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REGULATION OF NADH NITRATE REDUCTASE
OF TOBACCO XD CELLS BY SUBOPTIMAL
CONCENTRATIONS OF NITRATE AND SULFATE

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PHILIP MICHAEL TRINITY

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REGULATION OF NADH NITRATE REDUCTASE OF TOBACCO XD CELLS BY SUBOPTIMAL CONCENTRATIONS OF NITRATE AND SULFATE

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Philip Michael Trinity

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ABSTRACT

REGULATION OF NADH NITRATE
REDUCTASE OF TOBACCO XD CELLS BY
SUBOPTIMAL CONCENTRATIONS OF NITRATE
AND SULFATE

By

Philip Michael Trinity

NADH nitrate reductase was studied in suspension cultures of the XD cell line of Nicotiana tabacum L. cv. Xanthi. When cultured on suboptimal concentrations of either nitrate or sulfate, the overt nitrate reductase, i.e. that which is assayable without an activation treatment, decreases with decreasing initial concentrations of the suboptimal nutrient.

As XD cells growing on nitrate nitrogen progress from exponential phase into stationary phase due to depletion of nitrate, the overt nitrate reductase activity decreases and a latent, i.e. activable, form becomes detectable. The latent enzyme spontaneously activates when stored at -20°C in 0.1 M potassium phosphate, 1 mM ethyleneglycol-bis (ß-amino ethyl ether) N,N'-tetraacetic acid, 1 mM L-cysteine, 1 µM flavin adenine dinucleotide, pH 7.5. Incubation with ferricyanide

also activates the latent form of the enzyme. Nine-fold activations were observed in extracts from stationary phase cultures. Replacement of overt by latent activity closely parallels depletion of nitrate from the medium. Whereas overt activity per culture decreases, the sum of overt plus latent activities per culture increases.

In vitro, nitrate reductase can be partially inactivated by incubation with NADH or cyanide, but essentially complete inactivation can be achieved by incubation with cyanide plus NADH. Incubation with ferricyanide can reverse this inactivation. In many respects, these characteristics resemble those of the nitrate reductase system in green algae (L. Solomonson, Biochim. Biophys. Acta, 334, 297-308 (1974)) in which cyanide binds reversibly to reduced nitrate reductase thereby inactivating it. However, a kinetic test indicates that inactivation by cyanide cannot fully account for the latent form of the enzyme extractable from XD cells.

Addition of ammonium to nitrate grown XD cells results in a 60% decrease in the overt activity after 11 hours without concomitant formation of latent activity. This is also at variance with the algal system.

Future studies of the "induction" or "repression" of nitrate reductase in plants will have to take this phenomenon into account.

A latent but ferricyanide-activable form of nitrate reductase was also observed during sulfur starvation of XD cells.

A method for maintaining constant but suboptimal concentrations of nitrate was devised employing the equilibrium between the insoluble salt of nitrate, nitron nitrate, and free nitrate.

TO LORI

A habit of keeping the eyes open to everything that is going on in the ordinary course of the business of life has oftener led, as it were by accident, or in the playful excursion of the imagination . . . to useful doubts, and sensible schemes for investigation and improvement, than all the more intense meditations of philosophers, in the hours expressly set aside for study.

Benjamin, Count of Rumford
1798

"An Inquiry Concerning the Source of Heat Which Is Excited by Friction." (Read before the Royal Society, January 25, 1798.)

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TABLE OF CONTENTS

Page

LIST OF TABLES ix

LIST OF FIGURES x

LIST OF ABBREVIATIONS xii

INTRODUCTION 1

LITERATURE REVIEW 5

- The Nitrate Assimilatory Pathway 5
- The Sulfate Assimilatory Pathway 8
- Properties of Nitrate Reductase 10
- Nitrate Reductase Mutants 12
- Induction of Nitrate Reductase 13
- Effects of Amino Acids Upon Nitrate Reductase 13
- Effect of Ammonium on Nitrate Reductase Activity 14
- Factors Promoting Inactivation of Nitrate Reductase 15
- Role of Cyanide in the Inactivation of Nitrate Reductase . . . 16
- Biological Synthesis of Cyanide 17
- Metabolism of Cyanide 18
- Effects of Metals Upon the Abundance of Cyanide 18
- Protein Factors Which Inactivated or Inhibited Nitrate Reductase 19
- Spontaneous Activation of Nitrate Reductase 20
- Chemical Activation of Nitrate Reductase 20
- Activating Factor of Nitrate Reductase 21
- Coordinated Regulation Between the Nitrate and Sulfate Assimilatory Pathways 22

