0.1 M K_2HPO_4 (pH 9.0) 10^{-2} M EDTA + 10^{-4} M cysteine	3:1	As above, for soybean but omit alumina.	Nicholas and Nason (1955a)
Yeast Hansenula anomala	0.2 M phosphate buffer (pH 7.5)		Raytheon sonic Oscillator for 40 min., centrifuged at 107,000 x g. 95-100% activity recovered.	Silver (1956)
Cauli- flower	0.1 M phosphate buffer (pH 7.0) 10^{-4} M EDTA + 10^{-4} cysteine	3:1	Ground in mortar at 0°C with sand. Centrifuged at 20,000 x g.	Candela et. al. (1957)
N. crassa	0.1 M K_2HPO_4 (pH 7.5)		Homogenized in Servall Omnimixer 0-4°C, centrifuged at 10,000 x g. 90% activity obtained.	Kinsky and McElroy (1958)

Table II (cont'd)

Source of Enzyme	Extraction Medium	Ml medium/ g tissue	Method of Extraction	Reference
Wheat embryo	0.1 M phosphate buffer (pH 7.5) 10^{-3} M glutathione	10:1	Ground in chilled mortar, centrifuged at 5000 x g.	Spencer (1959)
Wheat leaves	0.025 M K_2HPO_4 5 x 10^{-4} M EDTA	3:1	90 sec. in Blendor, 3 min. in Potter-Elvehjem Macerator at 4°C	Anacker and Stoy (1958)
Maize leaves	0.1 M Tris buffer (pH 7.3 - 7.8) 3 x 10^{-4} M EDTA and 10^{-2} M cysteine	4:1	Ground in Omnimixer, centrifuged at 30,000 x g.	Hageman and Flesher (1960)
Tomato leaves	0.1 M Tris (pH 7.5) 10^{-3} M EDTA	4:1	Ground in chilled mortar, centrifuged at 1750 x g for 20 min. 80-100% activity recovered.	Sanderson and Cocking (1964)

2. Purification of Enzyme

NRase enzyme purification has been accomplished by
ammonium sulfate precipitation and calcium phosphate gel
adsorption. Column chromatography and electrophoresis have
also been used.

Nason and Evans (1953) purified Neurospora NRase by
ammonium sulfate fractionation and calcium phosphate gel
adsorption. All steps of purification procedure were carried
out at 0-4° C. Centrifugation was performed at approximately
3000 x g. The various percentages of saturation were
obtained by the addition of a saturated ammonium sulfate
solution to the extract. The first fraction, obtained with
0-43% saturation, contained 50% of the initial activity with
a twofold increase in purity. The second ammonium sulfate
fractionation used 24-46% saturation. The activity of second
fraction (24-46% saturation) represented 20% of the units in
the crude starting material and a sevenfold purification.
The second fraction was treated with calcium phosphate gel,
aged 9 months. The gel-protein ratio was about 2:5. The
precipitate was collected by centrifugation and washed twice
with cold 0.1 M pyrophosphate buffer, pH 7.0. The pyrophos-
phate eluate showed about 10% of the total activity of the
original crude extract, and an overall purification of 40 to
50 times. The third ammonium sulfate fractionation involved

0-60% saturation. This final fraction contained 10% of the units in the crude starting material and the purification was 60- to 70-fold.

Purification of NRase from soybean leaves (Evans and Nason, 1953) employed calcium phosphate gel adsorption followed by ammonium sulfate precipitation. Ninety-five ml of calcium phosphate gel (11 mg per ml) was added to 190 ml of crude extract. The enzyme was then eluted from the gel by 0.1 M pyrophosphate buffer at pH 7.0. This fraction contained 40% of the total activity. The purification was 24-fold. After two ammonium sulfate precipitations, the enzyme preparation contained 7% of the total activity and was purified 65-fold.

Charcoal (Darco G 60) has been used by Nicholas and Scawin (1956) to treat Neurospora NRase preparations which had already been fractionated by ammonium sulfate precipitation and calcium phosphate gel adsorption. When the enzyme preparation was treated with "Darco G 60" (30 mg/ml enzyme) and centrifuged at 3000 x g for 15 minutes, the enzymatic activity was present in supernatant. This fraction contained 0.8% of the initial activity and represented an overall purification of 84-fold.

Anacker and Stoy (1958) used a column of calcium phosphate to fractionate wheat leaf NRase. The adsorption column was 35 mm in diameter and contained 5 g of calcium phosphate in 30 ml. The enzyme was eluted from the column by increasing concentrations of K_2HPO_4 buffer, pH 7.0. The most active fraction was obtained by elution with the phosphate buffer at 0.05 M. Rechromatography and gradient elution with sodium pyrophosphate 0.001-0.1 M, pH 7.0, resulted in a 100-fold increase in activity. The enzyme was then precipitated by ammonium sulfate 0-35% saturation, with a further two-fold increase in specific activity. The preparation was not homogeneous in the ultracentrifuge, but the activity was associated with protein having a sedimentation of 19S and a molecular weight of 500,000-600,000.

Kinsky and McElroy (1958) demonstrated that nucleic acid in enzyme preparations from Neurospora could be successfully removed with protamine sulfate. In the subsequent ammonium sulfate precipitation, they obtained a highly purified enzyme, with specific activity about 10,000. They also reported that when this purified preparation of NRase was examined in the Perkin-Elmer electrophoresis apparatus, a minimum of three distinct peaks were discernible.

Spencer (1959) purified wheat embryo NRase with three ammonium sulfate precipitations (0-55, 28-40 and 29-35% saturation). The final fraction showed a sevenfold increase in specific activity with a recovery of 58% of the activity of the crude cell-free extract.

Sanderson and Cocking (1964) passed the crude enzyme preparation from tomato leaves through Sephadex column containing 10^{-3} M cysteine. From 80 to 100% of the enzyme was normally recovered in the 6 ml of eluate following the first 12 ml. The eluate represented twofold purification.

3. Measurements of Activity

Three methods have been used to assay NRase activity: colorimetric, manometric, and spectrophotometric.

Colorimetric method: This method is based on the diazotization of an aromatic amino compound by nitrite in acid solution and coupling with a suitable reagent to give an intensive red-purple color. This method for quantitative determination of nitrite was originally described by Snell and Snell (1949). The procedure is as follows: allow 5 minutes of incubation of reaction mixture; add 0.9 ml of H_2O and 0.5 ml of 1% sulfanilamide in N HCl to stop the reaction; follow by 0.5 ml of 0.02% N-(1-naphthyl)-ethylene-diamine hydrochloride to develop the color. After 10 minutes,

read the density of the color in a colorimeter with a green (540 mu) filter.

Using this method, Evans and Nason (1953) found that there was a small non-enzymatic disappearance of nitrite if TPNH and nitrite were allowed to remain in contact with the acid sulfanilamide reagent for a few minutes before adding the solution of N-(1-naphthyl)-ethylene-diamine hydrochloride.

Medina and Nicolas (1957) reported that DPNH and TPNH reacted with the diazotised product of nitrite and sulphanilamide, in acid solution. It is supposed that DPNH in acid solution reduces diazotised sulfanilamide to a phenylhydrazide derivative which is not able to couple with the naphthyl reagent. This source of interference can be overcome by removal of residual DPNH and TPNH at the end of the reaction. Barium acetate and ethanol were used to precipitate the pyridine nucleotides.

Evans and Nason (1953) found that the NRase present in the reaction mixture also caused the disappearance of nitrite. 10^{-3} M hydroxylamine hydrochloride effectively inhibited the slight nitrite reductase action in soybean extracts but also caused a 12% inhibition of NRase. Silver and McElroy (1954) included cyanide at about 3×10^{-5} M to inhibit the enzyme. Nicholas et al. (1954) also used cyanide of a low concentration (4×10^{-5} M).

A variety of methods employing colorimetry are summarized in Table III.

Table III

Summary of Measurements of Assimilatory NRase Activity

by Colorimetric Method

Plant Material	Reaction Mixture	Time (min.)	Temp. (0°)	Unit	Reference
Soybean leaves	10 uM KNO_3, 55 uM phosphate buffer, pH 7.0, 1.3 muM FAD, 80 muM TPNH, 0.2 ml enzyme extract in 0.5 ml.	10	28	1 muM NO_2^-/5 min.	Evans and Nason (1953)
N. crassa	10 uM KNO_3, 52 uM pyrophosphate buffer, pH 7.0, 0.75 muM FAD, 80 muM TPNH, 0.05 ml enzyme extract in 0.5 ml.	5	23-28	1 muM NO_2^-/5 min.	Nason and Evans (1953)
N. crassa	10 uM KNO_3, 48 uM pyrophosphate buffer, pH 7.0, 50 uM FMN, 80 muM TPNH, 0.05 ml enzyme extract.	10	Room temp.	1 muM NO_2^-/10 min.	Nicholas et al. (1954)
N. crassa	10 uM KNO_2, 23 uM Tris buffer, pH 7.5, 0.02 ml boiled Tris-extract of pig heart, 0.02 ml acetone powder. 100 muM TPNH in 0.5 ml.				Nicholas and Scawin (1956)
Hansenula anomala	10 uM KNO_3, 0.05 ml boiled extract of pig liver, 0.01 - 0.05 ml enzyme extract. 0.2 M phosphate buffer, pH 7.5, in 0.5 ml.	10	Room temp	1 muM NO_2^-/10 min.	Silver (1956)

Table III (cont'd)

Plant Material	Reaction Mixture	Time (min.)	Temp. (0°)	Unit	Reference
N. crassa	10 uM NaNO$_3$, 20-29 uM phosphate buffer, pH 7-7.5, 50 muM FAD 100 uM TPNH, 0.1-1.0 ml enzyme extract in 0.5 ml.	10	Room temp	0.19 muM NO$_2^-$/30 min.	Kinsky and McElroy (1958)
Wheat	10 uM KNO$_3$, 100-250 uM phosphate buffer, pH 7.5, 0.5 muM FAD, 200 muM DPNH, 0.05-0.2 ml enzyme extract water to 0.5 ml.	30	30	1 muM NO$_2^-$/30 min.	Spencer (1959)
Maize	20 uM KNO$_3$, 100 uM phosphate buffer, ph 7.3, 0.68 uM DPNH, 0.2 ml enzyme extract water to 2.0 ml.	15	27	1 muM NO$_2^-$/hr./g fresh wt.	Hageman and Flesher (1960)
Azoto-bacter vin. - landii	30 uM KNO$_3$, 1.5 uM DPNH, 0.2-0.6 ml enzyme extract, 0.05M phosphate buffer to 3 ml in a Thunberg tube, use CuSO$_4$ to reduce interference of DPNH	10 30	30	1 uM NO$_2^-$/hr.	Taniguchi and Ohmachi (1960)
Tomato leaf	10 uM KNO$_3$, 50 uM phosphate buffer, pH 7.3, 270 muM DPNH, 0.05-0.2 ml enzyme extract, water to 0.8 ml	20	27	1 muM NO$_2^-$/20 min.	Sanderson and Cocking (1964)

Manometric method: Robinson (1954) described a method

of manometric determination of NRase activity.   It is based on

the reaction of nitrite with sulfanic acid in sulfuric acid

solution with subsequent liberation of molecular nitrogen in

accordance with the following reaction:

$$NH_2SO_3H \quad + \quad HNO_2 \longrightarrow H_2SO_4 \quad + \quad H_2O \quad + \quad N_2$$
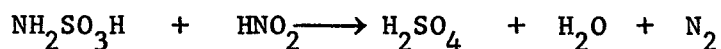
Nitrite was determined manometrically using Warburg

equipment.   The vessels contained the $NaNO_2$ in 2.0 ml 0.05 M

phosphate buffer, pH 7.6, with 0.2 ml of the sulfanic acid

solution in the side arm.   After equilibration at 30° C the two

solutions were mixed and nitrogen evolution was determined.

The same procedure was used for the determination of NRase

activity in the halophilic bacterium, Vibrio costicolus.

Double-arm Warburg reaction vessels contained the cell suspen-

sion or cell-free extract in phosphate buffer, pH 7.6, with

appropriate concentrations of NaCl.   Sodium succinate (10 uM)

as hydrogen donor and $NaNO_3$ (5.0 uM) were pipetted into one

side arm and the sulfanic acid solution into the other.   After

equilibration, the substrate and enzyme were combined, the

reaction mixture was incubated for 30 min. in nitrogen, and the

sulfanic acid solution was finally tipped into the reaction

mixture.

The manometric method of Hewitt and Hallas (1959)

depended on nitrite reaction with azide in acid solution as follows:

$$NO_2^- \quad + \quad N_3^- \quad + 2H^+ \longrightarrow N_2O \quad + N_2 \quad + \quad H_2O$$
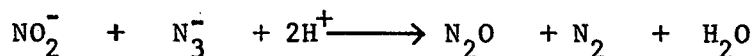
In this method, one uM nitrite yielded 44.8 ul of gas. Using this method, Hewitt and Hallas obtained 82-86 ul of gas from 2 uM nitrite in a fluid volume of 4.5 ml at $27^\circ C$ in an atmosphere of nitrogen.

Spectrophotometric method: Nason and Evans (1953) measured the changes in optical density at 340 mu associated with oxidation of TPNH or DPNH to follow the reaction in the NRase system of Neurospora. The assay system contained 0.83 uM TPNH, 0.3 uM FMN, 100 uM KNO_3, and 424 units of enzyme in 3.6 ml of 0.03 M pyrophosphate buffer. A change of optical density of 0.06 per minute, at 340 mu, was produced over a period of 15 minutes.

Evans and Nason (1953) observed a very rapid oxidation of TPNH in presence of the NRase system and a fairly rapid endogenous rate when all constituents, except KNO_3 were present. Since nitrite was formed in the complete system only, the oxidation of TPNH was caused by some factor other than nitrate.

Owing to the presence of TPNH or DPNH oxidase system in the partially purified enzyme, this method of assay is not sufficiently sensitive.

4. Stability of Enzyme

Most NRase preparations are relatively thermolabile and are easily denatured. The enzyme from Neurospora, obtained by Nason and Evans (1953) lost about 50% of its activity in 5 minutes at 40°C, and all of its activity at 50°C in 5 minutes. Dialysis of the enzyme against phosphate buffer, running tap water, or triply-distilled water resulted in a complete loss of activity within one hour, the shortest time interval tried. When the enzyme was exposed to washed cellophane dialyzing membrane (5.0 ml of enzyme, 0.5 gm of shredded membrane) for 2 minutes, 95% of the original activity was lost. Attempts to restore the activity by addition of boiled crude extract, ashed crude extract, glutathione, or inorganic ions such as MoO_4^{2-}, Fe^{2+} etc. were unsuccessful.

The partially purified enzyme from soybean leaves obtained by Evans and Nason (1953) was very heat-sensitive. Twenty-eight, 46, 71 and 100% inactivation resulted from 5-minute exposures at 20°, 30°, 40° and $50^\circ C$ respectively. The enzyme activity was reduced to 50 to 70% of its original activity during a storage period of one week at -15° C.

Spencer (1959) reported that wheat embryo NRase was inactivated 38, 96 and 100% by heating to 30°, 45°, and 60° C, respectively. Approximately 50% of the enzymatic activity was lost after storage for one week at -15° C.

It has been shown that in order to obtain maximal
specific activity and good yields, the addition of protective
agents such as cysteine, glutathione and EDTA to the extraction
medium is necessary.

Nicholas and Nason (1955a) reported that the addition
of cysteine and EDTA, each at final concentration of 10^{-4} M,
improved the stability and yield of the enzyme by protecting
it against inactivation by heavy metals or oxidation of
sulfhydryl groups. Extraction from cauliflower leaf by Hewitt
et al. (1955) was carried out with 10^{-4} M cysteine and EDTA.
Spencer (1959) demonstrated that when the extracting medium
included 10^{-3} M glutathione a slight increase in activity re-
sulted. Thus it is advantageous to maintain low concentrations
of these protective agents during stages of fractionation.

5. Electron Donors

In a fungus such as Neurospora the NRase is essentially
TPNH-specific (Nason and Evans 1953), but in many higher plants,
e.g. soybean, barley cauliflower and vegetable marrow, DPNH and
TPNH function equally well (Evans and Nason, 1953; Candella et al.
1957; Cresswell et al. (1962). The wheat embryo system (Spencer
1959) and maize leaf system (Hageman and Flesher, 1960) are
DPNH-specific but the wheat leaf system functions with DPNH
(Anacker and Stoy, 1958) or TPNH (Spencer, 1959). The activity

in corn roots was obtained with TPNH or DPNH in the work of
Evans and Nason (1953). Nason and Evans (1953) indicated that
succinate, cysteine, glutathione or ascorbic acid could not
replace TPNH as electron donors for NRase from Neurospora.
Vaidyanathan and Street (1959), however, obtained slow
reduction of nitrate by a crude preparation from excised tomato
roots when ascorbate and ferrous iron were present. Hageman
et al. (1962) have shown that NRase obtained from vegetable
marrow leaves can function with reduced benzyl viologen in
place of pyridine nucleotide as the primary electron donor.

Nason and Evans (1953) presented evidence that energy
for nitrate reduction can be derived from those substrates, for
the most part intermediates of carbohydrate metabolism, which
are oxidized by TPNH enzyme systems. Spencer (1959) used
crystalline alcohol dehydroganase at pH 7.5 in the presence of
ethanol and DPNH for assays on crude wheat embryo extract.
Hageman et al. (1962) linked glucose-6-phosphate and its
dehydrogenase and catalytic amounts of TPN to NRase obtained
from vegetable marrow.

Evans and Nason (1953) were able to link soybean NRase
with photoreduction of TPNH, through the mediation of chloro-
plast grana. Stoy (1956) has shown that photochemical
reduction of riboflavin can be linked directly to NRase action
without the addition of a pyridine nucleotide.

6. FAD as Prosthetic Group

The necessity of flavin as a prosthetic group of NRase was demonstrated by Evans and Nason (1953). Their report showed that the activity of the twice-precipitated soybean NRase was markedly stimulated by FAD, but very slightly by FMN. Tests with D-amino acid oxidase and fluorometric analyses indicated that enzyme solutions contained FAD and that 89 to 98% of the total flavin present in the enzyme extract was FAD. They concluded that the enzyme extract contained a maximum of 3.4×10^{-5} uM FMN per ml. This or greater quantities of FMN were not sufficient to produce an appreciable stimulation of the activity of twice-precipitated enzyme in the standard assay procedure. A similar conclusion applied to Neurospora NRase obtained by Nason and Evans (1954).

Spencer (1859) showed that the activity of NRase of crude cell-free extracts of embryos from germinating wheat was doubled by the addition of FAD. After isolation of the enzyme by ammonium sulfate fractionation, addition of FAD caused a three-fold stimulation at a final concentration of 10^{-6} M. FMN was ineffective as the cofactor.

In the work of Hageman and Flesher (1960), no response to added flavins was obtained with the crude preparation of NRase from corn seedlings. It is probable that the flavin and protein were not easily dissociated as was also the case with the enzyme from E. coli (Nicholas and Nason, 1955b).

Table IV summarizes the nucleotide specificity of assimilatory NRases from several sources.

Table IV

Summary of Properties of Assimilatory NRase

Plant material	Original form	Pyridine nucleotide specificity	Flavin nucleotide specificity	References
Soybean leaves	Soluble	Non-specific	FAD	Evans and Nason (1953)
Neuro-spora	Soluble	TPNH-specific	FAD	Nason and Evans (1953)
E. coli	Soluble	DPNH-specific	FAD	Nicholas and Nason (1954)
Hansenula	Soluble	Non-specific	FAD	Silver (1956)
Wheat leaves	Soluble	Non-specific	FAD	Anacker and Stoy (1958)
Wheat embryo	Soluble	DPNH-specific	FAD	Spencer (1959)
Azoto-bacter	Particulate	Non-specific	FAD	Taniguchi and Ohmachi (1960)
Tomato leaves	Soluble	DPNH-specific	-	Sanderson and Cocking (1964)

7. Sulfhydryl Property

The sulfhydryl nature of NRase was shown by p-
chloromercuribenzoate inhibition and its reversal by
sulfhydryl reagents such as glutathione and cysteine
(Evans and Nason, 1953).