MATERIALS AND METHODS 24

- Cultures of Tobacco XD Cells 24
- Culture Medium 24
- Harvesting of Tissue 25
- NADH Nitrate Reductase Assay 25
- Assay of ATP Sulfurylase Activity 26
- Protein Determination 27
- Assay for Nitrate 28
- Gel Filtration Apparatus 28
- Ferricyanide Activation of Nitrate Reductase 29
- Partial Purification of Nitrate Reductase 29

Page

Preparation of Nitron Nitrate. 30
Nitron Assay 31
Assay of Tetrazolium Violet. 32
Preequilibration and Recycling of the Ionac Ion Exchange Membranes. 32
Preparation and Sterilization of Bio Fiber 20 Filters. 33

PART I: CONTROL OF NUTRIENT CONCENTRATIONS IN LIQUID SUSPENSION CULTURE

Introduction 35
Batch Cultures 36
Chemostat Cultures 37
Equilibrium Chemostat Culture. 38
Proposed Method to Maintain Constant Nitrate Concentrations in Cultures of Tobacco Cells 39
Properties of Nitron 42
Nitron Toxicity. 42
Attempts to Select Cell Lines Tolerant of Nitron 45
Segregation of Nitron From Living Tissue 48
Ion Exchange Membranes 48
Flux of Nitrate Through MA-3475 Membranes. 49
Ultrafiltration of Nitrate and Nitron. 51
Flux of Nitrate and Nitron Across the Bio Fiber 20 Filters. 51
Toxicity of the Hollow Fiber Filter Assembly 58
Effects of Various Types of Tubing Upon Growth of Tobacco Cells. 59
Toxicity of the Bio Fiber 20 Filters 59
Conclusion 62

PART II: REGULATION OF NADH NITRATE REDUCTASE

Coupled Regulation of Nitrate Reductase and ATP Sulfurylase. 64
Dependence of Nitrate Reductase Activity Upon Sulfate Nutrition. 66
Dependence of ATP Sulfurylase Activity Upon Nitrate Nutrition. 69
Stabilization of Nitrate Reductase Activity. 72
Stability of Nitrate Reductase Activity in Various Buffers. 72
Stability of Nitrate Reductase at Various Temperatures 78
Partial Purification of Nitrate Reductase. 78
Ammonium Sulfate Fractionation of Nitrate Reductase. 82
Long Term Stability of Nitrate Reductase 82
Purification by DEAE - Sephadex Chromatography and Stability of Nitrate Reductase 82
Activation and Inactivation of Nitrate Reductase 88

Page

Inactivation of Nitrate Reductase by NADH. 90
Effects of Various Reagents Upon Nitrate Reductase 90
Ferricyanide Activation of Nitrate Reductase 93
Inactivation of Nitrate Reductase by Cyanide Plus NADH and Reversal of Inactivation by Ferricyanide 97
Relationships Among Culture Age, Nitrate Reductase Activity and the Activable Nitrate Reductase Activity. . . . 99
Induction of Nitrate Reductase 105
Influence of Nitrate Upon the Formation of Latent Nitrate Reductase Activity 107
Response of Nitrate Reductase Activity Upon Inoculation Into Conditioned Medium. 108
Effects of Mixing Extracts from Old and Young Tissue Upon Nitrate Reductase Activity 111
Effects of Protein Upon Nitrate Reductase Activity 111
Kinetic Evaluation of the Binding of Cyanide to Nitrate Reductase. 114
Additional Factors Which Would Promote the Formation of Inactive Nitrate Reductase 119
Role of Threonine in the Regulation of Nitrate Reductase Activity 120
Influence of Ammonium Upon Nitrate Reductase Activity. 120
Effects of Inadequate Sulfate Nutrition Upon Nitrate Reductase Activity 123

DISCUSSION. 125

APPENDIX. 145

BIBLIOGRAPHY. 155

LIST OF TABLES

Table		Page
1	Liters of growth medium required to grow tobacco XD tissue with only a 20% decrease in the initial concentration of nitrate in the medium.	37
2	Toxicity of various types of tubing to tobacco XD cells.	60
3	Effect of Ca^{+2} and Mg^{+2} upon nitrate reductase activity in modified Zielke buffer	81
4	Partial purification of nitrate reductase.	85
5	Stability of partially purified nitrate reductase after DEAE-Sephadex chromatography	89
6	Relative effects of various reagents upon nitrate reductase activity	94
7	Effects upon nitrate reductase activity of mixing extracts from young and old tissue and of addition of protein to the extraction buffer.	112
8	Effect of threonine upon nitrate reductase activity.	121
9	Effect of ammonium upon nitrate reductase activity	122
10	Effect of inadequate sulfate nutrition upon nitrate reductase activity	124