Studies of the possible role of sulfhydryl groups by
Nicholas and Nason (1954) indicated that these groups are
necessary for the enzymic transfer of electrons from TPNH
to flavin. This was indicated by the complete inhibition of
enzyme activity by the mercury reagent and its reversal by
glutathione. Subsequent electron transport to nitrate,
using $RMNH_2$ as the electron donor, is inhibited only 25% by
p-chloromercuribenzoate. These experiments have shown the
-SH groups of the protein are necessary largely for the
enzymatic reduction of FMN by TPNH, probably by the binding
of TPNH and unreduced flavin to the enzyme. That the
sulfhydryl groups are probably not serving as electron
carriers, was indicated by the fact that they do not serve as
electron donors in this enzymatic system.

NRase has not been purified to the homogenous protein
level, hence the number of sulfhydryl groups which are
involved in enzyme function is not known.

8. Molybdenum Constituent

Nicholas and Nason (1954) presented the first evidence
that molybdenum is the metal consituent of NRase in Neurospora.

1) When <u>Neurospora</u> NRase was fractionated with
ammonium sulfate, the highest activity occurred in the 24 to
40% ammonium sulfate fraction which also had the greatest
molybdenum content. Increased enzyme activity in various
protein fractions was accompanied by increased molybdenum
content.

2) Dialysis of NRase against cyanide resulted in a
decrease of molybdenum content and a deactivation of the
enzyme. The addition of molybdenum trioxide or sodium
molybdate reactivated the enzyme to 85% of the original value.
Preincubation with other trace metals, including iron, zinc,
manganese, nickle, cobalt, mercury, tungsten, uranium,
vanadium, and boron, respectively, did not restore the activity
of nitrate reductase.

In their study of the mechanism of the <u>Neurospora</u> NRase
reaction, Nicholas and Nason (1954) found that the molybdenum-
free enzyme had lost its ability to catalyze the reduction of
nitrate to nitrite by either TPNH or reduced flavin. It still
possessed the ability to catalyze the reduction of flavin by
TPNH. They also indicated that dithionite-treated molybdate
can reduce nitrate to nitrite anaerobically in the presence of
NRase without the participation of TPNH or reduced flavin.

Evans and Hall (1955) presented direct evidence of
association of molybdenum with nitrate reduction from soybean
leaves. They germinated soybean seeds in a flat of sand.

Four days after the seeds were planted, the flats were treated

with a solution containing 3.5 mc of Mo^{99} per gram. When the

seedlings were 10 days old, leaves were harvested. The

extracts were fractionated with ammonium sulfate precipitation

and calcium phosphate adsorption. The activity of NRase from

each fraction was assayed, using DPNH as the hydrogen donor.

An aliquot from each fraction was ashed in a muffle furnace and

counted for radioactivity with a Geiger-Muller counter. The

results indicated that a close correlation between enzyme

activity and radioactivity of the fraction of highest purity

existed.

In order to obtain further evidence concerning the

metal requirement of NRase, Evans and Hall (1955) dialyzed

partially purified soybean NRase against phosphate buffer con-

taining glutathione and KCN, followed by dialysis for 8 hours

against a similar buffer solution containing glutathione but

no KCN. They found that NRase activity was stimulated by the

addition of molybdenum.

The most reduced valence state of Mo in the dithionite

reduced solution of sodium molybdate was shown by paper

chromatography to be +5, (Nicholas and Stevens, 1955). Mo^{+5}

pentachloride, freed from the other valence states of Mo by

column chromatography, acts as an effective electron donor for

the enzymic reduction of nitrate. Nicholas and Stevens (1955)

found that Mo^{4+} is stable in strong ethanol only, and that it

dismutes readily in the presence of a trace of water to give Mo^{5+} and Mo^{3+}. It is most unlikely that Mo^{4+} is involved in any physiological process. Mo^{3+} reduced nitrate non-enzymatically. The rate of chemical reduction was approximately twice that of NRase action. Mo^{3+} is oxidized instantaneously by Mo^{6+}. Reduction of nitrate by Mo^{3+} in Neurospora is unlikely since Mo^{3+} has not been detected in this organism. It would thus appear that one of the functions of Mo in the enzyme is to couple the flavin to nitrate by a one electron transport charge whereby Mo^{5+} is oxidized Mo^{6+}.

Candela et al. (1955) reported that cauliflower grown without molybdenum possessed much lower total and specific NRase activity than the normal plants. Infiltration of molybdenum into leaves of deficient plants resulted in increased activity of the enzyme in 18 to 24 hours.

Nicholas and Nason (1954) reported that Fe^{2+} or Fe^{3+} treated with $Na_2S_2O_4$ failed to serve as an electron donor for the enzymatic reduction of nitrate in the presence or absence of molybdate. This fact rules out the role of iron as an electron carrier in the Neurospora NRase system.

9. Phosphate Stimulation

Phosphate stimulation of Neurospora NRase has been observed by Nicholas and Scawin (1956) and Kinsky and McElroy (1958). A similar action by phosphate on the wheat embryo NRase was reported by Spencer (1959).

Nicholas and Scawin (1956) fractionated <u>Neurospora</u>
NRase by ammonium sulfate precipitation, calcium phosphate gel
absorption, and carbon absorption. Maximal enzyme activity
was obtained by the addition of 2.5 uM phosphate to 0.1 ml of
the enzyme. The phosphate could be replaced by selenate,
tellurate or arsenate, partially by sulfate or silicate, but
not by pyrophosphate, adenosine triphosphate, borate, vanadate
molybdate and tungstate. The investigators suggested that one
of the roles of phosphate in NRase is to bind the Mo to the
apoenzyme because the anions which can act as did phosphate
have similar atomic radii to phosphate (2.76 $\overset{o}{A}$), being within
the range 2.4 to 2.8 $\overset{o}{A}$. They also form complexes with
molybdates.

Kinsky and McElroy (1958) observed that arsenate and
tungstate, but not silicate, could substitute for phosphate in
the case of <u>Neurospora</u> NRase. They also presented evidence
that phosphate had no effect on the TPNH-to-flavin nucleotide
reaction, whereas the second step, from flavin nucleotide, is
definitely stimulated by phosphate. They suggested that
molybdenum combines with phosphate, arsenate and tungstate, to
form phosphomolybdo-, arsenomolybdo- and tungstomolybdo-
complexes which presumably have oxidation-reduction potentials
different from that of the molybdenum ion. This alternation
of potential due to complexes may permit interaction with
enzymatically or chemically reduced flavin nucleotide.

Spencer (1959) also reported that inorganic phosphate was essential for maximum enzymatic activity. The presence of 10^{-2} M potassium phosphate caused a 30% increase in the activity of wheat embryo NRase.

10. **Inhibitors**

NRase is very sensitive to metal-chelating agents. Evans and Nason (1953) demonstrated that KCN at a final concentration of 10^{-3} and 10^{-4} M inhibited the activity of soybean NRase 83 and 27% respectively; and NaN_3 at these concentrations inhibited 89 and 39% respectively. A concentration of 2×10^{-4} M potassium ethyl xanthate inhibited the activity by 49%, and 10^{-4} M $CuSO_4$ inhibited it by 32%. There was no inhibition by 10^{-3} M NaF. Other metal-chelating agents such as 8-hydroxyquinoline, diethyldithiocarbamate, EDTA, a,a-dipyridyl and phenanthroline also inhibited at different rates. A complete lack of inhibition by 100% CO suggests the absence of cytochrome in the enzyme system.

Sulfhydryl reagents, such as p-chloromercuribenzoate and heavy metals, strongly inhibited NRase. This inhibition could be reversed by the addition of glutathione.

Nicholas and Scawin (1956) showed that the *Neurospora* NRase was not inhibited by uncoupling reagents, e.g. dinitrophenol at 10^{-5} and 10^{-4} M, Aureomycin at 5×10^{-4} M, or sodium arsenate at 10^{-2} M. Thus, there was no evidence for a

phosphorylation mechanism during nitrate reductase action.

Vanadate ion has been shown to be a powerful inhibitor of the enzyme from wheat embryo (Spencer, 1959). A final concentration of approximately 3×10^{-5} M sodium orthovanadate caused 50% inhibition. This inhibition was not reversed by a 25-fold excess of sodium molybdate.

11. Mechanism of Action

In the classic paper "Mechanism of action of nitrate reductase from Neurospora", Nicholas and Nason (1954) suggested the following sequence of electron transfer for the enzyme:

$$TPNH \longrightarrow FAD \text{ (or FMN)} \longrightarrow Mo \longrightarrow NO_3^-$$

2, 3, 6-trichloroindophenol
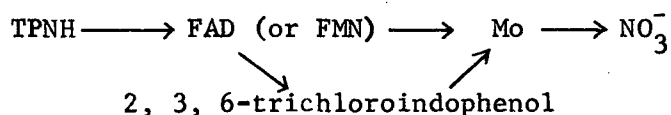
The enzyme catalyzes the transfer of electrons from TPNH to nitrate via electron carriers FAD (or FMN) and molybdenum. When introduced into this system, 2,3,6-trichloro-indeophenol may act as an artificial carrier between flavin and molybdenum. Molybdenum is essential for the final step in electron donation to nitrate. If it is separated from the enzyme by dialysis against cyanide, neither TPNH nor $FADH_2$ can reduce nitrate to nitrite. Reduced molybdate prepared with either the reducing agent $Na_2S_2O_4$ and H_2 or palladium and H_2 can reduce nitrate to nitrite anaerobically in the presence of NRase. Iron, copper, vanadate or chromate when treated with

$Na_2S_2O_4$ and H_2 failed to substitute for dithionite-treated molybdate.

Fewson and Nicholas (1961), by measurement of electron spin resonance signals, confirmed the formation of Mo^{5+} during NRase action in P. aeruginosa. Valence changes which occurred were presumed to involve Mo^{5+} and Mo^{6+}.

According to Kinsky and McElroy's (1958) suggestion, phosphate and other anions combine with molybdenum to form a complex. FAD or FMN may behave as one of the specific reducing agents capable of reducing only molybdenum complexes with phosphate, arsenate, tungstate and silicate.

In support of their suggestion for the electron transfer sequence of NRase, Nicholas and Nason (1954) were able to show that DPNH can be replaced by $FADH_2$. $FADH_2$ prepared enzymatically by the DPNH oxidizing system from Clostridium kluvveri, could serve, under anaerobic conditions, as electron donor for the reduction of nitrate by Neurospora NRase. Chemically reduced flavins also were active as electron donors.

The following scheme is a summation of various contributions discussed above:

DPNH (TPNH) + H^+ / DPN^+ (TPN^+) — FAD (FMN) / $FADH_2$ ($FMNH_2$) — $2Mo^{5+}$ $+2H^+$ / $2Mo^{6+}$ — cyanide inhibition — NO_3^- / NO_2^- + H_2O

-SH dependent   Phosphate stimulation
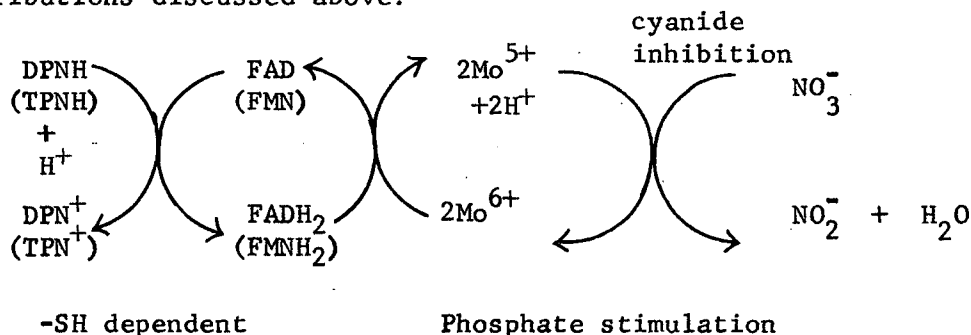
12. Localization of Enzyme

With the exception of some bacterial enzymes reducing
nitrate, all the assimilatory NRase studied thus far seem to
be soluble.

Nason and Evans (1953) indicated that 85% or more of
the NRase of the homogenate from Neurospora was present in
the cell-free extract.

Spencer (1959) ground embryos from germinating wheat
in 0.4 M sucrose containing 0.05 M potassium phosphate, pH
7.4 and 10^{-3} M glutathione. The grindate was centrifuged at
low speed (x 100 g) to remove unbroken cells. The supernatant
solution was further centrifuged at 105,000 x g in a Spinco
ultracentrifuge for 30 minutes. He found that all the NRase
present in the supernatant solution from the low speed centri-
fugation was recovered in the supernatant solution following
high speed centrifugation. The enzyme was thus located in
soluble, cytoplasmic fraction of the cell, rather than in the
particulate fraction.

In studies of distribution of NRase in cell-free
extracts of Azotobacter vinelandii, Taniguchi and Ohmachi
(1960) found that 90% of NRase activity resided in large
particles (2000 - 14,000 x g) regardless of the electron
donors, and 10% in small particles (14,000 - 105,000 x g) and
soluble supernatant. It is apparently a particulate assimilatory
NRase.

The soluble or particulate nature of NRases from various sources is summarized in Table IV (page 34).

13. Adaptive Formation of Enzyme

Evans and Nason (1952) first reported that Neurospora NRase appeared to be adaptive since little or no activity could be detected in extracts of mycelia grown on an ammonium nitrogen medium in the absence of exogenous nitrate. In 1953, their studies of effects of different sources of nitrogen on the activity of Neurospora NRase indicated that the enzyme activity occurred only in mycelia grown in the presence of nitrate or nitrite and that no activity existed in mycelia grown with ammonia or alanine as a sole nitrogen source. They suggested that some of the nitrite was oxidized to nitrate which was responsible for the induction of the enzyme.

Tang and Wu (1957) obtained more direct evidence of the adaptive property of the enzyme in rice seedlings. They demonstrated that no NRase activity was present in 4- to 6-day old seedlings when grown in quartz sand with either 0.033 or 0.066 M phosphate buffer. This activity could be demonstrated in both etiolated and green seedlings when nitrate was added to culture solution, or when the grains were treated with nitrate solution before germination. Under optimal conditions such as pH 5.0, 30° C, and 6 mM nitrate, the NRase formation could be induced in 1 day, in 5 hours or even in 3

hours, in etiolated intact seedlings, intact green seedlings, and excised roots of those seedlings, respectively. When the seedlings were transferred back to a nitrate-free medium, de-adaptation occurred in about 2 days. Ammonium sulfate is ineffective in inducing the formation of NRase and is inhibitory in the presence of nitrate. Low oxygen tension of 1 to 5% does not significantly impede NRase formation as compared with 10% oxygen tension.

Hewitt and Afridi (1959) have found in numerous tests that formation of NRase activity can be rapidly induced in small fragments of leaf excised from cauliflower, white mustard and sunflower. Their results showed that when nitrate or molybdate, or both, were introduced by vacuum filtration into excised fragments of leaves of cauliflower, rapid production of enzyme activity occurred.

The introduction of molybdenum into leaves of plants deficient in Mo and grown with nitrate had a similar effect. Tissues of plants grown with ammonium sulfate but without molybdenum, responded little or not at all to either factor alone, but developed activity rapidly when both were present. Their results also indicated that some of the inhibitors of protein synthesis, such as 1,2-dichloro-4-(p-nitrobenzene-sulfonylamide)-5-nitrobenzene (DCDNS), actidione, and

polymyxin-b-sulfate, markedly inhibited the synthesis of NRase. They suggested that protein synthesis was necessary for development of enzyme activity and the process seemed analogous to enzyme induction in other systems.

The presence of ammonia in the nutrient medium may cause suppression of enzyme activity in some organism, e.g. in the fungus Scopulariopsis, even when nitrate is also given (Marton, 1956).

Kinsky (1961) studied induction and repression of nitrate reduction by Neurospora. He found that the nitrate-induced enzyme formation was completely inhibited by citrate and acetate, and that the rate of enzyme formation was dependent on the pH of the induction medium. Kinsky used "feed-back repression" to explain ammonia repression of NRase formation.

14. Effect of Plant age on Enzyme Activity

It is well known that the metabolic activity of tissues varies with their age, as well as with other physiological factors.

Evans and Nason (1953) reported that NRase appeared to be more active in the meristematic and young trifoliated leaves of 17-day-old soybean plants and suggested that the enzyme is concentrated in young, metabolically active tissue.

Candela et al. (1957) showed that the activity of cauliflower NRase was greatest in mature fully expanded

leaves, adjacent to the oldest two or three leaves remaining
on the plant at the time. The net total activity in leaves
of cauliflower was considerably less for plants aged 20 weeks
than those aged 12 weeks sampled at the same time. The
specific activity, however, was similar in both age groups
due to the lower protein extracted from the older plants.

Spencer (1959) found a relative high activity of NRase
in wheat embryo. It was observed that, on the basis of fresh
weight, 2300 muM nitrate was produced per hour. A value of
about 200 muM was obtained by Sanderson and Cocking (1964) for
33-old wheat leaves.

15. Nitrate Reductase Activity in Roots

Based on his finding that nitrate was present only in
the finest parts of the roots of the apple tree, Thomas (1927)
suggested that most of nitrate reduction takes place in root
tissues of this plant.

The reduction of nitrate in excised roots and leaves
of wheat led Burstrom (1946) to conclude that in the intact
plant nitrate reduction occurs in both these organs.

Bonner (1952) indicated that in asparagus and narcissus
nitrate reduction is ordinarily confined to the root, and that
upward translocation of nitrate was observed only when it was
supplied to the root in large amounts. In still other species
such as tomato, tobacco, curcurbits and cereals the roots are

not particularly active in the reduction of nitrate but on the contrary, nitrate is translocated through the tissue of the entire plant with reduction taking place in the leaves as well as in the roots.

Evans and Nason (1953) reported the presence of NRase activity in extracts of roots of seven species of plants namely: potato, barley, muskmelon, wheat, tomato, corn and soybean. A relatively high rate of activity was found in the extract of tomato roots. When TPNH was used as hydrogen donor in the assay system, the activity of NRase in the roots of these plants was as high as in their leaves. The presence in roots of enzyme systems capable of reduced pyridine nucleotide production would make the reduction of nitrate possible.

Vaidyanathan and Street (1959) have shown that extracts from excised nitrate-grown roots did not reduce nitrate to nitrite in spite of addition of Mo, DPNH and FMN. However, the further addition of aldehyde-free ethanol, lactate, succinate or glutamate led to the formation of nitrite.

Sanderson and Cocking (1964) reported that the activity of NRase in the root extracts of such higher plants as tomato, barley, corn, kidney bean, potato, tobacco and wheat, was one-quarter of the activity in their leaf extracts. In order to demonstrate with certainty that this activity was due to enzymic activity endogenous to the roots, excised roots were grown in sterile nutrient solution culture. The results showed

clearly that tomato roots do contain NRase and that the level

of activity is dependent on the level of nitrate supplied during

culture.

16. Light and Nitrate Reductase Activity

Although the effect of light on the nitrate reduction

in green plants had been observed by plant physiologists for

over a century, the mechanism of the reaction is still unknown.

That photochemical processes may furnish the hydrogen

donors, i.e. reduced pyridine nucleotides, for nitrate reduction

was shown by Evans and Nason (1953). In their historical experi-

ment, they showed that the combination of NRase and grana from

soybean leaves could reduce nitrate to nitrite in light if

catalytic amounts of TPN were added. The system was inactive in

the dark unless TPN was replaced by TPNH in stoichiometric amounts.

In this photochemical reduction, nitrate accepts the hydrogen of

the reduced carrier formed during the photolysis of water. The

following reactions were used to explain this mechanism.

$$H_2O + TPN \xrightarrow{\text{Light and grana}} 1/2\ O_2 + TPNH + H^+$$

$$TPNH + H^+ + NO_3^- \xrightarrow{\text{Nitrate reductase}} NO_2^- + TPN^+ + H_2O$$
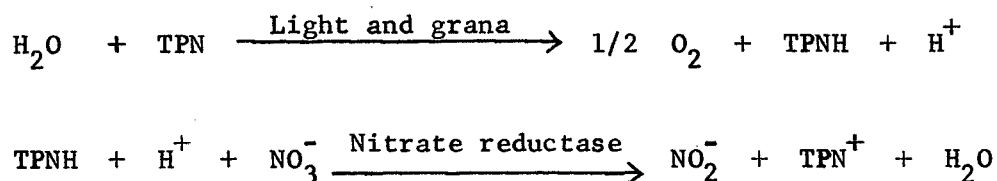
Jagendorf (1956) showed that the highly purified chloro-

plasts are capable of reducing dyes, the usual Hill oxidants,

such as ferricyanide and TPN, and that the reduction of TPN was

coupled to spinach NRase using the system of Evans and Nason (1953).

However, chloroplasts do not reduce DPN under conditions where grana are perfectly capable of carrying out this photoreduction of TPN.

Recently, Ramirex et al. (1964) reported the findings of a new type of non-cyclic photosynthetic electron flow, in which flavin-nucleotides mediate the direct transfer of electrons from illuminated grana to nitrate with the aid of NRase. In their experiment, the photoevolution of oxygen had been suppressed by heating the grana at 55° C for 5 minutes and the electrons were supplied by the system of ascorbate-dichlorophenol-indophenol (DPIP). The reaction did not proceed in the dark and required FAD in addition to NRase. Manadione and ferredoxin were not capable óf replacing the flavin nucleotide, but benzyl viologen wàs an effective electron carrier in the system. They suggested the following sequence of electron transfer in photosynthetic reduction of nitrate.

$$\text{Water} \xrightarrow{} \text{Chlor. I.} \xrightarrow{} \text{Chlor. II} \xrightarrow{\text{Reduced DPIP}} \text{FAD or FMN} \xrightarrow{\text{Nitrate reductase}} \text{Nitrate}$$

(light)   (light)
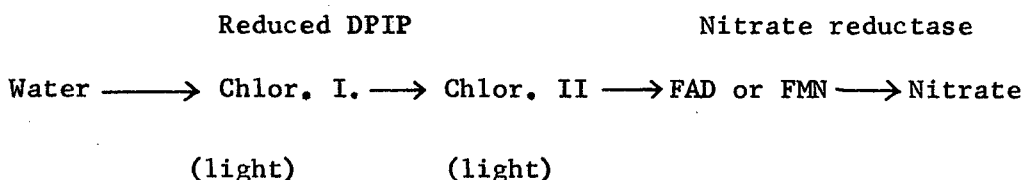
A concept based upon competition between nitrate and carbon dioxide for reducing power has been proposed by Van Neil et al. (1953). They observed that in Chlorella at low light intensity the uptake of carbon dioxide, but not the production

of oxygen was decreased in the presence of nitrate.

Stoy's experiments gave another clue for the under-
standing of the photoreduction of nitrate. He first observed
(1955) nitrate reduction by detached wheat leaves in the blue
and violet parts of the spectrum and suggested involvement of
a yellow pigment. He also observed that photosynthesis and
nitrate assimilation paralleled each other in red and green
light. Based on this, he suggested that riboflavin served as
a light-absorbing catalyst and hydrogen carrier in biological
photoreduction as described by Branner and Branner (1954).
Stoy's experiment demonstrated that photoreduced riboflavin was
actually a more efficient reducing agent than DPNH. EDTA was
required for the riboflavin-coupled system and could not be
replaced by cystein. The following reaction scheme was
proposed.

$$
\begin{array}{c}
\qquad\qquad hv \\
DPN \xrightarrow{\hspace{2.5cm}} DPNH \xrightarrow{\hspace{1.5cm}} FAD \rightarrow Mo \longrightarrow NO_3^- \\
\qquad grana \qquad\qquad\qquad \nearrow \\
\qquad\quad hv \qquad\qquad\quad / \\
Riboflavin \xrightarrow{\hspace{1cm}} Reduced\ flavin
\end{array}
$$

(EDTA riboflavin)
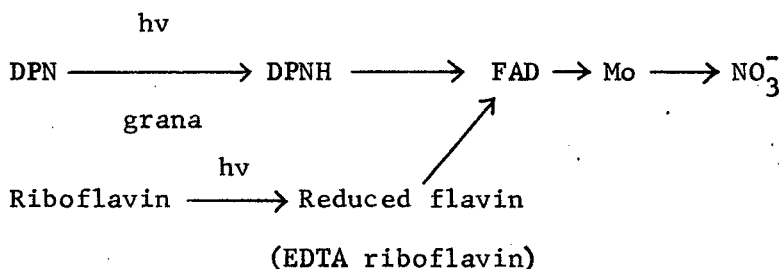
No direct light activation of the flavin-containing
nitrate reductase was observed.

Candela et al. (1957) found that when cauliflower
plants were transferred to darkness, NRase activity in the
extract decreased to nil after 5 to 6 days. It was found

that when normal light was restored there were rapid increases in the total and specific activities.

Hageman and Flesher (1960) demonstrated that when corn seedlings were placed in the dark, the NRase dropped to less than 10% of the original level of activity after 48 hours. NRase was not detectable in the extracts from corn seedlings after 90 hours of darkness. No increase in NRase was detectable within 2 hours after the plants were returned to full sunlight after 65 hours of darkness.

The extraction, purification and characterization of sugar beet NRase, described in this thesis, was undertaken as part of the overall study of sugar beet metabolism conducted at the University of British Columbia over a period of years and supported by research grants from the National Research Council of Canada and the British Columbia Sugar Refining Co., Vancouver, B. C. A knowledge of the properties of the enzyme was particularly desirable as an entry into the broader field of nitrogen metabolism of the beet as related to late season growth which results in a depletion of sucrose stored in the root.

MATERIALS AND METHODS

1. Source of Enzyme

The crude enzyme was obtained from the blades of
leaves of 40- to 60-day-old sugar beet (Beta vulgaris L.)
plants. The seeds were supplied by the British Columbia Sugar
Refining Co., Vancouver, British Columbia, Canada. The plants,
grown in wooden flats filled with vermiculite, were supplied
once every day with nutrient solution containing 0.005 M
$Ca(NO_3)_2$, 0.0005 M KH_2PO_4, 0.002 M $MgSO_4$, 0.05 M KNO_3, 0.5 ppm
Fe as iron chelate, 0.04 ppm of Cu as $CuSO_4$, 0.25 ppm of Mo
as Na_2MoO_4, 2.0 ppm of B as H_3BO_4, 0.2 ppm of Zn as $ZnSO_4$ and
0.5 ppm of Mn as $MnCl_2$. The pH was adjusted to 6.5.

The plants were grown in a controlled environment room
with the following conditions: photoperiod, 16 hrs.; temperature,
day 21-26° C, night 18-22° C; relative humidity, day 62-70%,
night 65-80%; light intensity 1800 foot candles at the top of
the plants. Slimline cool white fluorescent tubes were supple-
mented by 60 watt incandescent lights.

2. Extraction of Enzyme

Crude cell-free extracts were prepared by grinding one
weight of fresh leaf blades with four weights of cold 0.1 M
K_2HPO_4 buffer (pH 7.8 approximately) containing 10^{-3} M of
reduced glutathione, in a Waring Blendor (at full speed) for

1 to 2 minutes, at 0 - 4° C. The homogenate was strained
through four layers of cheesecloth and then centrifuged in
a Servall centrifuge at 20,000 x g for 20 minutes at 0 to 4° C.
The resulting green, cell-free supernatant solution (crude
enzyme) which was shown to contain 100% of the NRase activity
present in the whole homogenate, was used for purification.

3. Cofactors and Other Substances

DPNH (approximately 97.5% pure), TPNH (96% pure),
FAD (80% pure) and FMN (100% pure) were obtained from the
Sigma Chemical Company of St. Louis, Missouri. The concentra-
tions of DPNH and TPNH were determined spectrophotometrically
with 6.22×10^6 sq. cm. x $mole^{-1}$ and 6.24×10^6 sq. cm. x
$mole^{-1}$ as the extinction coefficient at 340 mu respectively
(Horecker and Kornberg, 1948). The concentration of flavin
solutions was determined spectrophotometrically by the use
of the extinction coefficient of 1.13×10^7 sq. cm. x $mole^{-1}$
at 455 mu (Warburg and Christian, 1938). Benzyl viologen
was obtained from Mann Research Laboratories of New York,
N.Y. Methylene blue and the sodium salt of 2,6-dichlorophenol
idophenol was obtained from Kodak Company of Rochester, N. Y.
Phenazine methosulfate and all other substances were obtained
from the Nutritional Biochemical Corporation of Cleveland,
Ohio.

4. <u>Preparation of Calcium Phosphate Gel</u>

The tricalcium phosphate gel was prepared by the
method of Keilin and Hartee (1938). One hundred fify ml of
$CaCl_2$ solution (88.5 g $CaCl_2$ per liter) was diluted to about
1600 ml with demineralized water and shaken with 150 ml
trisodium phosphate solution (152 g $Na_3PO_4.12H_2O$ per liter).
The pH of the mixture was brought to 7.5 with acetic acid and
allowed to stand for 10 minutes. The supernatant was decanted
and the gel centrifuged. The gel was then washed three times
with large volumes of distilled water to remove Cl^- ions.
After final centrifugation it was resuspended in 500 ml
distilled water and the dry weight per ml of the suspension
determined. Distilled water was added to bring the concen-
tration to 20 mg per ml of suspension. After three months
storage at 4^oC the gel was centrifuged down and resuspended
in the same volume of phosphate buffer, pH 7.5.

5. <u>Preparation of Alumina C_r Gel</u>

Alumina C_r gel was prepared by a modification of the
method of Colowick (1955). A hot solution of 340 grams of
aluminum sulfate in 500 ml of water was poured all at once
into 3.25 liters of ammonium sulfate-ammonia water at 60° C.
The latter reagent contained 100 g of ammonium sulfate and
215 ml of 20% ammonia. During the precipitation and for an
additional 15 minutes, the mixture was stirred vigorously and
the temperature was kept at 50° C. After the first voluminous

precipitate gradually became flocculent, the mixture was diluted
to 10 liters with demineralized water and the supernatant
fluid decanted as soon as the precipitate had settled out. The
washing with demineralized water and decantation was repeated
six times. To solubilize any remaining basic aluminum sulfate
40 ml of 20% ammonia was added during the fourth washing.
After repeated washings, the gel was suspended in 200 ml
distilled water and kept at 4° C for three months before using.

6. Preparation of Electron Donors

Reduced flavins were obtained by the method of Nicholas
and Nason (1954b). Two mg of sodium hydrosulfite was added to
0.1 to 0.5 uM FAD or FMN contained in 3 ml of 0.1 M pyro-
phosphate buffer, pH 7.5, in a Thunberg tube. Hydrogen gas was
then bubbled through the solution for 2 minutes at 4° C, followed
by evacuation. The dyes were reduced to their leuco form by the
same procedure.

7. Determination of Protein

The protein content of enzyme preparation was measured
according to the method of Lowery et al. (1951). To 0.4 ml of
the sample was added 2 ml of alkaline copper solution. This
solution was prepared by the mixture of 50 ml of 2% Na_2CO_3 in
0.1 N NaOH and 1 ml of 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium
tartrate. The contents were mixed. After 10 minutes 0.2 ml
of 1 N Folin-Ciocalteau phenol reagent was added and the

contents mixed. Optical density readings at 500 mu were taken with a Beckman Model B Spectrophotometer. Protein content, as mg per ml, was obtained by comparison with a standard curve prepared by the use of crystallized bovine albumin.

8. Determination of Chlorophyll Content

Chlorophyll content was measured by the method described by Arnon (1949). 0.1 ml of crude enzyme extract was diluted to 20 ml with 80% acetone (20 ml of H_2O made to 100 ml with acetone), and filtered through Whatman No. 1 filter paper. The optical density was read against 80% acetone at 625 mu (1-cm light path) in a Beckman Model B Spectrophotometer. Chlorophyll solutions in acetone were protected from ambient light, O.D. x 5.8 gave mg of chlorophyll per ml of crude enzyme extract.

9. Determination of Nitrate Content

Nitrate content was estimated by the method of Woolley et al. (1960). Four ml of extract was diluted to 20 ml. To 1 ml of this diluted solution was added 9 ml of 20% acetic acid solution containing 0.2 ppm Cu as $CuSO_4$. By the use of a measuring scoop 0.8 g of an intimate mixture of 75 g of citric acid, 10 g manganous sulfate dihydrate, 4 g. of sulfanilic acid, 2 g of zinc powder, and 2 g of 1-naphtylamine was added. The

sample was then shaken for about 15 seconds and was again

shaken three minutes later. After three minutes more the

sample was shaken for the third time and was centrifuged for

three minutes at 1000 x g.

The supernatant solution was poured through a small

loose plug of borosilicate glass wool, the light absorbance

was measured at 520 mu, and the amount of nitrate was calcu-

lated from a standard curve.

10. Standard Nitrate Reductase Assay

The NRase was measured by a modification of the method

described by Evans and Nason (1953). At zero time 0.05 - 0.2

ml of enzyme added to a reaction mixture containing 0.1 ml of

0.1 M KNO_3, 0.05 ml 2 x 10^{-5} M FAD, 0.05 ml 2 x 10^{-3} M DPNH,

and 0.1 M phosphate buffer, pH 7.0, to give a volume of 0.5 ml.

After 15 minutes incubation at 30° C, 1 ml of H_2O and 1 ml of

1% W/V sulfanilamide reagent were added to stop the reaction.

One ml of 0.22 % W/V N-(1-naphthyl)-ethylene diamine

hydrochloride reagent was added and the contents mixed by in-

verting the tubes. The color was allowed to develop for 15 min-

utes. A Beckman Model B spectrophotometer was used to determine

the O.D. of each sample and its blank (complete except for DPNH)

at 540 mu. The enzyme exhibited no difference in activity when

the assay was conducted under anaerobic or aerobic conditions.

There was no chemical reduction of nitrate to nitrite by DPNH.

One unit of NRase is defined as that amount of enzyme which results in the formation of 10^{-3} uM of nitrite under the above conditions of the assay. Specific activity is expressed as units per mg of protein.

11. Determination of DPNH-Menadione Reductase Activity

DPNH-menadione reductase was determined by a modification of the procedure of Wosilait and Nason (1954). The reaction was started by the addition of enzyme to a mixture containing 0.3 uM menadione, 0.35 uM DPNH and 200 uM phosphate buffer, pH 8.3, to give a final volume of 3.0 ml. After the addition of the enzyme, the decrease in optical density at 340 mu was measured at 30 second intervals for the first three minutes. One unit of menadione reductase is defined as that amount of enzyme which results in a change in optical density of 0.001 per minute.

The non-enzymatic rate was also determined spectrophotometrically at 340 mu as above, except that the enzyme was omitted from the reaction mixture. The specific activity is expressed as units per mg of protein.

12. Determination of DPNH-Quinone Reductase Activity

DPNH-quinone reductase activity was determined by a modification of the procedure of Wosilait and Nason (1954). The reaction was started by the addition of enzyme to a mixture containing 0.45 uM of p-quinone solution, 0.35 uM DPNH and 200

uM phosphate buffer, pH 7.0, to give a final volume of 3.0 ml.

After the addition of the enzyme, the decrease in optical

density at 340 mu was measured at 30-second intervals for the

first three minutes. One unit of quinone reductase is defined

as that amount of enzyme which results in a change in optical

density of 0.001 per minute. The non-enzymatic rate was also

determined spectrophotometrically at 340 mu as above, except

that the enzyme was omitted from the reaction mixture. The

specific activity is expressed as units per mg of protein.

13. Determination of DPNH-Cytochrome c Reductase Activity

Activity of DPNH-cytochrome c reductase was determined

by a modification of the procedure of Mahler et al. (1954).

The reaction mixture contained 0.2 uM DPNH, 0.03 uM FMN, 0.1 uM

of cytochrome c and 150 mM of Tris buffer, pH 7.5, in a final

volume of 3 ml. The reaction was started by addition of enzyme

and the optical density at 550 mu was followed with a Beckman

Model B spectrophotometer. The unit of enzyme activity is

defined as an optical density change of 1.00 per minute under

the above conditions.

14. Determination of DPNH-Diaphorase Activity

DPNH-diaphorase activity was determined by a modifi-

cation of the procedure of Avron and Jagendorf (1956). The

reaction mixture contained 0.2 uM DPNH, 15 uM of Tris buffer,

pH 8.0, and 0.3 uM 2, 6-dichlorophenol indophenol and 0.05 ml

of enzyme in a final volume of 3 ml. The reaction was started

by the addition of the enzyme. The decrease in optical density

at 620 mu was measured at 30-second intervals for three minutes.

One unit is defined as that amount of enzyme which resulted in

a net change in optical density of 1.00 per minute at 620 mu

under the above conditions.

15. Isolation and Identification of Enzyme-bound Flavin

The flavin of NRase was released by the method des-

cribed by Rao et al. (1962). Five ml of Fraction V enzyme was

heated in a boiling water bath for 5 minutes, followed by

cooling to 5° C and acidification with perchloric acid to a

final concentration of about 10%. After centrifugation at

5000 x g for 10 minutes, the precipitate was discarded and the

yellow supernatant fluid was adjusted to pH 7.8 with 6 N KOH.

The potassium perchlorate was removed by centrifugation at 5000

x g for 10 minutes. The supernatant was condensed in the dark

with a vacuum pump. Descending paper chromatography of flavins

was performed on 20 x 40 cm sheets of Whatman No. 1 paper, with

n-butanol-acetic acid-water (4:1:5, volume for volume) or 5%

Na_2HPO_4 as the solvent system. The flavins were identified under

an ultraviolet lamp.

16. Fractionation of Cellular Components

Fractionation of cellular components was carried out

by the modification of the method of Pierpoint (1963). Five g

of sugar beet leaf blades with midribs removed was cut into

small pieces and ground for 1 minute in a homogenizer with 20

ml of extraction medium. The latter consisted of 0.4 M

sucrose, 0.2 M Tris, 0.01 M phosphate buffer, pH 7.5, 0.01 M

EDTA and 0.02 M sodium nitrate, pH 7.5. The homogenate was

strained through two layers of cheesecloth and the filtrate

was centrifuged at 200 x g for two minutes to remove intact

cells, cell wall and nuclei. The decanted supernatant

solution was further centrifuged for 7 minutes at 1000 x g to

give a sediment containing chlorophyllous materials. This is

referred to as the "Chloroplast fraction". The supernatant

was further centrifuged for 30 minutes at 10,000 x g to give

a green "mitochrondrial" fraction and a relatively clear

supernatant. The mitochondria-free supernatant was centri-

fuged in a Spinco Model L preparative centrifuge for 30 minutes

at 105,000 x g, to obtain microsomal and supernatant fractions.

The temperature was kept at 0-4° C.

Each fraction, viz., nuclei, chloroplast, mitochondria

and microsome, was washed with 5 ml of the extraction medium

and the washings were added to the supernatant fraction. The

washed particulate materials were suspended in 10 ml of 0.01 M

potassium phosphate buffer at pH 7.5.

17. Photochemical Reduction of Flavin Nucleotides

The photochemical reactions described in this experi-

ment were performed under anaerobic conditions. In order

that precise spectroscopic measurements could be made under anaerobic conditions, Thunberg tubes were modified in such a fashion that a pyrex absorption tube was joined to the Thunberg Tube. This allowed direct measurement of optical density to be made with a Bausch & Lamb spectrophotometer. Anaerobic conditions were obtained by means of evacuation with vacuum pump.

For intense illumination, a 375 watt Sylvania floodlight at distances of 10 to 15 cm was used. To filter out infrared waves, the light beam was passed through a blue light filter (Klett-Summerson Light Filter No. 42). The Thunberg tubes were immersed in a 30° C water bath during illumination.