LIST OF FIGURES

Figure		Page
1	The nitrate and sulfate assimilatory pathways	7
2	Toxicity of nitron to tobacco XD cells with nitrate or ammonium as the nitrogen source.	44
3	Toxicity of nitron to tobacco XD cells growing on M-1D medium	47
4	Inhibition of growth of tobacco XD cells by Ionac MA-3475 membrane.	53
5	Equilibration of nitrate and nitron across a Bio Fiber 20 hollow fiber filter.	57
6	Dependence of nitrate reductase and ATP sulfurylase activities upon the initial sulfate concentration of the medium.	68
7	Dependence of ATP sulfurylase and nitrate reductase activities upon initial nitrate concentration	71
8	Stability of nitrate reductase in various buffers	74
9	Effects of EDTA and EGTA upon spontaneous activation and stability of nitrate reductase.	77
10	Stability of nitrate reductase activity at various temperatures.	80
11	Long term stability of nitrate reductase activity in modified Zielke buffer.	84
12	Elution profile of nitrate reductase activity from DEAE-Sephadex column.	87
13	Inactivation of nitrate reductase by NADH	92
14	Activation of nitrate reductase by ferricyanide	96

Figure Page

15 Inactivation of nitrate reductase by cyanide plus NADH and reactivation by ferricyanide 98

16 Variation in overt and latent nitrate reductase activity as a function of the age of tobacco XD tissue cultures 101

17 Total nitrate reductase activity and protein precipitable by 50% saturated ammonium sulfate per liter of tobacco XD cultures as a function of culture age 103

18 Comparison of spontaneous activation and ferricyanide dependent activation of nitrate reductase as a function of culture age. 104

19 Changes in overt and total activation of nitrate reductase in tobacco XD cells upon subculturing 106

20 Effect upon nitrate reductase activity of subculturing tobacco XD cells into medium lacking nitrate or phosphate or into conditioned medium 110

21 Kinetic evaluation of the NADH plus cyanide induced inactivation of nitrate reductase 118

22 Proposed model for the binding of an effector and cyanide to nitrate reductase. 138

23 Biosynthesis and metabolism of cyanide. 141

LIST OF ABBREVIATIONS

ADP - Adenosine-5'-diphosphate

AMP - Adenosine-5'-phosphate

APS - Adenosine-5'-phosphosulfate

ATP - Adenosine-5'-triphosphate

DCPIP - 2,6-Dichlorophenolindophenol

DEAE - Diethylaminoethyl

EDTA - Ethylene diamine tetraacetic acid

EGTA - Ethyleneglycol-bis(ß-amino ethyl ether) N,N'-tetraacetic acid

FAD - Flavin adenine dinucleotide

$FMNH_2$ - Flavin mononucleotide (reduced)

MVH_2 - Methyl viologen (reduced)

NAD^+ - Nicotinamide adenine dinucleotide

NADH - Nicotinamide adenine dinucleotide (reduced)

$NADP^+$ - Nicotinamide adenine dinucleotide phosphate

NADPH - Nicotinamide adenine dinucleotide phosphate (reduced)

PAPS - 3'-phosphoadenosine-5'-phosphosulfate

TRIS - tris (hydroxymethyl) aminomethane

INTRODUCTION

For a plant, survival is often difficult at best. Not the least of its difficulties is the procurement of adequate nutrients to meet its metabolic needs. Nitrogen and sulfur are among the most important of these. They are often available in soil only at suboptimal concentrations or in forms which are not readily assimilatable. Thus for a plant to survive and propagate, it must be adept at scavenging these two elements from its environment.

Nitrogen is required for the synthesis of amino acids, protein and scores of other nitrogen-containing compounds. The assimilation of nitrate, the form of nitrogen most generally available to plants, is considered to proceed via the reaction catalyzed by nitrate reductase, which is a well regulated enzyme (1). It has been estimated that up to 30% of the photosynthetic energy of a plant may be used for the 8-electron reduction of nitrate to ammonium (2). An understanding of the regulation of this vital and energetically costly metabolic pathway is therefore of critical importance for our understanding of how plants function and survive.

In the field, plants are generally subjected to conditions of suboptimal nutrient concentrations. The assimilatory pathways probably do not run at the maximum possible rates, but the fluxes of nutrients

are generally adequate for survival and growth. The availability of nutrients is influenced by several processes which occur in the soil, such as ion exchange and nutrient cycling by the soil biota. For much of the typical growing season, soil nitrate is often present at sub-optimal concentrations. Therefore, to better understand how plants cope with inadequate nitrate, the nitrate pathway, especially the well regulated nitrate reductase, should be studied under conditions of sustained suboptimal nitrate concentrations.

Because of the occurrence of nitrogen and sulfur in protein, there is a functional convergence of nitrogen and sulfur assimilatory pathways. In maize, for example, over 85% of the nitrogen content of the plant is contained in its protein (2). However, protein synthesis also requires the sulfur-containing amino acids cysteine and methionine. If sulfur nutrition is inadequate (cysteine and methionine are inadequate), how does the nitrate assimilatory pathway respond? In several plants (summarized in reference 3), nitrate accumulated during sulfur inadequacy, suggesting that the nitrate pathway was being modulated at nitrate reduction. Furthermore, P. Filner (personal communication) observed that when sulfate was withheld from tobacco XD cells, nitrate reductase was not induced by nitrate. This observation made it of interest to investigate the response of nitrate reductase to sustained suboptimal concentrations of sulfate.

Sulfate is generally the source of sulfur in plants. As with the reduction of nitrate, the reduction of sulfate requires 8-electrons. However, the reduction of sulfate requires much less of the total photosynthetic energy of the plant than does the reduction of nitrate.

The primary reason is that on a molar basis, the nitrogen content of a plant is about 30- to 40- fold greater than the sulfur content (4). Ten to fifty percent of the total sulfur in the plant, can be accounted for as protein (3).

Were the nitrogen nutrition of the plant inadequate and as a consequence the rate of protein synthesis diminished, then sulfur-containing compounds, particularly cysteine and methionine, would accumulate in the plant if the sulfate assimilatory pathway were not modulated. Thus, one would predict that the sulfate assimilatory pathway should be modulated during periods of inadequate nitrogen nutrition. In fact, it was reported that, in corn, sulfate does accumulate when the nitrogen nutrition is inadequate (5). Furthermore, when tobacco XD cells were starved for nitrogen, ATP sulfurylase, the enzyme which activates sulfate for subsequent reduction and assimilation, was not derepress under conditions of sulfur inadequacy which otherwise were sufficient to derepress the enzyme (6). These results are consistent with the hypothesis that the sulfate assimilatory pathway is modulated during conditions of inadequate nitrogen nutrition, at least in part by modulation of the activity of ATP sulfurylase. These observations made it of interest to investigate the response of ATP sulfurylase to sustained, suboptimal concentrations of nitrate.

Therefore, in an effort to learn more about how plants cope with suboptimal nitrate and sulfate nutrition, three sets of questions were posed and investigated.

1) Does a plant modulate the activity of nitrate reductase during conditions of suboptimal nitrate nutrition? If there is modulation, by what mechanism does it occur?

2) Does nitrate reductase respond to inadequate sulfur nutrition? If so, by what mechanism does it occur?

3) Does ATP sulfurylase respond to inadequate nitrogen nutrition? If so, by what mechanism does it occur?

Answers to some, but not all of these questions have been obtained through the research reported in this thesis.