I. PURIFICATION OF ENZYME

1. First Ammonium Sulfate Fractionation

The crude enzyme was divided into four portions by fractional precipitation with ammonium sulfate. The fractions were 0-30, 30-50, 50-70 and 70-90% of saturation, and supernatant. The activity was found in the 0-30 and 30-50 fractions:

Saturation of Ammonium Sulfate (%)	Activity of Enzyme (Units/ml)	Total Volume (ml)	Total Activity (Units)
0-30	177	20	3540
30-50	122	30	3660
50-70	0	20	0
70-90	0	20	0
Supernatant	0	80	0

In another experiment using 0-20, 20-45 and 45-90% saturations with ammonium sulfate the major activity was concentrated in the 0-20 and 20-45 fractions:

Saturation of Ammonium Sulfate	Activity of Enzyme (Units/ml)	Total Volume (ml)	Total Activity (units)
0-20	51.3	20	1026
20-45	145.0	40	5800
45-90	0	20	0
Supernatant	0	80	0

2. Second Ammonium Sulfate Fractionation

The first ammonium sulfate fraction (0-45% saturation) was divided into four portions by fractionation with ammonium sulfate. The fractions were 0-20, 20-30, and 30-40% saturation, and supernatant. The activity was found to be in the 20-30 and 30-40 fractions:

Saturation of Ammonium Sulfate (%)	Activity of Enzyme (Units/ml)	Total Volume (Ml)	Total Activity (Units)
0-20	12.3	20	246
20-30	189.0	20	3600
30-40	75.0	20	1500
Supernatant	5.4	40	216

In another experiment using 0-22, 22-35, and 35-50% saturation with ammonium sulfate the major activity was found in the 22-35 fraction:

Saturation of Ammonium Sulfate (%)	Activity of Enzyme (Units/ml)	Total Volume (ml)	Total Activity (units)
0-22	15.1	20	302
22-35	210.0	20	4200
35-50	31.3	20	626
Supernatant	0.0	40	0

3. <u>Calcium Phosphate Gel Adsorption</u>

The fraction which was brought down between 22-35% saturation was adsorbed by calcium phosphate gel. To each 2.0 ml of the enzyme solution, various amounts of calcium phosphate suspension (10 mg dry weight per ml) and phosphate buffer (pH 7.0) were added. The total volume was 4.0 ml. The mixtures were kept in an ice bath and stirred with stirring rod for 5 minutes, after which they were centrifuged and enzyme activity and protein were determined on the supernatant. The results are given in Table V. It was found that 1.0 ml of calcium phosphate gel had adsorbed most of the enzyme present in the mixtures.

Table V

Adsorption of Nitrate Reductase by Calcium Phosphate Gel

Enzyme Preparation	Calcium Phosphate	Phosphate Buffer	Total Volume	Enzyme Activity
(ml)	(ml)	(ml)	(ml)	(units/ml)
2.0	0.00	2.00	4.00	751
2.0	0.10	1.90	4.00	710
2.0	0.20	1.80	4.00	650
2.0	0.50	1.50	4.00	201
2.0	1.00	1.00	4.00	78
2.0	2.00	0.00	4.00	33

4.   Third Ammonium Sulfate Fractionation

The enzyme eluted from calcium phosphate gel by 0.1 M
pyrophosphate buffer, pH 7.0, was divided into four portions
by fractional precipitation with ammonium sulfate. The fractions
were 0-25, 25-50, 50-90 and supernatant. The activity was
found to be in the 0-25 and in the 25-50 fractions.

Saturation of Ammonium Sulfate (%)	Activity of Enzyme (units/ml)	Total Volume (ml)	Total Activity (units)
0-25	184	5	920
25-50	275	5	1350
50-90	0	5	0
Supernatant	0	20	0

As a result of the above experiment, the method of
purification adopted was as follows. Unless otherwise specified,
all steps of the fractionation were carried out at 0-4° C. The
precipitates were centrifuged at approximately 5000 x g at 0° C
for 10 minutes.

Approximately 80 g of the leaves of 50-day-old sugar
beet plants was ground in a Waring Blender with 320 ml of 0.1 M
potassium phosphate buffer, at pH 7.8, containing 10^{-3} M
reduced glutathione. The homogenate was centrifuged for
20 minutes at 20,000 x g. The clear, brownish yellow super-
natant solution comprised the crude, cell-free extract
(Fraction I).

To this Fraction I was added 100.80 g solid ammonium

sulfate, the suspension was allowed to stand for 10 minutes,

then was centrifuged for 10 minutes. This 0-45% ammonium

sulfate precipitate is referred to as Fraction II. The pre-

cipitate was dissolved in 160 ml of cold 0.1 M potassium

phosphate buffer, pH 7.0, containing 10^{-3} M reduced gluta-

thione. To the solution of Fraction II was added 24.60 g solid

ammonium sulfate, and after standing 10 minutes the precipitate

was centrifuged down and discarded. The supernatant solution

was treated with a further 14.6 g ammonium sulfate and again

centrifuged. This precipitate, which is the 22-35% ammonium

sulfate precipitate (Fraction III), was dissolved in 40 ml of

0.1 M potassium phosphate buffer, pH 7.0, containing 10^{-3} M

reduced glutathione, and stored at -17° C.

After thawing, the Fraction III solution was diluted

to 80 ml with potassium phosphate buffer and treated with 40

ml of calcium phosphate gel (10 mg dry weight per ml). After

15 minutes, the precipitate was collected by centrifugation,

washed once with a 5 ml portion of cold 0.1 M phosphate buffer,

pH 7.5, and eluted twice with 20 ml of cold 0.1 M pyrophosphate

buffer, pH 7.0. The pyrophosphate eluate (Fraction IV) showed

about 17.3% of the total activity of the original crude

extract and an overall purification of 20 times.

To Fraction IV (40 ml) 14 g of solid ammonium

sulfate was added to give 50% saturation. After standing

for 15 minutes at 0° C, the precipitate was collected by

centrifugation and dissolved in 20 ml of cold 0.1 M

phosphate buffer, pH 7.0, containing 10^{-3} M reduced

glutathione. The enzyme activity on this final fraction

(Fraction V) was 15% of the unit in the crude starting

materials. The overall purification was 60-fold.

Table VI

Summary of Purification of Sugar Beet NRase

Fraction	Total volume (ml)	Total protein (mg)	Total units	Specific activity	Percent recovery
I. Crude enzyme	320	1856	39,000	21	100
II. 0-45% Ammonium sulfate ppt.	160	518	26,400	51	67.7
III. 22-35% Ammonium sulfate ppt.	80	112	14,000	125	35.9
IV. Calcium phosphate gel eluate	40	15	6,750	450	17.5
V. 0-50% ammonium sulfate ppt.	20	5	6,250	1250	16.0

II. CHARACTERIZATION OF NITRATE REDUCTASE

1. Stability of Enzyme

The effect of protectants during the extraction of the enzyme from sugar beet leaves is shown in Fig. 1. It indiates that in the presence of 10^{-3} M cysteine in the extraction medium, the activity of NRase is three times greater than in the media which contained no protectant and that glutathione is as effective as cysteine.

The data relative to loss of the enzyme activity during storage are presented in Fig. 2. The percentage residual activity of the enzyme when stored at the various temperatures has been plotted against time. In general, the enzyme was found to be extremely unstable, particularly at the higher temperatures. The higher the storage temperature, the faster the rate of inactivation. The extent of inactivation was also directly related to the length of storage time.

When the enzyme was stored in a frozen state (-15° C), a loss of only 10 to 20% of its activity was observed in three weeks, revealing that it was quite stable in the frozen state. Storage at 4^{0} C resulted in a loss of 30 and 60% of its activity in 6 and 9 hours, respectively. At 25° C, the enzyme lost its activity almost completely in 6 hours.
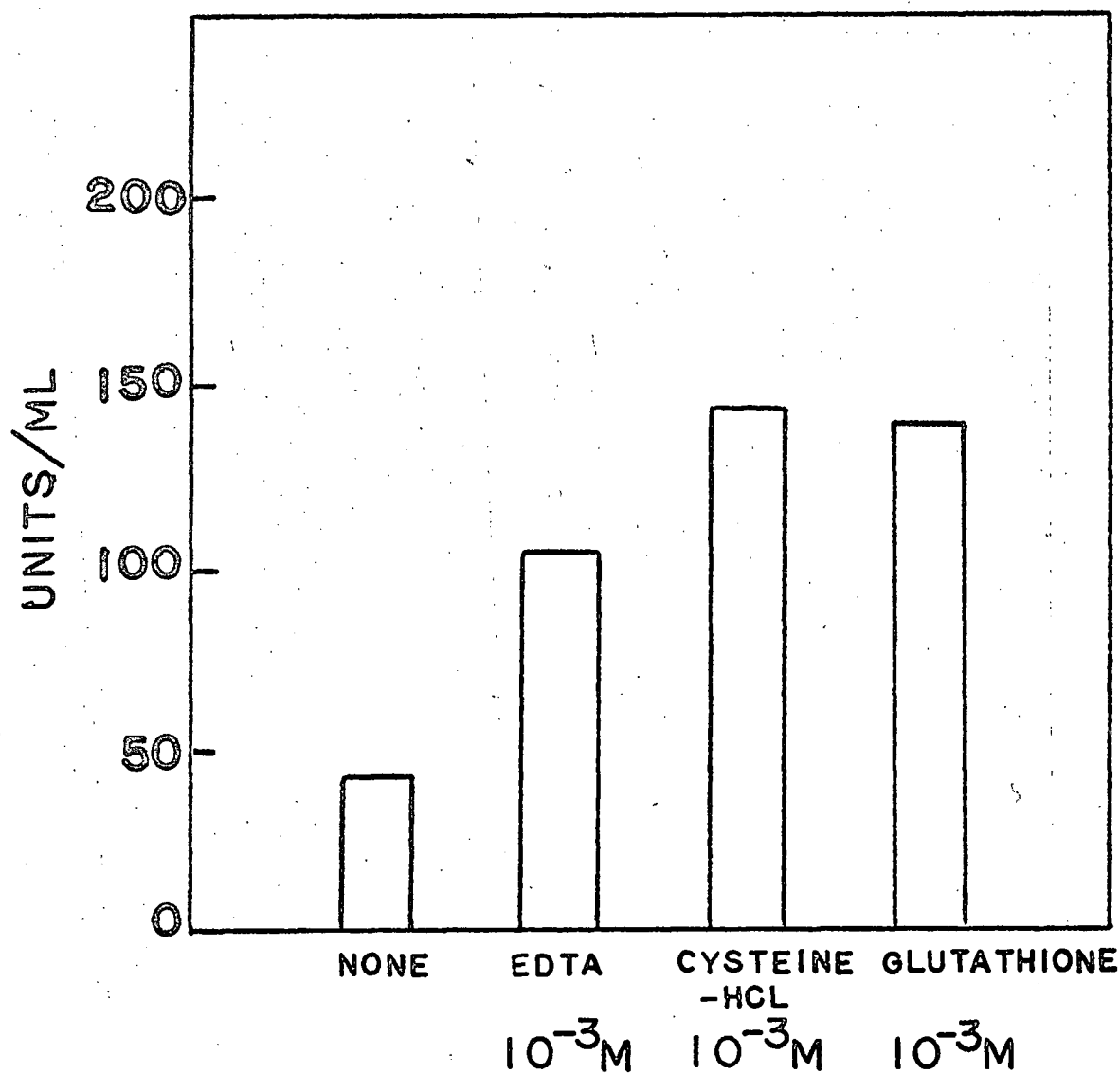
Fig.1. Effect of protectants in the extraction media
on the level of nitrate reductase activity
in sugar beet extracts.
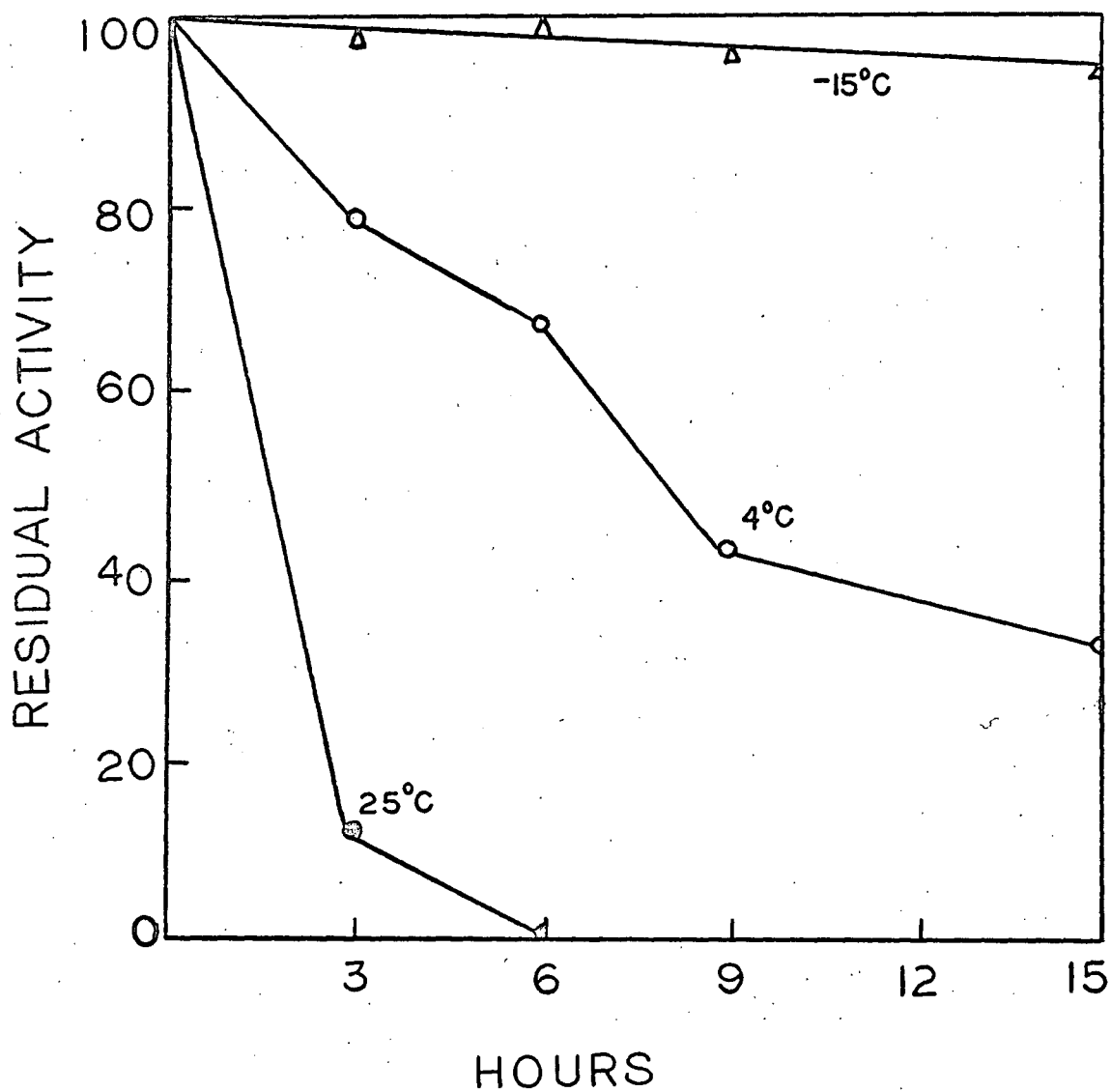
Fig. 2. Stability of nitrate reductase (Fraction V)
in phosphate buffer, pH 7.0, containing
10^{-3} M glutathione stored at various
temperatures for different lengths of
time.

2. Time Course of Enzymatic Reaction

A typical time-course of the nitrite formation from nitrate is shown in Fig. 3. In the presence of the complete system the rate of nitrate reduction is essentially constant within 25 minutes.

3. Enzyme Activity and Enzyme Concentration

Fig. 4 illustrates the relationship of the concentration of enzyme to the amount of nitrite formed. It can be seen that activity is linear for quantities of enzyme up to 80 units.

4. pH Optimum

The effect of pH on the activity of the enzyme is shown in Fig. 5. The enzyme catalyzed the reduction of nitrate over a wide pH range, 6.0 to 10.0, with a maximum activity at pH 7.0. The curve indicates that the enzyme has a very sharp optimum peak. Below Ph 5.0 no enzyme activity could be detected.