LITERATURE REVIEW

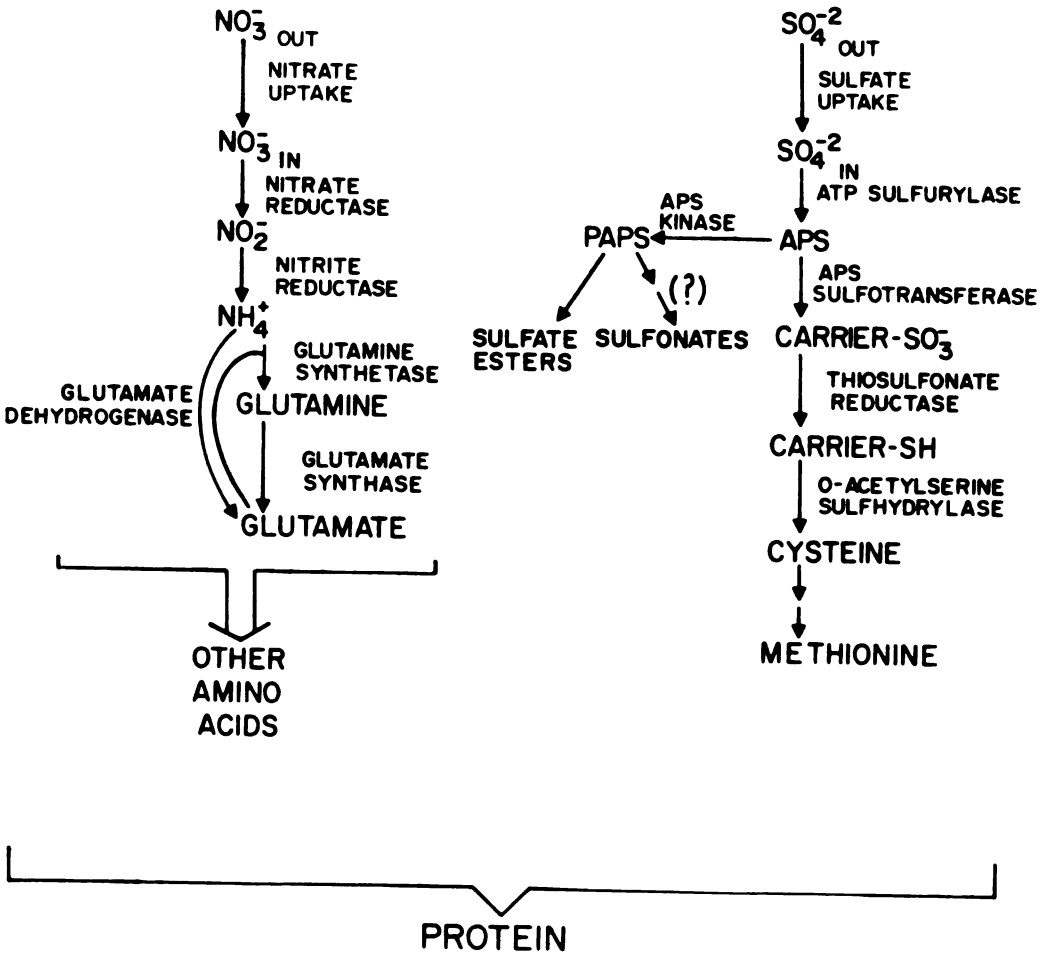
The Nitrate Assimilatory Pathway

The nitrate assimilatory pathway is illustrated in Figure 1. (See references 7 and 8 for extensive reviews of this pathway.) This assimilatory pathway begins with the uptake of nitrate from the environment. The uptake system is energy dependent (9) and is inducible by nitrate (9, 10). Protein synthesis is required for both induction (11) and for persistence (12) of this activity.

Once in the cell, nitrate is reduced to nitrite by the action of nitrate reductase, an activity which is apparently inducible by nitrate (7). The two electron reduction uses NADH or NADPH as the electron donor (13) and is generally considered to be the rate-limiting step of the nitrate assimilatory pathway (1). NADH is overwhelmingly the preferred reductant for the enzyme from higher plants. (A more detailed review of literature concerning nitrate reductase can be found in subsequent sections of this literature review.)

The six electron reduction of nitrate to ammonium is catalyzed by nitrite reductase with ferredoxin thought to be supplying the reducing power (13). Like the nitrate uptake and nitrate reductase activities, nitrite reductase activity is apparently inducible by nitrate (14, 15, 16) and develops in conjunction with these two other activities (17).

Figure 1. The nitrate and sulfate assimilatory pathways.


NO_3^- OUT
NITRATE UPTAKE
NO_3^- IN
NITRATE REDUCTASE
NO_2^-
NITRITE REDUCTASE
NH_4^+
GLUTAMINE SYNTHETASE
GLUTAMATE DEHYDROGENASE
GLUTAMINE
GLUTAMATE SYNTHASE
GLUTAMATE
OTHER AMINO ACIDS
SO_4^{-2} OUT
SULFATE UPTAKE
SO_4^{-2} IN
ATP SULFURYLASE
APS
APS KINASE
PAPS
(?)
SULFATE ESTERS
SULFONATES
APS SULFOTRANSFERASE
CARRIER-SO_3^-
THIOSULFONATE REDUCTASE
CARRIER-SH
O-ACETYLSERINE SULFHYDRYLASE
CYSTEINE
METHIONINE
PROTEIN

Ammonium can be assimilated further by one of two routes. Glutamine synthetase catalyzes the reaction between ammonium and glutamate to form glutamine. An ATP is hydrolyzed to ADP to provide energy for this reaction. The enzyme glutamate synthase can then catalyze the 2-electron reduction of α-ketoglutarate to glutamate, utilizing the amide-nitrogen of glutamine. An alternative route for the assimilation of ammonium is by the enzyme glutamate dehydrogenase which converts ammonium and α-ketoglutarate into glutamate with NADH supplying the reductive energy. The primary route of assimilation of ammonium is considered to be via glutamine synthetase and glutamate synthase as opposed to glutamate dehydrogenase (18, 19). However, glutamate dehydrogenase can catalyze a significant proportion of the assimilation when ammonium is available at elevated concentrations (19).