5. Substrate Affinity

The effect of nitrate concentration upon the reaction velocity is shown in Fig. 6. The maximum rate was obtained at 10 uM per ml, and the amount necessary for the one-half maximal rate was approximately 0.5 uM per ml. The Michaelis
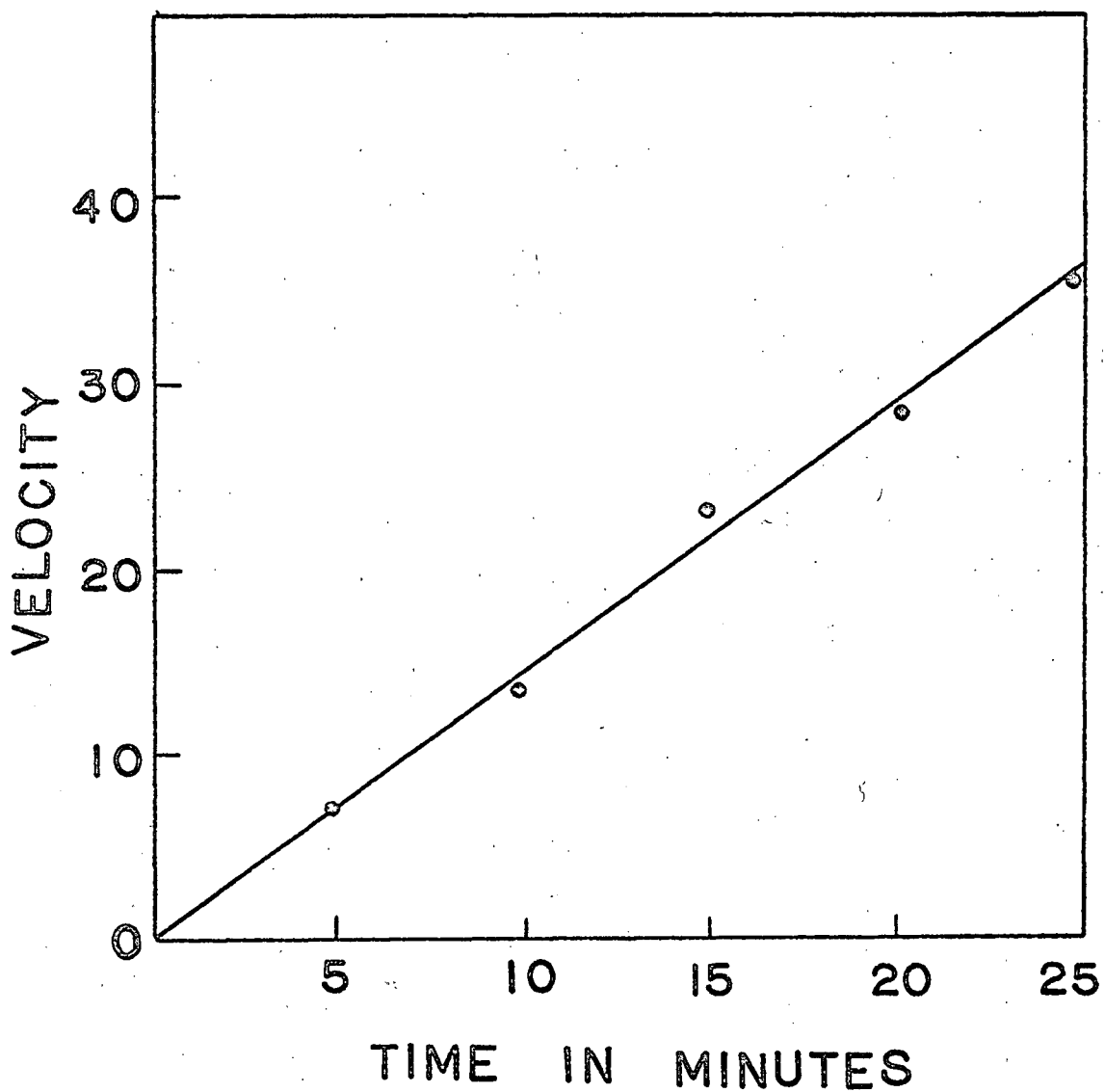
Fig. 3. Proportionality of enzyme activity with
time. The standard assay procedure was
used with 35 units of Fraction V enzyme.
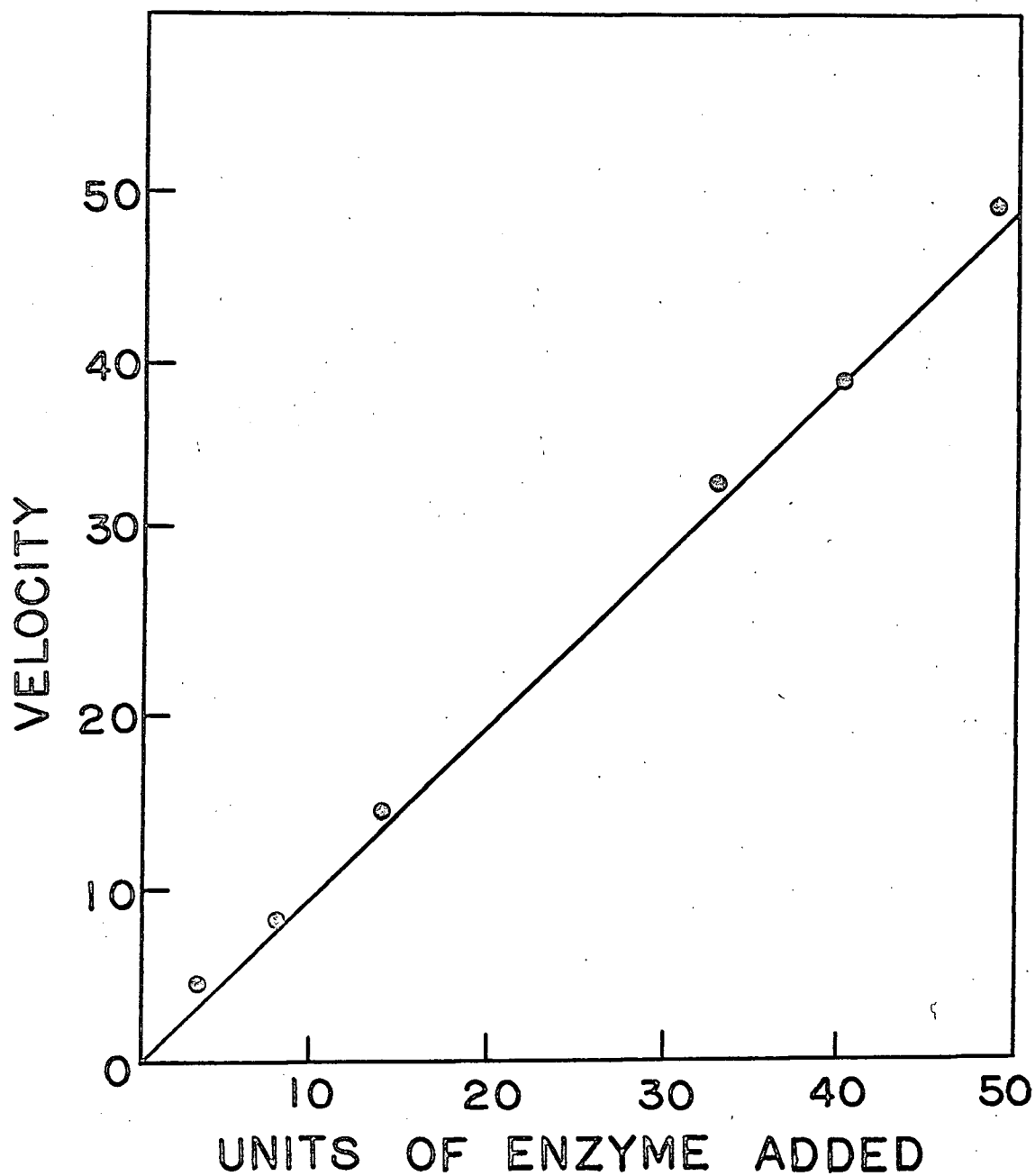
Fig. 4. Proportionality of enzyme activity with
enzyme concentration. The standard assay
procedure was used with variation in
volume of enzyme (Fraction V).
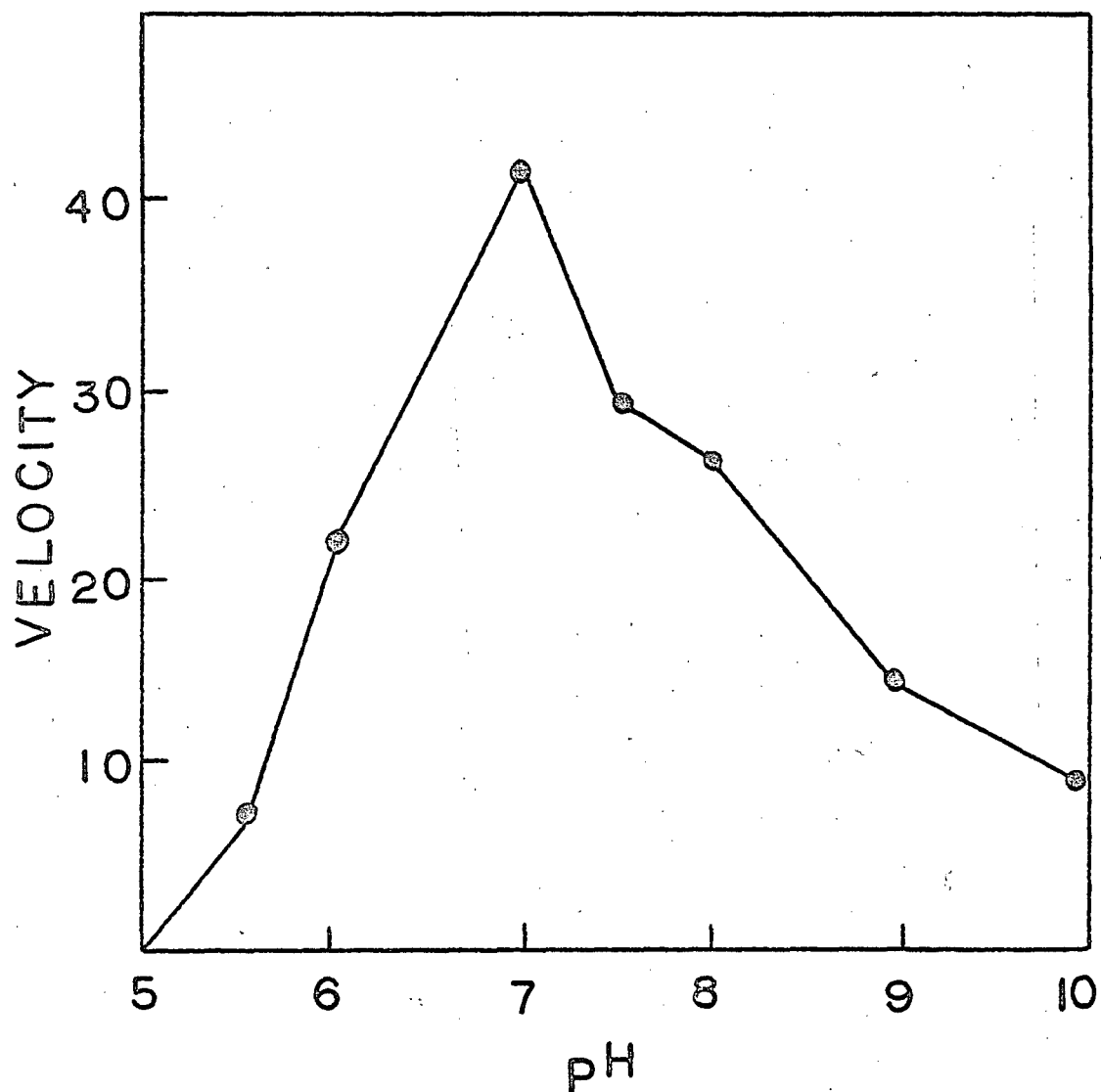
Fig. 5. Effect of pH on nitrate reductase activity.
The standard assay procedure was used,
except for varying pH values. 42 units
of enzyme (Fraction V) was used.
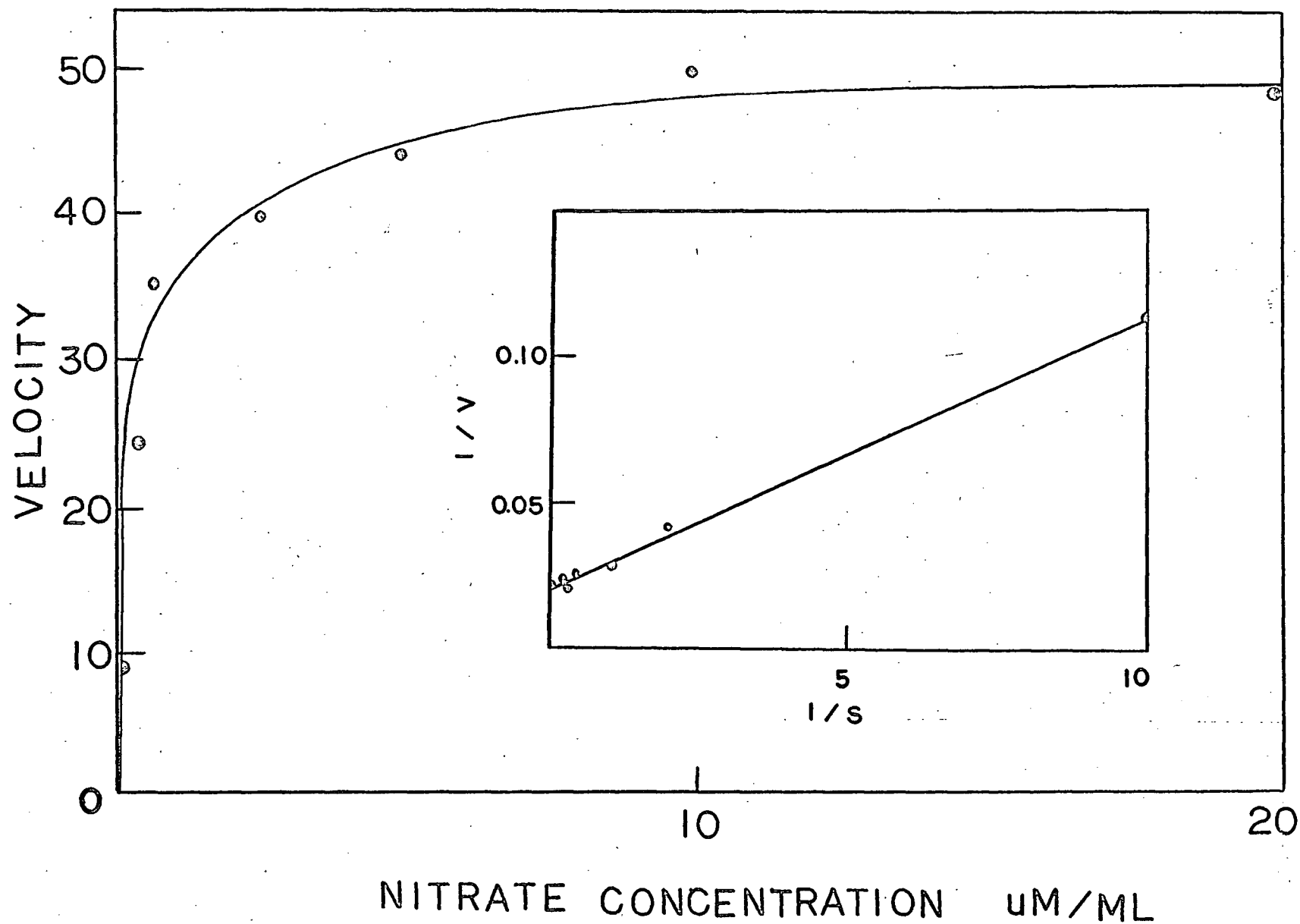
Fig. 6. Effect of nitrate concentration on nitrate reductase activity.

constant, obtained by the method of Lineweaver and Burk
(1934), was $Km = 4.5 \times 10^{-4}$ M per liter.

6. Specificity for Electron Donor

Fig. 7 shows that the highly purified nitrate
reductase from sugar beet leaves appeared to be specific for
DPNH as a source of electron TPNH was completely inactive as
an electron donor. The Km value for DPNH as estimated from the
curve in Fig. 7 is 5.0×10^{-5} M.

As shown in Table VII, reduced FAD, FMN, and molybdate
were all effective electron donors for nitrate reductase.
Among the artificial donors, pyocyanide and phenazine metho-
sulfate, both one-electron donors with very low redox potential,
give a low rate of enzymic activity. Reducing agents as
glutathione and ascorbate were inactive as electron donors.

7. Effect of Temperature on Activity

In order to evaluate the activity of the enzyme at
different temperatures, the reaction mixtures were incubated
for 15 minutes at various temperatures. The results are
shown in Table VIII. It is shown that at 0° C there is no
detectable enzymatic activity. From 10° to 30° C, enzymatic
activity is proportional to the temperature increase. At
50° C, 33% of the activity remained, at 60° C only 4%.
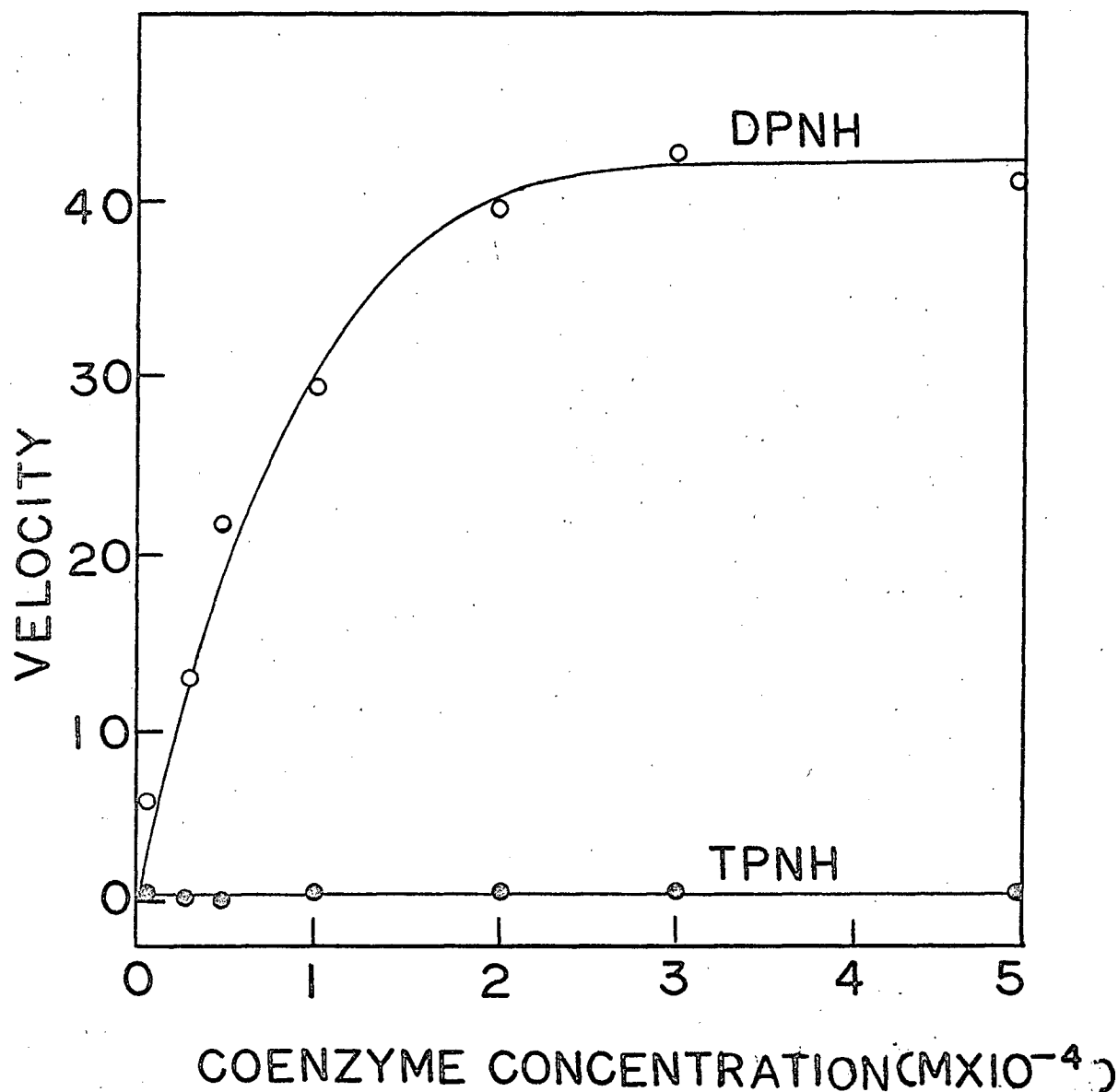
Fig. 7. Effect of reduced pyridine nucleotide
concentration on nitrate reductase
activity. The standard assay procedure
was used with 42 units of Fraction V
enzyme.

Table VII

The Effect of Various Hydrogen Donors on NRase Activity

Fraction V enzyme was used. The reaction mixtures were incubated
anaerobically with the appropriate hydrogen donor in Thunberg tubes.
The hydrogen donor was dissolved in 0.1 M phosphate buffer, pH 7.0.

Hydrogen donor	Hydrogen donor final concentration	Relative activity
	(M)	(%)
DPNH	2×10^{-4}	100
FAD*	2×10^{-4}	85
FMN*	2×10^{-4}	80
Sodium molybdate*	2×10^{-4}	80
Benzyl viologen*	2×10^{-4}	10
Methylene blue	2×10^{-4}	15
Pyocyanime*	1×10^{-3}	5
Phenazine methosulfate*	1×10^{-3}	5
Mammalian cytochrome c*	2×10^{-4}	10
Glutathione	1×10^{-3}	0
Sodium ascorbate	1×10^{-3}	0

* Reduced by sodium hydrosulfite

Table VIII

Effect of Temperature on Activity of NRase

Temperature	Nitrite formed	Relative activity
(^{o}C)	(muM)	(%)
0	0.00	0
10	8.40	37
20	16.00	74
30	22.80	100
40	28.00	122
50	7.60	33
60	1.00	4
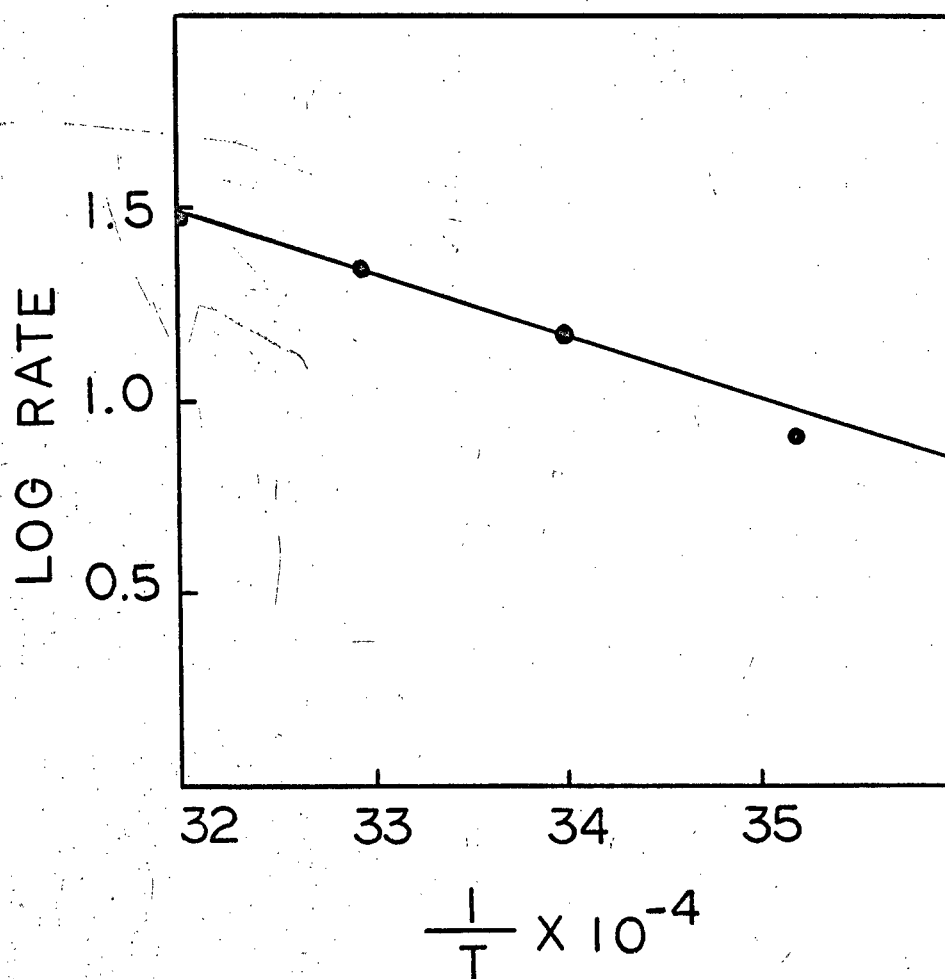
Fig. 8. Variation of activity with
temperature.