The Sulfate Assimilatory Pathway

The sulfate assimilatory pathway is also illustrated in Figure 1 and is reviewed in more detail in references 20 and 21. Sulfate is transported into the cell by sulfate uptake activity. In plant tissue culture this activity is under negative feedback regulation by end-products of the assimilatory pathway; addition of cysteine or methionine results in an inhibition of the uptake activity (22, 23). Smith attributed this inhibition in tobacco XD cells to the accumulaion of sulfate within the cells (24) probably as a result of the action of cysteine desulfhydrase which can generate H_2S from cysteine; the H_2S could then be oxidized to sulfate (25).

After entering the cell, sulfate is activated by ATP sulfurylase to form adenosine 5'-phosphosulfate (APS). This enzyme can be repressed in tobacco XD cells by readily assimilatable sulfur compounds such as sulfate, cysteine or methionine (6). The apparent derepression of the enzyme during sulfur starvation has been observed in both heterotrophic tobacco XD cells (6) and in another line of tobacco cells which contains green chloroplasts (L. Bergmann - personal communication).

APS is apparently a branch point in sulfate assimilation in plants and algae. Some of the APS is converted to 3'-phosphoadenosine 5'-phosphosulfate (PAPS) by APS kinase. PAPS is believed to be a substrate for the synthesis of sulfate esters and sulfonates (26). On the other hand, APS is believed to be the substrate for assimilatory sulfur reduction leading to the synthesis of cysteine and methionine (20).

The sulfo-moiety of APS is transferred to a carrier molecule by APS sulfotransferase. This transfer utilizes APS specifically as opposed to PAPS (27). The activity of the enzyme is decreased by addition of H_2S or cysteine, end products of the pathway, to duckweed (28) or to cultured tobacco cells (29), thus supporting the suggestion that this reaction is in the pathway of reductive sulfate assimilation.

The carrier-bound sulfur is reduced by thiosulfonate reductase to the level of sulfide. Reduced ferredoxin can supply the reducing potential for this reaction in cell free preparations containing

broken chloroplasts (30). Although glutathione can serve as the sulfur carrier during the *in vitro* assay of thiosulfonate reductase, the identity of the *in vivo* carrier in higher plants is uncertain.

After reduction to the level of sulfide, the sulfur is assimilated into cysteine by O-acetylserine sulfhydrylase with O-acetylserine providing the carbon skeleton. Although in *Salmonella typhimurium* (31) the enzyme is repressed by cysteine, in the higher plants, *Lemna minor*, L. (28) and tobacco XD cells (32), the enzyme does not seem to be modulated by end products of the sulfur assimilatory pathway.

Properties of Nitrate Reductase

The regulation of the nitrate assimilatory pathway occurs primarily at the level of nitrate reductase (1). Nitrate reductase (NADH: nitrate oxidoreductase, EC 1.6.6.1) catalyzes the two electron reduction of nitrate to nitrite using primarily NADH as the electron donor.

$$NO_3^- + NADH + H^+ \longrightarrow NO_2^- + NAD^+ + H_2O$$

At pH = 7, the change in free energy is

$\Delta G^{\circ\prime}$ = 34 kcal / mole (13).

Estimates of the molecular weight of the enzyme vary considerably. In *Chlorella vulgaris* for example, the native species has a molecular weight of 356,000 daltons as estimated by gel electrophoresis and appears to be composed of three subunits (34, 34). In soybean, 330,000 daltons is the reported mass (35), whereas in spinach, the value is 240,000 daltons (36). In the fungus, *Aspergillus nidulans*, the estimated molecular weight is 190,000 daltons (37).

In these large proteins, several components and cofactors have been identified. The sequence of electron transfer is believed to involve several steps (13). The electron donor NADH or NADPH donates electrons to the flavin moiety, FAD, of the enzyme. A cytochrome b_{557} then accepts the electrons from FAD and subsequently transfers them to a molybdenum-containing component. In this component, molybdenum is reduced from a +6 to a +5 valence (7). Nitrate is the ultimate electron acceptor in the sequence.