Figure 8 is an Arrhenius plot of the variation of the activity of the enzyme (Fraction V) with temperatures. Despite the fact that NRase is unstable at higher temperatures a linear relationship was obtained between 20° to 40° C because of the short duration of assay (15 minutes).

8. FAD as Prosthetic Group

The enzyme activity of Fraction I was not appreciably affected by the addition of FAD or FMN at a final concentration of 10^{-5} M. A very marked response to FAD, however, was demonstrated by Fraction V. The addition of FAD at a final concentration of 10^{-6} M caused a threefold stimulation. One half maximum reaction of the enzyme was obtained with FAD at approximately 1×10^{-7} M and with FMN at about 5×10^{-7} M. This greater effectiveness of FAD in activating the sugar beet nitrate reductase suggested that the prosthetic group of the enzyme is FAD.

The release and chromatography of the flavin from Fraction V by the method of Rao et al. (1962) resulted in a spot which showed a greenish fluorescence. The Rf values of this material and those of riboflavin, FMN, and FAD run in two solvent systems (Table IX) suggest that the substance released from Fraction V NRase is not riboflavin or FMN and that it may be FAD.
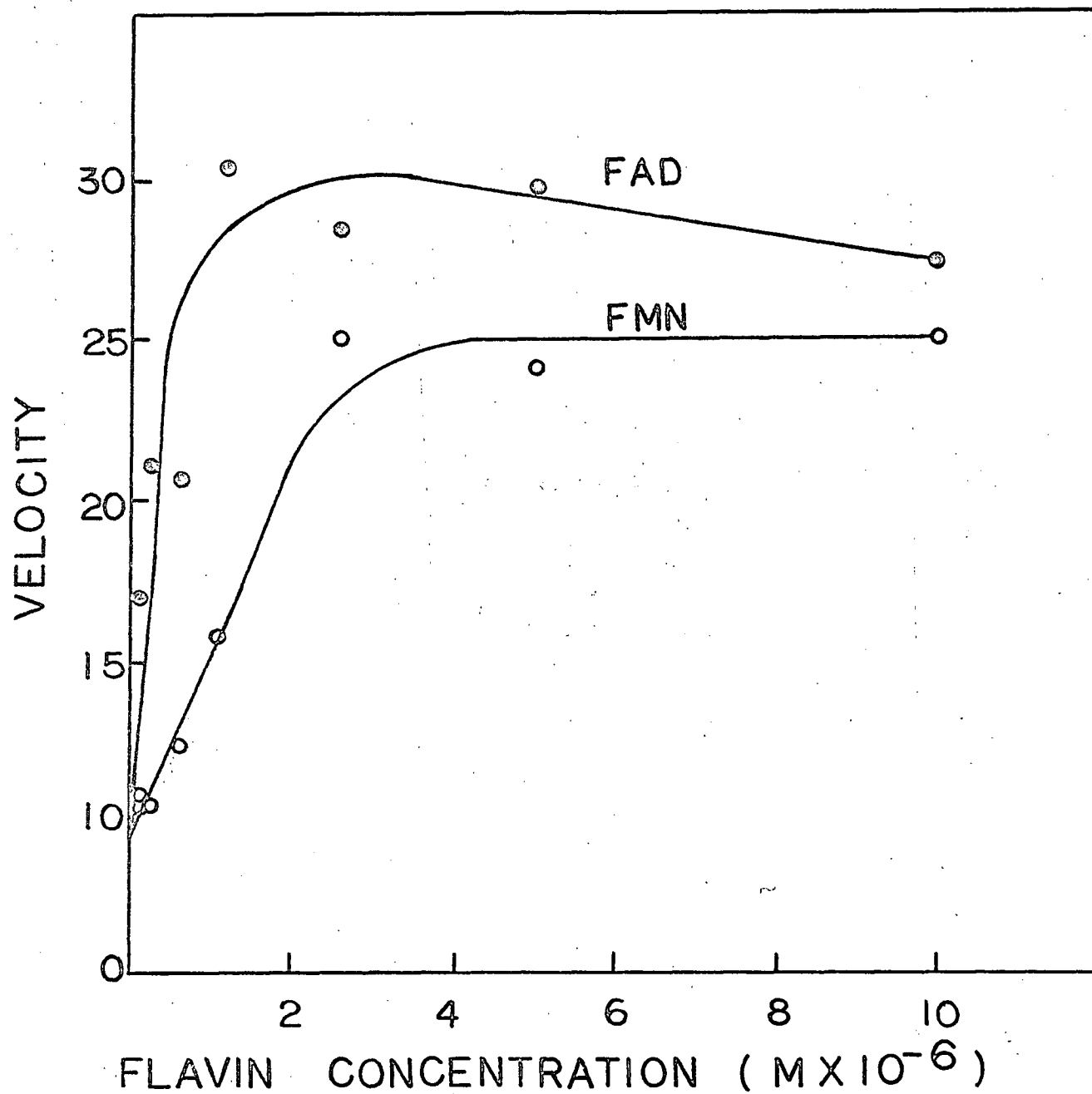
Fig. 9. Effect of flavin nucleotide concentration on nitrate
reductase activity. Preincubated for 5 minutes at
4° C with all components of the reaction mixture
except DPNH prior to the standard assay.

Nitrate reductase is not the only flavin-containing protein present in the enzyme preparation, hence precise identification of the prosthetic group must await further purification of the enzyme.

As shown in Table XV, the enzyme activity was strongly inhibited by atebrin and riboflavin, known flavoprotein inhibitors (Haas, 1944; Wright and Sabine, 1944; Helleman, Lindsay and Bovarmick, 1948), at a final concentration of 10^{-4} M. The inhibition by atebrin could be reversed by the addition of FAD.

Table IX

Paper Chromatography of Nitrate Reductase Flavin

Solvent System	Riboflavin	Rf x 100		Compound from Fraction V sugar beet NRase
		FMN	FAD	
n-Butanol acetic acid water (4:1:5)	50	32	19	20
Na_2HPO_4 (5%)	33	55	38	40

9. Sulfhydryl Nature

Partially purified NRase of the sugar beet was found to be sharply inhibited by p-chloromercuribenzoate. This is

similar to the results obtained by Evans and Nason (1953)

for NRase of soybean leaves. Table X summarizes the results

of the experiment demonstrating the inhibition by p-

chloromercuribenzoate and its reversal by cysteine and gluta-

thione. Preincubation of the enzyme with concentrations of

10^{-3} M and 10^{-5} M p-chloromercuribenzoate resulted in 100 and

78% inhibition respectively.

Table X

Inhibition of NRase by p-chloromercuribenzoate and its Reversal

by Sulfhydryl Compounds

Reaction mixture contained p-chloromercuribenzoate indicated,
nitrate reductase (Fraction IV) and all constituents with the
exception of the sulfhydryl compound and electron donor. The
mixtures were preincubated for 10 minutes in ice bath. Cysteine
hydrochloride or glutathione was added as indicated, followed by
an equivalent amount of Na_2CO_3. When no sulfhydryl reagents
were used, water was added to maintain equal volume. The
mixtures were again incubated for 5 minutes at 4° C and then
assayed for nitrite reductase activity in the usual manner.

Reaction	p-chloromer-curibenzoate	Cysteine-HCl	Glutathione	Inhibition
No.	Final Conc.(M)	Final Conc.(M)	Final Conc.(M)	(%)
1	10^{-3}	-	-	100
2	10^{-5}	-	-	78
3	10^{-3}	10^{-3}	-	48
4	10^{-5}	10^{-3}	-	18
5	10^{-3}	-	10^{-3}	36
6	10^{-5}	-	10^{-3}	11

10. <u>Dialysis of Enzyme</u>

Nicholas and Nason (1953) have indicated that the dialysis of Neurospora NRase against cyanide resulted in an inactivation of the enzyme and that the dialyzed enzyme was reactivated by molybdenum salts. This was taken to indicate that the metal constituents of this NRase was molybdenum.

In order to study the metal constituent of sugar beet nitrate reductase, the following experiments were carried out:

(a) Dialysis of Nitrate Reductase

As recommended by Nicholas and Nason (1953), some of the cellophane dialyzing tubes to be employed in the dialysis of the enzyme were first used to dialyze a 2% solution of egg albumin for 12 hours against a phosphate buffer, pH 7.0, and then were bathed for 12 hours in the various dialyzing solutions listed in Table XI. Three ml of the partially purified enzyme (Fraction III) was dialyzed against 500 ml of the dialyzing solution at 4^0 C. Table XI summarizes the results of the experiments.

Dialysis resulted in a higher activity of the enzyme. This could have been the consequence of the removal of ammonium sulfate or of an endogenous inhibitor from the preparation. Pre-treatment of the dialyzing tubes did not result in more effective dialysis. The presence of 10^{-3} M glutathione in the dialyzing solution resulted in a much greater activity of the enzyme at the end of 6 and 12 hours dialysis.

Table XI

Influence of Dialysis Against Various Reagents on the Activity of NRase

Dialysis	Activity of enzyme[*] Period of dialysis (hrs)			
	0	3	6	12
Experiment A				
No dialysis	25	18	15	9
Untreated tube. Dialysis against 0.1 M phosphate	25	38	21	5
Treated tube.[**] Dialyzed against 0.1 M phosphate containing 10^{-3} M glutathione	25	40	32	18
Experiment B				
No dialysis	23	15	12	10
Treated tube.[**] Dialyzed against 0.1 M phosphate containing 10^{-3} M glutathione and 10^{-3} M cyanide	10	6	0	0

[*] Units of enzyme activity per 0.1 ml of Fraction III enzyme

[**] Tube bathed for 12 hours in solution listed and 3 ml of enzyme preparation subsequently dialyzed against 500 ml of the same solution at pH 7.0, at 4° C.

(b) Attempt to reactivate the Cyanide-dialyzed

Enzyme

The partially purified enzyme (Fraction III) was

dialyzed for 6 hours at 4° C against 0.1 M phosphate buffer,

pH 7.0, containing 10^{-3} M glutathione and 10^{-3} M potassium

cyanide. It was then redialyzed for 3 hours against 0.1 M

phosphate buffer, pH 7.0, containing 10^{-3} M glutathione to

remove KCN. Distilled water, which was used to prepare this

buffer solution, had been treated to remove metals by the

method of Nicholas (1952).

At the end of dialysis, various metals were added to

the enzyme solution at a final concentration of 1 mM and it

was preincubated at 4° C for 10 minutes before testing its

activity. The following compounds were added to the reaction

mixture:

$FeSO_4$, $AgNO_3$, $Na_2Cr_2O_7$, $CoCl_2$, $NiSO_4$, $MnSO_4$, $FeCl_3$, Na_2WO_4

$ZnSO_4$, Na_2MoO_4, MoO_3. It was found that the addition of

these compounds failed to restore enzyme activity.

11. Phosphate Stimulation

In order to identify the requirement of phosphate in

the reduction of nitrate, the NRase was extracted with 0.1 M

Tris buffer, pH 7.8, and subjected to 0-45 and 22-35% ammonium

sulfate fractionations. The final preparation was dissolved

in 0.1 M Tris buffer, pH 7.0, and dialyzed against 0.01 M

Tris buffer, pH 7.0, for one hour at 4° C.

The effect of adding graded amounts of phosphate to

0.1 ml of the partially purified enzyme is shown in Table

XII.

In the experiment, maximal activity was obtained at a

level of approximately 1.0 uM phosphate. It is not certain

whether the requirement for phosphate is absolute, a

preparation dialyzed to reduce phosphate may contain enough

to account for the residue activity.

The results in Table XIII show that the phosphate

can be replaced partially by arsenate and selenate. Instead

of stimulating, vanadate and silicate inhibited the enzyme

activity.

Table XII

Activation of NRase by Phosphate

Phosphate final concentration	Nitrite formed
(uM)	(muM)
0.00	40.8
0.05	52.4
0.10	58.0
0.20	60.8
0.50	64.0
1.00	65.2

Table XIII

The Substitution of Other Anions for Phosphate in the Activation of NRase

Addition	Final concentration	Nitrite formed	Substitution
	(uM)	(uM)	(%)
None		40.0	
Potassium phosphate	1.0	62.4	-
Sodium sulfate	10.0	36.8	0
Sodium tungstate	10.0	32.0	0
Sodium arsenate	10.0	52.0	53
ATP	10.0	34.0	0
Sodium borate	10.0	21.0	0
Sodium selenate	10.0	42.4	10
Sodium orthovanadate	10.0	10.0	0
Sodium silicate	10.0	16.0	0
Sodium molybdate	10.0	24.0	0

12. Inhibition

(a) Inhibition by Heavy Metals

Sugar beet NRase activity was inhibited in the presence of heavy metals (Table XIV). The addition of cupric sulfate at 10^{-4} M resulted in an inhibition of 100% in the rate of reaction. Sodium vanadate at 10^{-4} M also caused a complete inhibition. Other metal compounds such as sodium chromate, cobalt chloride, zinc sulfate and sodium asenate also strongly inhibited the activity of this enzyme.

(b) Inhibition by Metal-chelating Agents

Effects of various metal-chelating agents are summarized in Table XV. Sugar beet NRase is very sensitive to KCN and is inhibited approximately 60% at 10^{-5} M final concentration. A number of other chelating agents at higher concentration inhibited the enzyme, indicating the presence of metal as an active constituent in the system. No inhibition was observed on addition of EDTA or sodium fluoride and only slight inhibition was obtained with thiourea.

(c) Inhibition by Amytal and Atebrin

Amytal, a well-known inhibitor of mitochondria electron transport, at 10^{-4} M final concentration caused approximately 50% inhibition. Atebrin is a competitive inhibitor with respect to flavin in the assay system of nitrate reductase. Its inhibition could be reversed by the addition of FAD, as shown in Table XVI.

(d) Inhibition by Phenolic Compounds

Several quinones have previously been shown to serve as electron carriers for flavoprotein enzymes. Indeed, menadione was found to mediate anaerobic reduction of nitrate by E. coli respiratory NRase. However, the analogy between respiratory NRase and assimilatory NRase failed in this instance, since menadione is strikingly effective as an inhibitor of sugar beet NRase, an assimilatory enzyme. The enzyme was 50% inhibited by a menadione concentration of 10^{-4} M.

In view of the unexpected inhibition of sugar beet NRase by manadione, it appeared of interest to assay the effects of various phenolic compounds on NRase of this plant. Of particular interest were the findings with benzoquinone and pyrocatechol. As shown in Table XVII, the presence of 10^{-6} M p-benzoquinone or pyrocatechol resulted in 50% inhibition of nitrate reduction. Other phenolic compounds such as hydroxyquinone, 4-carboxycatechol, phloroglucinol and pyrogallol all inhibited the enzyme activity at different rates.

Table XIV

The Effect of Heavy Metals on the Activity of NRase

Reaction mixtures contained the compounds indicated, Fraction V enzyme, and all constituents of the standard assay mixture with the exception of DPNH. These were preincubated for 10 minutes at $4^{\circ}C$; then the standard assay was initiated by addition of the electron donor.

Additions*	Inhibition
	(%)
$NiSO_4$	37
Na_2CrO_7	61
$FeSO_4$	21
$CoCl_2$	40
$AgNO_3$	89
$CuSO_4$	100
Na_2WO_4	35
Na_2VO_4	100
$NaAsO_2$	68
$ZnSO_4$	71
$MnSO_4$	45

* At a final concentration of 1×10^{-4} M.

Table XV

The Effect of Various Metal-chelating Agents on the Activity of NRase

Reaction mixture contained the inhibitors indicated, Fraction V, and
all the constituents of the standard assay mixture with the exception
of electron donor. These were preincubated for 10 minutes at 4°C;
then the standard assay was initiated by the addition of DPNH.

Addition	Final concentration	Inhibition
	(M)	(%)
Potassium cyanide	1×10^{-3}	100
	1×10^{-4}	94
	1×10^{-5}	65
Sodium azide	1×10^{-3}	100
	1×10^{-4}	91
	1×10^{-5}	52
Tiron	1×10^{-4}	45
Thiourea	1×10^{-3}	40
a, a-Dipyridyl	1×10^{-4}	71
8-Hydroxyquinoline	1×10^{-4}	64
Phenylhydrazine	1×10^{-4}	42
o-Phenanthroline	1×10^{-4}	38
Sodium diethyl-dithiocarbamate	1×10^{-3}	35
Sodium citrate	1×10^{-3}	37
EDTA	1×10^{-3}	0
Sodium fluoride	1×10^{-3}	0

Table XVI

The Effect of Non-specific Inhibitors on the Activity of NRase

Reaction mixtures contained the inhibitors indicated, Fraction V, and all constituents of the standard assay mixture with the exception of electron donor. These were preincubated for 10 minutes at 4° C; then the standard assay was initiated by addition of electron donor.

Addition	Final concentration	Inhibition
	(M)	(%)
Ethyl urethane	10^{-4}	24
Hydroxylamine hydrochloride	10^{-4}	41
Sodium amytal	10^{-4}	48
Atebrin	10^{-4}	73
Atebrin	10^{-5}	51
Atebrin, FAD	10^{-5}, 10^{-4}	0
2,4-Dinitrophenol	10^{-4}	30
Sodium sulfite	10^{-4}	0
Riboflavin	10^{-4}	41
Iodoacetate	10^{-4}	54

Table XVII

The Effect of Phenolic Compounds on the Activity of NRase

Reaction mixtures contained the phenolic compound indicated, Fraction V enzyme, and all constituents of the standard assay mixture with the exception of electron donor. These were pre-incubated for 10 minutes at 4° C; then the standard assay was initiated by addition of DPNH.

Addition	Final concentration (M)	Inhibition (%)
p-Benzoquinone	1×10^{-4}	100
	1×10^{-5}	94
	1×10^{-6}	58
Pyrocatechol	1×10^{-4}	100
	1×10^{-5}	85
	1×10^{-6}	41
Menadione	1×10^{-4}	48
	1×10^{-5}	20
Hydroxyquinone	1×10^{-4}	52
4-Carboxycatechol	1×10^{-4}	45
Phloroglucinol	1×10^{-4}	38
Pyrogallol	1×10^{-4}	48

13. Separation of DPNH-nitrate Reductase from TPNH-nitrate

   Reductase

   During the course of this investigation each fraction

was examined for pyridine nucleotide specificity. It was

found that Fraction I, II, and III enzyme preparation could

use either DPNH or TPNH as electron donor. However, rates

with DPNH were greater than with TPNH in the range of two to

five times. When the enzyme was eluted from calcium phosphate

gel and then precipitated by ammonium sulfate (0-50% saturation),

only DPNH could be used as electron donor (Table XVIII).

   The ratios of activity between DPNH and TPNH for the

Fraction I, II, and III were 5.1, 2,4, and 3.7 respectively.

Due to the fact that the ratios in the three fractions differed,

it seems logical to postulate that more than one enzyme was

involved.

14. Other Enzymatic Activity Present in Partially Purified Enzyme

   The partially purified nitrate reductase preparations

contain other enzymatic activity. This included DPNH-quinone

reductase, DPNH-manadione reductase, DPNH-cytochrome c reductase

and DPNH-diaphorase. Other pyridine nucleotide enzymes such as

DPNH-glutathione reductase (Racker, 1955) and nitrite reductase

(Roussos and Nason, 1960) are not present in the enzyme pre-

paration Fraction V.

   As indicated in Table XIX, the first two enzymes,

DPNH-quinone reductase and DPNH-menadione reductase, seem to have been purified together with nitrate reductase. Each step in the purification of NRase resulted in a purification of other enzymes, although the degree of purification in each case is less than that achieved for the NRase.