The various stages of the electron transport can be assayed as one of several enzyme activities. The overall activity which is believed to function in vivo is the NADH nitrate reductase activity in which NADH supplies electrons for the reduction of nitrate. Alternatively, the reduced flavin, $FMNH_2$, or reduced methyl viologen (MVH_2) can serve as the electron donor to nitrate in which case the respective activities are $FMNH_2$ nitrate reductase and MVH_2 nitrate reductase. A diaphorase activity can also be detected in which NADH serves as the electron donor to acceptors, such as oxidized cytochrome c, dichlorophenolindophenol (DCPIP) or ferricyanide (1, 7).

The molybdenum-containing component is essential for the in vivo function of the enzyme. Substitution of molybdenum with tungstate (9, 36, 38, 39, 40), or vanadate (40) results in the loss of NADH nitrate reductase activity while retaining NADH cytochrome c reductase activity.

The preferred electron donor for the nitrate reductase of higher plants and green algae is NADH whereas in molds and yeasts, NADPH is the preferred species (13). In a few plants two species of nitrate reductase have been identified. One preferentially accepts NADH while

the other accepts NADPH (35, 41, 42). The reason why two forms might exist is unclear. However, it was observed in soybean that the species which preferentially utilized NADH was more active in the young cotyledon whereas the species which preferentially utilized NADPH was more active in older tissue (43). Unless purified nitrate reductase was used, reports of the utilization of NADPH by the nitrate reductase of plants should be viewed critically. For example, Wells and Hageman (44) have claimed that the NADPH associated activity of soybean was an artifact resulting from phosphatase activity.

Nitrate Reductase Mutants

There are several reports of nitrate reductase mutants in higher plants. A Nicotiana tabacum variety which possessed a constitutive nitrate reductase was used for the selection of mutants using 20 mM chlorate, an analogue of nitrate (45). Nine strains were obtained in this selection one of which exhibited simultaneous loss of both nitrate reductase and xanthine dehydrogenase activities (46). (These two enzymes apparently contain the same molybdenum-containing component as part of their reaction center). Furthermore, complete NADH nitrate reductase activity could be reconstituted by combining extracts of two mutants, one lacking NADH cytochrome c reductase activity and the other lacking the other component activities of nitrate reductase but exhibiting NADH cytochrome c reductase activity (47). In addition to the mutants of tobacco, a nitrate reductase mutant of Datura innoxia (Mill) was obtained by King and Khanna (14) using chlorate.

Mutants of barley have been obtained which have greatly lowered nitrate reductase activity or lack the activity completely as determined by both *in vivo* and *in vitro* assays. However, even though the apparent activity of nitrate reductase was minimal, the whole plants grew nearly as well as the wild-type plants when nitrate was the nitrogen source (48, 49). This report resurrected the questions of whether the assay methods for nitrate reductase genuinely reflect the *in vivo* activity of the enzyme (49) and of whether our understanding of the biochemistry of nitrate reduction is correct.

Induction of Nitrate Reductase

The induction of nitrate reductase in higher plants was first demonstrated conclusively in 1957 by Tang and Wu (50) using rice. Subsequently, induction has been demonstrated in many plants (See reviews in references 1 and 7). Using $^{15}NO_3^-$, Zielke and Filner (51) demonstrated that the activity in tobacco XD cells was attributable to enzyme synthesized after addition of the inducer, nitrate. However, they also detected synthesis and degradation of the enzyme when nitrate reductase activity was constant or falling. Johnson (52) also attributed at least part of the increased nitrate reductase activity in *Chlorella vulgaris* to newly synthesized protein as indicated by density labelling experiments. In addition, he attributed some of the increased activity to activation of latent enzyme.

Effects of Amino Acids Upon Nitrate Reductase

Amino acids have been shown to affect the activity of nitrate reductase in plants. In tobacco XD cells (11, 53) tomato cells (11)

and cotton root tips (54) alanine, glycine, leucine, methionine, serine and threonine all caused apparent repression of nitrate reductase activity. Arginine, cysteine, isoleucine and lysine seemed to overcome or antagonize repression of nitrate reductase activity in tobacco (53) but either caused apparent repression or had no effect in cotton (54) or tomato (11). Aspartic acid, glutamic acid and phenylalanine seemed to overcome or antagonize a repression of nitrate reductase in cotton root tips (54) but apparently repressed the nitrate reductase activity of tobacco (11, 53) and tomato (11).