15. Products and Stoichiometry of Reaction

The stoichiometry of sugar beet NRase was determined by measuring the concomitant oxidation of DPNH and the formation of nitrite in reaction mixtures with exactly the same composition as those employed in the standard assay. DPNH oxidation was observed at various intervals during the reaction period by measuring the decrease in absorption, at 340 mu, of aliquots of reaction mixture maintained in spectrophotometric cuvettes. The extinction coefficient 6.22×10^{6} sq. cm. x mole^{-1} at 340 mu was used to compute DPNH oxidation after first subtracting the endogenous activity observed in the absence of nitrate. At the same time, other aliquots of the reaction mixtures were removed and assayed.

The time course of the sugar beet NRase is presented in Fig. 10. There was a very rapid decrease in absorption in the presence of the complete system, and a moderate endogenous rate when all constituents except nitrate were

Table XVIII

Separation of DPNH-NRase from TPNH-NRase

The standard assay, with the exception of the final concentration
of electron donors being 1 uM, was used.

| Fraction | Enzyme Activity | | Ratio of Activity |
	DPNH	TPNH	DPNH/TPNH
	(units/mg protein)		
I. Crude extract	15.3	3.0	5.1
II. 0-45% Ammonium sulfate pp.	36.0	15.0	2.4
III. 22-35% Ammonium sulfate ppt.	81.0	21.9	3.7
IV. Calcium phosphate gel eluate	324.0	-	-
V. 0-50% Ammonium sulfate ppt.	975.0	-	-

Table XIX

Other Enzymatic Activities Present in the various NRase fractions

Enzyme	Enzyme fraction		
	I	II	V
Nitrate reductase			
Specific activity	21	125	1250
Purification		6	60
Quinone reductase			
Specific activity	175	1245	3750
Purification		7.1	21.3
Menadione reductase			
Specific activity	158	1016	2530
Purification		6.4	16.0
Cytochrome c reductase			
Specific activity	0.75	1.75	4.62
Purification		2.3	6.1
Diaphorase			
Specific activity	1.24	1.53	5.75
Purification		1.20	4.79

present. The addition of the DPNH-regenerating system
containing sodium lactate and yeast lactate dehydrogenase
restored the absorption of DPNH to the original level.
Thus, DPN$^+$ was shown to be one of the products of sugar
beet NRase. Table XX shows a mole for mole relationship
between DPNH oxidation and nitrite formation. These
results are in agreement with the conclusion that nitrate
reduction by this system proceeds according to the follow-
ing equation:

$$NO_3^- \quad + \quad DPNH \quad + \quad H^+ \longrightarrow NO_2^- \quad + \quad DPN^+ \quad + \quad H_2O$$
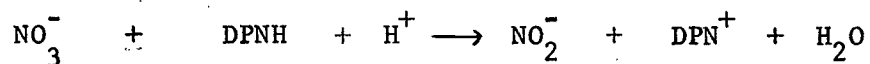
Table XX

Stoichiometry of NRase Reaction

The enzymatic rate of DPNH oxidation was calculated from Curves A
and B of Fig. 10. Nitrite formed was determined in 0.5 ml aliquots
of the complete reaction mixture under the condition of standard
assay.

Time of incubation	DPNH Oxidation	Nitrite formed	Ratio of DPNH oxidized / Nitrite formed
(min.)	(mu moles/ml)	(mu moles/ml)	
0	0.0	0.0	0.00
2	11.2	10.8	1.03
4	24.1	21.5	1.12
5	36.9	34.5	1.07
8	50.6	45.6	1.10
10	61.0	56.7	1.07
12	70.7	65.5	1.03
14	78.7	70.7	1.10
16	85.2	75.4	1.11
18	86.0	76.9	1.10
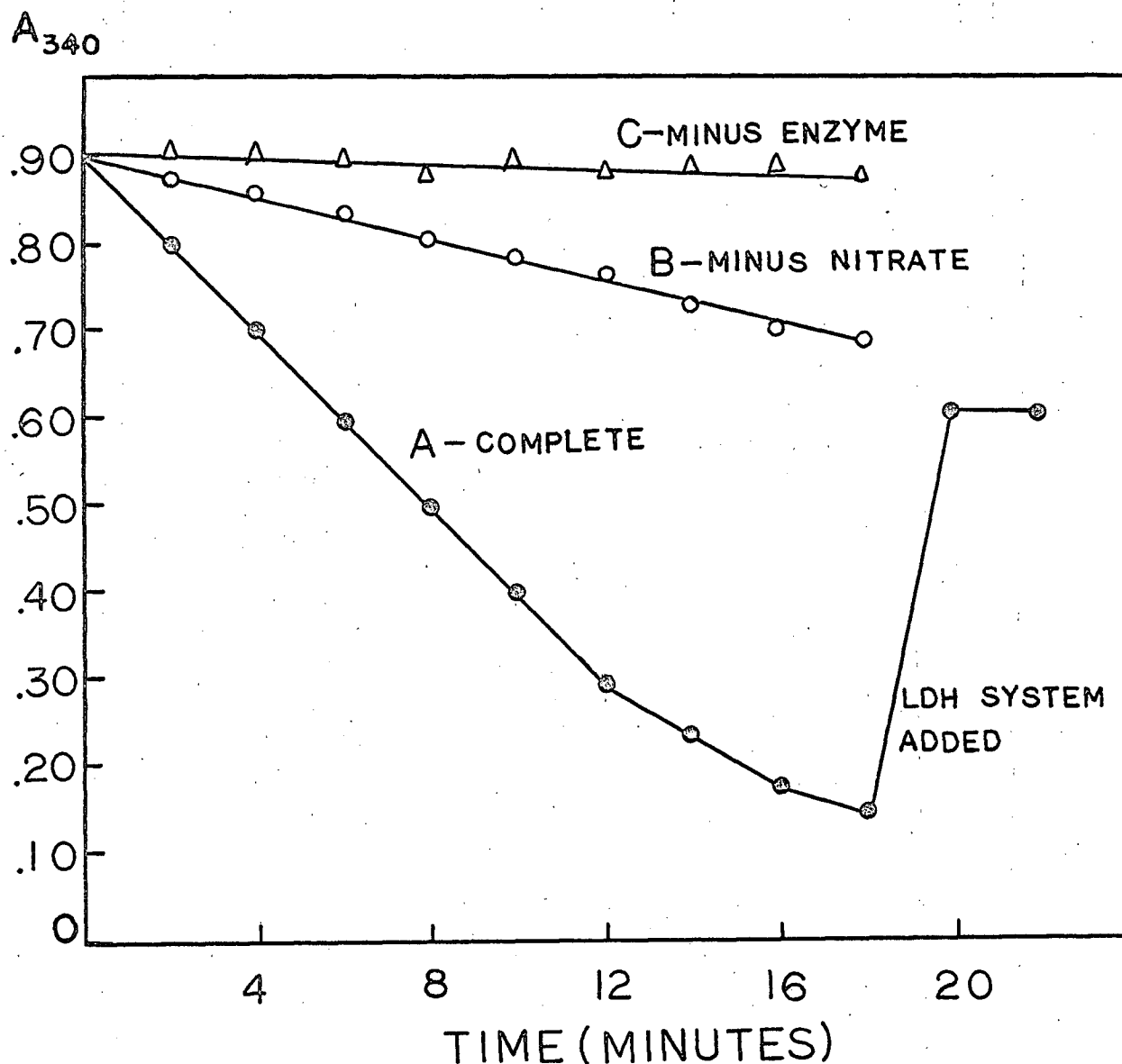
Fig. 10. Time course of nitrate reductase activity as followed
by the oxidation of DPNH. Reaction mixtures con-
tained the following in a final volume of 3.0 ml:
Curve A represents the complete reaction mixture
containing 220 units of the enzyme (Fraction V),
60 uM KNO$_3$, 0.6 uM DPNH, 60 muM FAD and 600 uM
phosphate buffer at pH 7.0; curve B represents
the same as Curve A, except that KNO$_3$ was omitted.
Curve C represents the same as curve A, except
that the enzyme was omitted. After 18 minutes,
the complete reaction mixture was poured into a
small beaker containing 2.5 ml of 0.1 M glycine
buffer, pH 10.2, 0.3 ml of 15% sodium D L-lactate
and 0.2 ml of lactic dehydrogenase and the reaction
mixture read again at 340 mu. Subsequently indi-
cated A 340 reading represents the original
readings x 2.

III. EFFECT OF AGE OF PLANT ON NITRATE REDUCTASE ACTIVITY

The effect of age of plant on the level of extractable
NRase is shown in Table XXI. The results indicate that the
crude extract from the leaf of two-weeks old sugar beet
seedlings contained a very low enzyme activity. The level of
enzyme activity increased rapidly between four and six weeks
of growing. During this time a concomitant increase in leaf
area was observed. The activity became constant from the
age of 8 weeks.

Table XXI

Effect of Age of Plant on NRase Activity in the Crude Extract of

Sugar Beet Leaves.

Age of Plant	Enzyme Activity	Protein Content	Specific Activity
(weeks)	(units/g f.wt.)	(mg/g f.wt.)	
2	71	9.5	7.4
4	212	14.4	14.7
6	457	18.3	24.4
8	504	22.7	21.3
10	465	21.2	21.4
12	495	24.3	20.3

IV. DISTRIBUTION OF NITRATE REDUCTASE IN THE SUGAR BEET PLANT

The distribution of nitrate and NRase activity in the sugar beet plant is given in Table XXII. In general, the concentration of nitrate in the petiole was five to ten times higher than that in the lamina. A very low content of nitrate was found in the roots.

Table XXII also shows that the activity of NRase of the lamina was roughly triple that of the petiole. No appreciable enzyme activity could be detected in the extracts of roots.

Table XXII

Distribution of NRase Activity in Beta vulgaris Seedlings

Regions	Dry wt.	Chlorophyll	Nitrate	Protein	NRase
	mg/g fresh wt.	ug/mg dry wt.	ug NO_3 N/mg dry wt.	ug/mg dry wt.	units/mg dry wt.
Lamina	98.5	9.71	3.55	167.51	3.60
Petiole	76.0	1.58	16.44	63.15	1.32
Root	185.0	0.00	1.02	29.12	0.00

V. SUB-CELLULAR LOCALIZATION OF SUGAR BEET NITRATE REDUCTASE

The sub-cellular distribution of NRase from sugar beet leaves (lamina) is summarized in Table XXIII.

An average of 60% of the total NRase was recovered after fractionation. Nearly 90% of the recovered enzyme activity was found in the microsome-free supernatant. Only a slight enzymatic activity resided in chloroplast and in mitochondrial fractions.

Chlorophyll was present in all cellular fractions. A concomitant release of NRase from ruptured chloroplasts could have occurred if the enzyme were indeed present within the chloroplast. The method of Stocking (1959) which employed a non-aqueous medium was used to clarify this point.

Following Stocking's procedure, 5 g of sugar beet leaves was removed and placed in a cellophane bag. This was stored at -15° C for 30 minutes. The midveins and the major lateral veins were then removed from the sugar beet leaves and discarded. The material was then ground in a homogenizer for 2 minutes with a cold hexane-carbon tetrachloride mixture of specific gravity 1.3, using 20 ml of hexane-CCl_4 per gram of tissue.

This suspension was filtered through a pad of glass wool and cheesecloth and centrifuged for 15 minutes at 12,000 x g. The green pellet, which accumulated near the surface

of the liquid, was collected. The green pellet was dissolved

in 100 ml of hexane, stirred with a glass rod, and centri-

fuged for 5 minutes at 1000 x g. The sediment obtained from

this centrifugation was dissolved in 10 ml of 0.01 M phosphate

buffer containing 10^{-3} M glutathione. After standing at a

temperature of -4° C for 10 minutes, the suspension was

centrifuged for 10 minutes at 1000 x g. The supernatant was

collected and tested for NRase activity. No enzymatic activity

could be detected in the extract of chloroplasts.

Table XXIII

Sub-Cellular Localization of Sugar Beet NRase

Gravity	Time	Fraction	Total chlorophyll	Total protein	Total NRase
(xg)	(min.)		(mg)	(mg)	(unit)
		homogenate*	13.00	265.00	1240
200	2	Cellular debris and nuclei	1.04	10.43	**
1,000	7	Chloroplasts	8.12	79.26	45
10,000	30	Mitochondria	3.49	6.40	27
105,000	30	"Microsome"	0.22	15.80	**
		Supernatant	0.13	114.75	839

* Starting material: 5 g of sugar beet lamina
** No detectable enzyme activity

VI. FLAVIN NUCLEOTIDE-CATALYZED ENZYMATIC PHOTOREDUCTION OF NITRATE

1. Photoreduction of Flavin Nucleotides

The time course of both FAD and FMN photoreduction under anaerobic conditions is presented in Fig. 11 and Fig. 12. From the data given it is noted that the FMN was photoreduced at a moderate rate in the absence of EDTA or methionine but that the addition of these activators markedly stimulated the rate of photoreduction of flavin nucleotide. Similarly, the presence of EDTA or methionine was required before any photoreduction of FAD was observed.

2. Characterization of Flavin Nucleotide-Catalyzed Enzymatic Photoreduction of Nitrate

Table XXIV shows that flavin nucleotide-catalyzed enzymic photoreduction of nitrate can couple with the flavin nucleotide photochemical reduction in which EDTA acts as an electron donor in an anaerobic photochemical reduction of flavin nucleotides. The reaction did not proceed in the dark and in the absence of nitrate or sugar beet NRase. FAD could be replaced by FMN at the same concentration. In the absence of EDTA or under aerobic condition, a slight photoreduction was observed. The highest activity was obtained by the addition of DPNH.
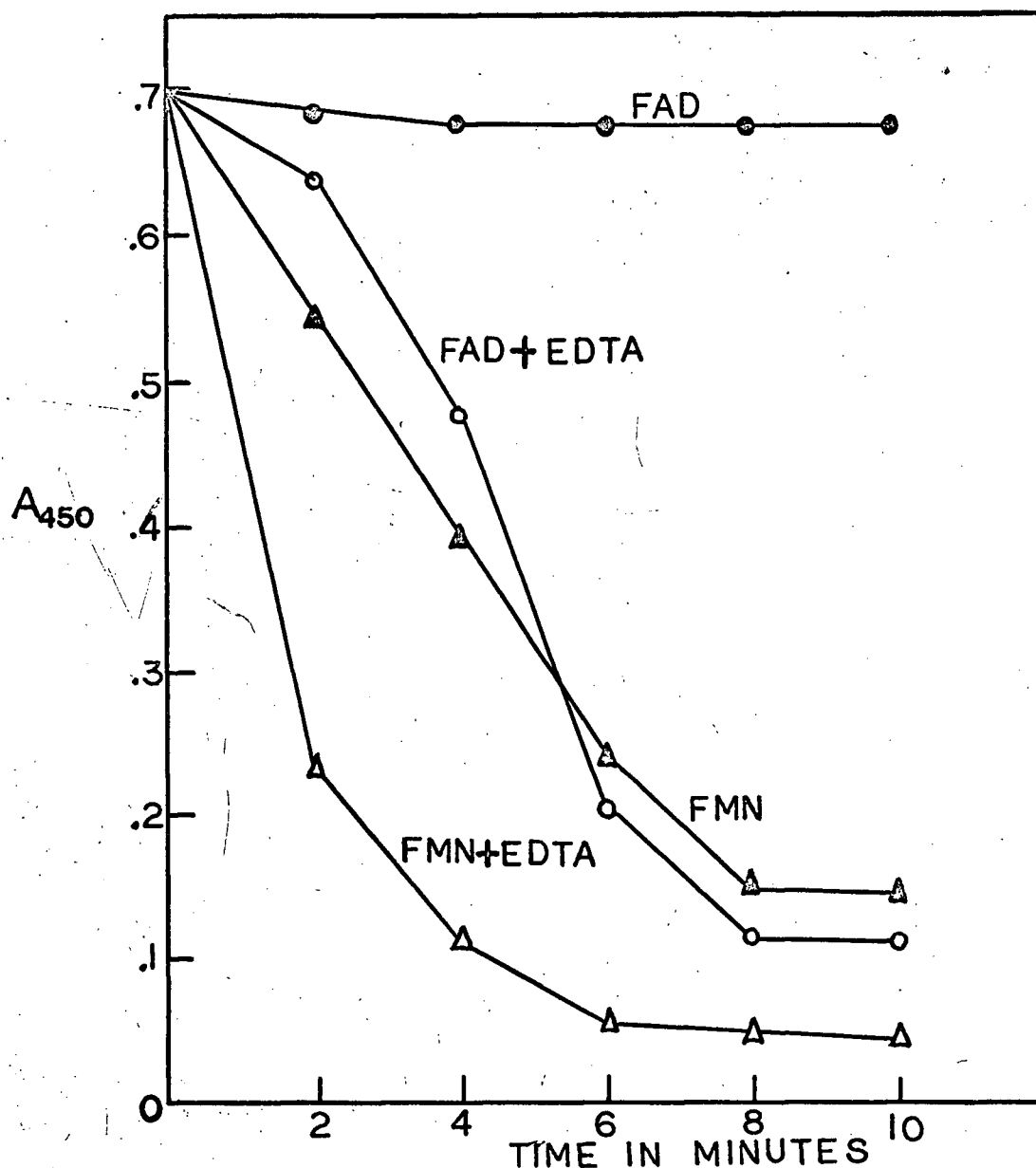
Fig. 11. Photoreduction of FAD and FMN with EDTA
under anaerobic conditions. The
reaction mixture consisted of 40 uM
phosphate buffer, pH 7.5, 0.1 uM FAD
of FMN, and 7.5 uM EDTA in a final
volume of 2.0 ml. The procedure for
obtaining anaerobicity and the adapted
Thunberg tubes used are described in
"Materials and Methods".
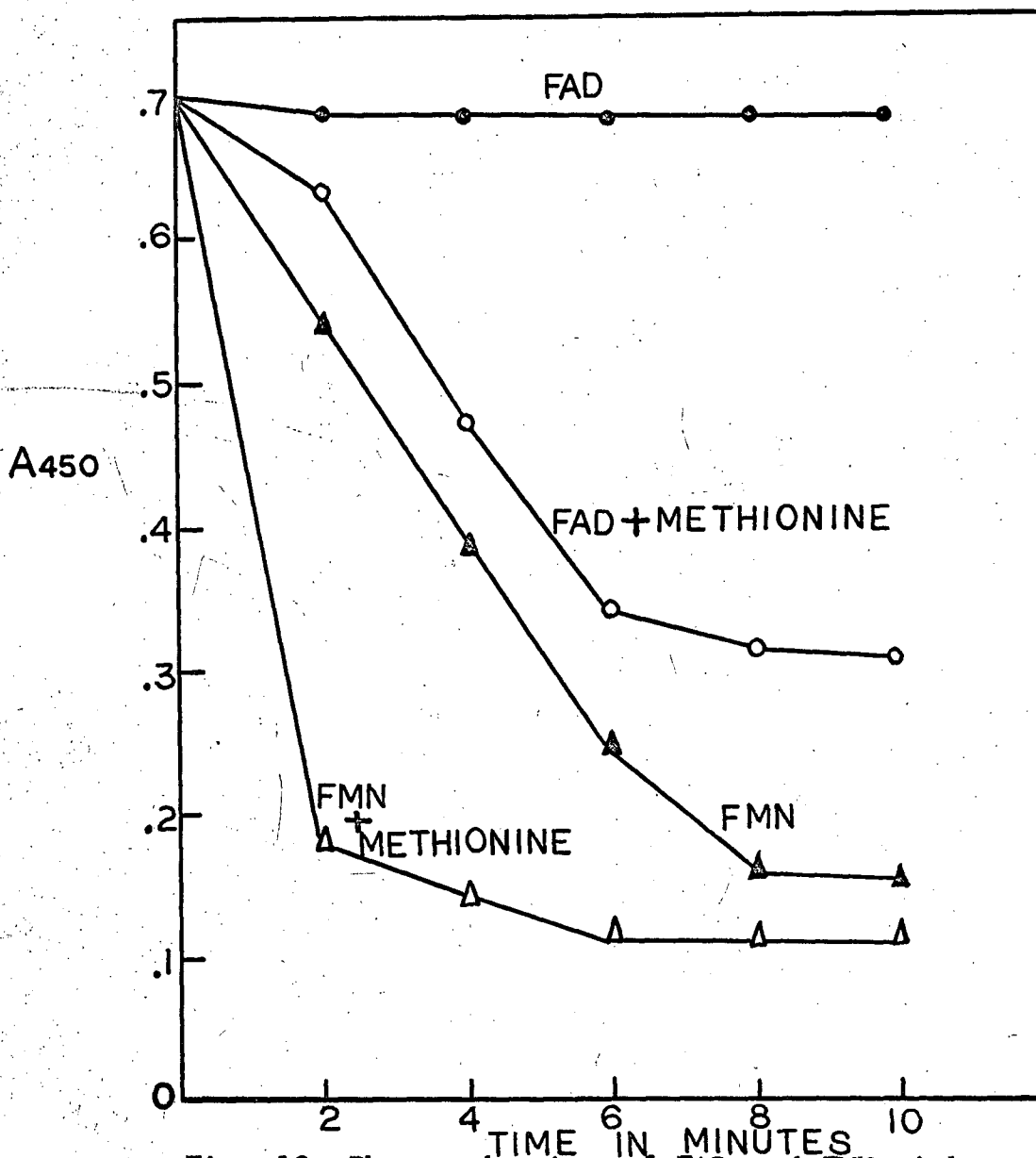
Fig. 12. Photoreduction of FAD and FMN with methionine under anaerobic conditions. The reaction mixture consisted of 40 uM phosphate buffer, pH 7.5, 0.1 uM FAD or FMN, and 7.5 uM methionine in a final volume of 2.0 ml. The procedure for obtaining anaerobicity and the adapted Thunberg tubes used are described in "Materials and Methods".

Table XXIV

Characterization of Flavin Nucleotide-Catalyzed Enzymic Photoreduction

of Nitrate

The complete reaction mixture consisted of 0.2 ml 0.1 M KNO_3, 0.3 ml
10^{-4} M FAD (FMN), 0.3 ml sugar beet NRase (Fraction IV), and 1.7 ml
0.1 M phosphate buffer, pH 7.5, containing 10^{-2} M EDTA (or methionine)
in a final volume of 2.5 ml. The adapted Thunberg tubes and the pro-
cedure for obtaining anaerobicity are described under "Methods".
Light intensity 375 foot candles, blue filter, temperature 30° C.

System	Nitrite Formed (muM)
Complete	56
NRase omitted	0
Nitrate omitted	0
EDTA omitted, methionine added	6
FAD omitted	0
FAD omitted, FMN added	60
FAD omitted, Riboflavin added	24
Complete, aerobic	5
Complete, dark	0
Complete, DPNH (1 mM) added	70

VII. DIURNAL CHANGES IN NITRATE CONTENT AND NITRATE

REDUCTASE ACTIVITY IN SUGAR BEET LEAVES

It was found that the sugar beet leaves harvested at the end of the dark period always contained a lower NRase activity than those harvested at the end of the light period. In order to determine the effect of the light and dark on the activity of NRase and the content of nitrate, the following experiment was carried out.

The plants were grown in a controlled room at a day (12 hours) temperature of 22-25° C and a night (12 Hours) temperature of 20-22° C. The relative humidity was kept 62-70% at day and 65-80% at night. Illumination was by slimline fluorescent tubes supplemented with 60 watt incandescent bulbs in order to give a light intensity of 1800 foot candles at the top of the plants.

When the plants were 50 days old, the lamina portion of the leaves were removed at 3, 6 and 12 hour intervals in light and in darkness. Twenty-five g of lamina from each harvest was used for the determination of nitrate content and NRase activity. The remainder (60 - 80g) after weighing, was dried in an oven at 75° to 80° C for 24 hours and the dry weight determined.

The results (Table XXV) indicated that NRase activity of sugar beet lamina varied diurnally with a minimum at end of dark period and maximum at end of light period. Maximum activity was almost three times minimum. The same degree of variation persisted when the activity was calculated on the basis of dry weight.

There was also a rhythm in nitrate content, which was contrary to that of the NRase activity, being highest at the end of the dark and lowest at the end of the light period.

When equal volumes of the homogenate from material collected at the end of the light and dark period were mixed, it was found that enzymatic activity of the mixed homogenate was close to the arithmetic mean of the individual activity of the two samples. This indicated that an accumulation of an inhibitor in the leaves had not occurred during the dark period.

The acidity of the juice of sugar beet leaves was followed during a 24-hour period. The acidity was higher at the end of the dark period (pH 5.7-6.0) and lower at the end of the light period (pH 6.5-6.7).

Table XXV

Dirurnal Change in Nitrate Content and NRase Activity in Sugar Beet Leaves

Time of collection of sample	Dry Wt.	Nitrate Content		NRase units per gram	
	mg/g fresh wt.	ug NO_3^--N/g. fresh wt.	ug NO_3^-N/mg dry wt.	fresh wt.	dry wt.
Light					
3 hours	96.2	598	6.21	158	1.6
6 hours	98.5	399	4.03	310	3.1
12 hours	103.4	345	3.33	384	3.7
Dark					
3 hours	94.8	474	5.01	245	2.6
6 hours	90.5	522	5.76	165	1.8
12 hours	91.1	786	8.26	125	1.3

VIII. OTHER METHODS OF ENZYME PURIFICATION

1. Extraction of NRase from Acetone Powder

Acetone-dried powders were prepared by the method described by Nason (1955) for soluble enzyme from higher plants. All steps in the following procedure were carried out at -10° C.

Twenty grams of sugar beet leaves was placed in a Waring Blendor with 200 ml of acetone and blended for one minute. The resulting slurry was filtered through a Buchner funnel and washed with an excess of acetone. The residue was spread out on filter paper, allowed to dry and was stored in an evacuated flask.

A crude extract of this leaf acetone powder was prepared by stirring the powder with 100 ml of 0.1 M K_2HPO_4 containing 10^{-3} M glutathione for 5 minutes at 0° C. A subsequent centrifugation was carried out at 4° C at a force of 20,000 x g for 15 minutes.

The results are given in Table XXVI. They indicate that the enzyme extracted from acetone powder had a low specific activity.

Table XXVI

Extraction of NRase from Acetone Powder

Experiment No.	Total Activity Units	Total Protein mg	Specific Activity Units/mg protein
1	1526	415	3.6
2	1780	323	5.5

2. Removal of Nucleoprotein from Enzyme Preparation

The 0-45% ammonium sulfate precipitate was dialyzed against 0.01 M phosphate buffer, pH 7.0, containing 10^{-3} M glutathione for one hour at 4° C. To the dialyzed enzyme preparation a 0.2% aqueous solution of protamine sulfate, pH 4.5, was slowly added until no further precipitation occurred. The precipitate was collected by centrifugation at 5000 x g for 10 minutes at 0° C and discarded. Enzyme activity and protein content of supernatant was determined by the usual methods. The results are given in Table XXVII. They show that the removal of nucleoprotein increased the specific activity of the enzyme slightly.

Table XXVII

Removal of Nucleoprotein from Enzyme Preparation

Fraction	Total volume	Total activity	Total protein	Specific activity	Recovery
	(ml)	(units)	(mg)		(%)
0-45% Ammonium Sulfate ppt.	50	5200	155	33	
Protamine Supernatant	58	3650	82	44	70

3. Absorption of NRase on Alumina C_r Gel

Thirty ml of dialyzed 0-45% ammonium sulfate precipitated enzyme was treated with 6 ml of alumina C gamma (C_r) gel (15 mg dry weight per ml) and stirred intermittently for 15 minutes at 0° C. The supernatant and gel precipitate was washed by resuspending it in 5 ml of 0.01 M potassium phosphate buffer pH 7.0, containing 10^{-3} M glutathione. After 5 minutes the precipitate was centrifuged down and washed in 0.5 M potassium phosphate buffer, pH 7.0, containing 10^{-3} M glutathione. The supernatant solution obtained by centrifugation after each of two washings is referred to as Supernatant 2 and Supernatant 3, respectively. The enzyme activity and protein content were determined by the usual methods. The results are given in Table XXVIII.

Table XXVIII

Absorption of NRase on Alumina C_r Gel

Fractions	Volume	Total activity	Total protein	Specific activity	Recovery
	(ml)	(Units)	(mg)		(%)
0-45% Ammonium Sulfate ppt.	30	2750	75	36	-
Supernatant I	36	1850	12	152	67
Supernatant 2	5	0	0.80	-	-
Supernatant 3	5	0	1.25	-	-

DISCUSSION

Experimental results reported here provide evidence that a soluble sulfhydryl NRase with a metalloflavoprotein nature is present in sugar beet leaves.

Almost all NRase activity of the homogenate of the leaves was recovered in the 20,000 x g supernatant. Fractionation by different speeds in 0.1 M sucrose-phosphate buffer containing 10^{-3} M glutathione showed that no particulate portion contained significant enzymatic activity. This intracellular distribution of sugar beet NRase is similar to that of the assimilatory NRases of most higher plants (Table IV).

The NRase was sharply inhibited by p-chloromercuribenzoate. The inhibition could be reversed by sulfhydryl reagents such as glutathione and cysteine. Inhibition also resulted from the use of the alkylating reagent iodoacetate and the oxidizing reagent cupric sulfate. These results indicate that the NRase is a sulfhydryl enzyme which loses its catalytic activity when some or all of its -SH groups undergo chemical modification.

The experiments demonstrating that chemically reduced molybdenum can serve as an electron donor suggested that enzyme systems which yield reduced molybdate may be capable of replacing pyridine nucleotide as the electron donor in the NRase sequence. It was not possible to establish that Mo was the metal prosthetic group as did Nicholas and Nason (1954) for Neurospora NRase.

Three lines of evidence suggest that FAD is the flavin nucleotide prosthetic group of the enzyme - the marked response of the partially purified enzyme to FAD addition, the low K_m for FAD compared with that for FMN, and the isolation and tentative identification of the enzyme-bound flavin as FAD. Because of the presence of some other flavoprotein enzymes, such as quinone reductase, in the enzyme preparation positive identification of the enzyme-bound flavin nucleotide will require further purification of the NRase. Table IV indicates the FAD specificity of the assimilatory NRases from several plant species.

The optimum pH of the sugar beet leaf enzyme Fraction V is 7.0 which is identical with that of the NRase from Neurospora (Nason and Evans, 1953). In contrast the pH optimum for crude NRase (Fraction I) was about 8.0. Apparently some physical changes took place during the purification procedure.

The inhibition by cyanide, azide, dipyridyl, vanadate and cupric sulfate, shown to occur with sugar beet NRase, has also been reported for the NRase of soybean leaves (Evans and Nason, 1953; Nicholas and Nason, 1955) and wheat embryo (Spencer, 1959).

The end products, nitrite and DPN^+, are also the same as those of other assimilatory NRases and indicate its role as an Assimilatory NRase rather than its involvement in nitrate respiration.

It is likely that the mechanism of electron transfer
is similar to that established for the enzyme in <u>Neurospora</u>
and soybean leaves:

$$\text{DPNH} \searrow \nearrow \text{FAD} \searrow \nearrow 2Mo^{5+} \searrow \nearrow NO_3^- $$
$$H^+ \quad\quad +2H^+ $$
$$\text{DPN} \swarrow \searrow \text{FADH}_2 \swarrow \searrow 2Mo^{6+} \swarrow \searrow NO_2^- + H_2O$$
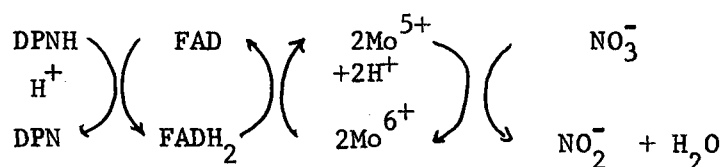
It is evident that the final step of the system
involved a one-equivalent donor Mo^{5+} and a two-equivalent
acceptor N^{5+}. This type of electron transfer is uncommon.
Generally speaking, metal is necessary for the reaction with
those acceptors which require only one equivalent of hydrogen
for their reduction and is not necessary for those requiring
two-equivalents (Mahler and Glenn, 1956). The reduction of
nitrate to nitrite requires two equivalents, and yet the
reduction invariably needs molybdenum.

The question whether the reduction of nitrate may
proceed in two one-equivalent steps, or nitrate as an
acceptor may require activation by complexing with the metal
molybdenum, is still not established since an additional
mechanism must be postulated to explain this special reaction.

In its pyridine nucleotide specificity purified NRase
of the sugar beet is similar to the assimilatory NRase from
<u>Escherichia</u> <u>coli</u> (Nicholas and Nason, 1955b), wheat embryo
(Spencer, 1959), tomato leaves (Sanderson and Cocking, 1964),

in that it is specifically linked to DPNH. The enzyme from

Neurospora (Nason and Evans, 1954) is TPNH-specific. Impure

NRase of the sugar beet resembles that from soybean leaves

(Evans and Nason, 1953), yeast (Silver, 1956), wheat leaves

(Anacker and Stoy, 1958), and the bacterium Azotobacter

vinelandii (Taniguchi and Ohmachi, 1960) which can be linked

to either DPNH or TPNH with equal effectiveness.

Spencer (1959) suggested that in higher plants DPNH-

specificity might be a characteristic of the NRase of the

embryo. He found that germinating soybean seeds appear to con-

tain only a DPNH-specific NRase while older tissue of soybean

(Evans and Nason, 1953) and wheat (Spencer, 1959) contain the non-

specific NRase.

From the evidence presented above it is possible that two

types of NRase occur in sugar beet leaves. One enzyme is speci-

fically linked to DPNH and the other to TPNH. During the course

of purification of the crude "enzyme" the two enzymes can be

separated. It is also possible that the leaves of sugar beet may

contain a DPNH-specific NRase and an enzyme which catalyzes a

reaction between TPNH and endogenous DPN to produce DPNH and TPN.

Keister et al. (1960) described such an enzyme in spinach,

pyridine nucleotide transhydrogenase, which catalyzes the

following reaction:

$$TPNH \quad + \quad DPN \longrightarrow DPNH \quad + \quad TPN^{+}$$
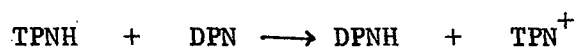
Shin et al. (1963) obtained a crystalline ferredoxin-TPN

reductase from spinach chloroplasts, which catalyzed the trans-

fer of the hydrogen from TPNH to a number of hydrogen or electron

acceptors such as DPN, menadione and FAD.

A comparison of some of the properties of the NRase

from sugar beet leaves with that from soybean, cauliflower,

wheat, and tomato, is given in Table XXIX.

The partially purified sugar beet NRase preparation con-

tained a number of other enzymatic activities. These included

DPNH-cytochrome c reductase, DPN-diaphorase, DPNH-manadione

reductase, and DPNH-quinone reductase. The latter three enzymes

have been designated "quinone reductases" which are characterized

by their ability to transfer hydrogen from reduced pyridine nuc-

leotide to suitable quinones (Martius, 1963).

Although the possible relationship between the DPNH-

NRase and quinone reductase activities present in purified sugar

beet NRase is obscure it is apparent that they are not identical.

Unlike the NRase quinone reductases are not inhibited by metal-

chelating agents such as cyanide, azide and o-phenothioline,

indicating the absence of metal in the enzymatic system, and they

are insensitive to sulfhydryl agents, such as p-chloromercuri-

benzoate and iodoacetate (Martius, 1963).

The experiments described above have revealed that in

the presence of EDTA flavin nucleotides can effect the transfer

Table XXIX

Comparison of Properties of Assimilatory NRase from <u>Beta</u> <u>vulgaris</u>

and Other Higher Plants

Source of enzyme	pH Optimum	Flavin Km	Enzyme specificity and Km	Substrate Km	Reference
Sugar beet leaves	7.0	FAD: 1×10^{-7} M FMN: 5×10^{-7} M	DPNH-specific. 5×10^{-5} M	4.5×10^{-4} M	
Soybean leaves	6.0	FAD: 1×10^{-7} M FMN: 3.7×10^{-6} M	Non-specific DPNH: 3.2×10^{-5} M TPNH: 2.3×10^{-5} M	7.5×10^{-3} M	Evans and Nason (1953)
Wheat leaves	7.0	FAD: 2 x FMN	Non-specific. DPNH-TPNH	–	Anacker and Stoy (1958)
Wheat embryo	7.4	FAD: 1×10^{-7} M	DPNH-specific	3.8×10^{-4} M	Spencer (1959)
Tomato leaves	7.5	–	DPNH-specific 2.3×10^{-6} M	4×10^{-4} M	Sanderson and Cocking (1964)

of electrons from some reducing agent to nitrate with the aid

of NRase. In this system flavin nucleotides may act as light-

absorbing catalysts. The participation of NRase and flavin

nucleotides in a new type of non-cyclic photosynthetic electron

flow has been described by Ramirex et al. (1964). They suggest

that pyridine nucleotide NRase may be in fact a mixture of

TPN-reductase and NRase.

The flavin nucleotide-catalyzed enzymatic photochemical

reduction of nitrate may be represented by the following:

Photoreduction

$$
\text{Flavin Nucleotide} \xrightarrow{\quad\quad\quad\quad} \begin{array}{c} \text{DPNH} \quad \text{DPN} \\[4pt] \text{AH}_2 \quad\quad \text{A} \end{array} \begin{array}{c} \text{Reduced} \\ \text{Flavin} \\ \text{Nucleotide} \end{array} \rightarrow \text{Mo} \xrightarrow[\text{Reductase}]{\text{Nitrate}} \text{NO}_3^-
$$

AH_2 = Reducing agent
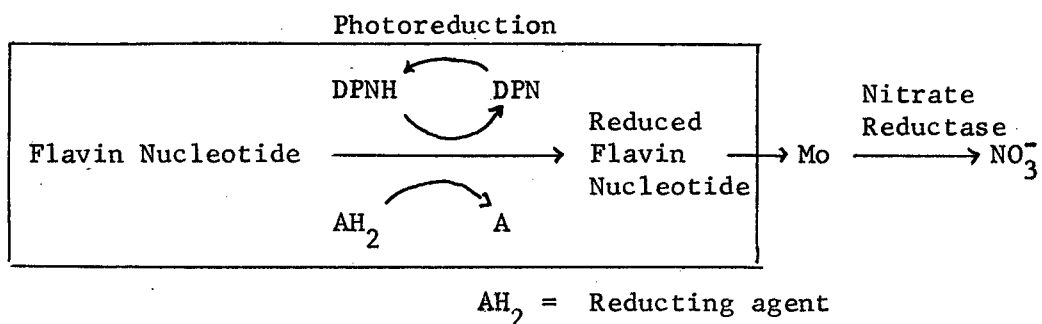
Just what physiological significance this reaction may

have in plant tissue remains to be shown. Certainly it seems that

flavin nucleotides should not be ignored when considering photo-

chemical reactions. The overall abundance of flavin nucleotides

in the plant tissue and the presence of natural reducing agents

which could serve to control these reactions, speak strongly in

favor of the theory that flavin nucleotides, enzyme-bound

flavins, play some role in light activated reactions.

It was found that sugar beet leaf NRase activity was high during the light period and low during darkness. A similar variation has been found to occur in maize (Hagemen and Fletcher, 1960) and tomato (Sanderson and Cocking, 1964). The former investigators suggested two possible reasons for the loss of activity during darkness - the accumulation of an inhibitory nitrogenous metabolite and the oxidation of the active sulfhydryl groups. However the occurrence of either was not proven. In the experiments with sugar beet NRase the accumulation of an inhibitor in the leaves during the dark period was not detected.

In common with the observations of Clevenger (1919), Ingalls and Shrive (1931), Bennet-Clark (1953) and others, who used various species of plants, it was found that growth in darkness and in light was associated with relatively low and high pH values of the expressed leaf sap. The sharp optimum of sugar beet NRase (pH 7.0) and its sensitivity to pH change coupled with the fall in leaf pH values in the dark (pH 5.7 - 6.0) and their rise in the light (pH 6.5 - 6.7) suggest a causal relationship between this rhythm in hydrogen ion concentration and the diurnal alternation in activity of NRase.

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