Effect of Ammonium on Nitrate Reductase Activity

In the alga Chlamydomonas reinhardii (55, 56), Chlorella fusca (57), Chlorella vulgaris (52, 58) and Cyanidium caldarium (59) addition of ammonium to nitrate-grown tissue resulted in a decrease in overt nitrate reductase activity, i.e. activity detectable prior to activation. Much of this decrease in activity was found to be attributable to inactivation of the enzyme. In C. fusca (57) and in C. reinhardii (56), this inactivation affected the NADH nitrate reductase and the $FMNH_2$ nitrate reductase activities but not the NADH diaphorase activity. When ammonium was replaced by nitrate as the source of nitrogen, in vivo activation of the enzyme was observed (52, 60).

In higher plants, as well, ammonium promotes a decrease in nitrate reductase activity. In Lemna minor, ammonium caused both inactivation and irreversible loss of activity (61). Likewise, in spinach, ammonium promoted the conversion of active to inactive enzyme as indicated by immunological techniques (62).

Factors Promoting Inactivation of Nitrate Reductase

In addition to ammonium, several other factors promote the formation of an inactive nitrate reductase. In vitro, NADH or NADPH alone cause inactivation of the enzyme. This inactivation was observed in a broad range of organisms including Chlamydomonas reinhardii (55), Chlorella vulgaris (63), Chlorella fusca (64), spinach (65, 66, 67), corn (68, 69) and rice (69, 70). However, other reports stated that these dinucleotides had little or no effect in Chlorella fusca (71), Chlorella vulgaris (72) or Neurospora crassa (68). These differences might be attributable to the purity of the enzyme used in the studies or to the presence of minute quantities of effectors (cyanide, for example) which might be present in the extract. The oxidized forms of the dinucleotides NAD^+ and $NADP^+$ did not inactivate the enzyme of Chlorella (63).

When air was withheld from Chlamydomonas, inactivation of nitrate reductase occurred. This inactivation did not depend upon the presence of CO_2 or light (55, 73). In contrast, the inactivation of nitrate reductase of Chlorella was stimulated by O_2 and light (60).

Chaparro et al. (74) suggested that the inactivation of nitrate reductase of Chlorella was modulated by the availability of the reducing power and high energy phosphate bonds. Uncouplers of photosynthetic phosphorylation such as ammonium or methylamine caused shifts in the relative concentrations of NADH and NAD^+ as well as of AMP, ADP and ATP. In vitro, similar shifts could promote inactivation of nitrate reductase.

Several other compounds were found to be effective inactivators of nitrate reductase. Thiols such as dithioerythritol, 2-mercaptoethanol

and thioglycollic acid, caused inactivation of spinach enzyme (67). Furthermore, in Chlorella, the reductants, dithionite, ferrocyanide and the chemicals sulfide and hydroxylamine also caused inactivation (75). In the alga Thalassiosira pseudonana, Cu^{+2} ions inactivated nitrate reductase but metal chelators such as EDTA or cysteine could protect against the inactivation (76).

Role of Cyanide in the Inactivation of Nitrate Reductase

Following the observation by Losada et al. (57) that an inactive nitrate reductase was formed in Chlorella upon addition of ammonium, a component extracted from this tissue was found to inactivate the enzyme in vitro (63). This factor was identified as cyanide which, when added along with NADH, caused inactivation (63,72). Cyanide was demonstrated to be the in vivo effector by purification of inactivated nitrate reductase from Chlorella and chemically identifying the bound inactivator (77).

Cyanide was found to bind to the reduced form of nitrate reductase of Chlorella with a dissociation constant (K_D) equal to 3.6×10^{-10} M (78). When bound to the enzyme, cyanide inhibited the reduction of nitrate non-competitively. However, when both cyanide and nitrate were present in the incubation mixture, cyanide competed competitively with nitrate for the active site of the enzyme (13). Inactivation of nitrate reductase by cyanide plus NADH was also reported in several other higher plants such as rice (79), corn (68), spinach (80) and barley (A. Oaks - personal communication).

Biological Synthesis of Cyanide

Several reports have discussed the possible biological origins of cyanide. Gewitz, et al. (81) reported that sonication of Chlorella stimulated the in vitro production of HCN. The production of HCN was also increased by addition of Mn^{+2} and the enzyme peroxidase to the extract or by a high intensity of light plus 0_2 (81). Addition of D-histidine, L-histidine or histamine to extracts of Chlorella also increased the generation of cyanide (82). Significantly, D-histidine generated ten-fold more HCN than did L-histidine (82). The aromatic amino acids D-phenylalanine, D-tyrosine and D-tryptophan could also promote the synthesis of HCN in Chlorella extracts (83).

One component of the extract of Chlorella which promoted the generation of HCN could be inactivated by heat and precipitated by ammonium sulfate (83). A flavin containing D-amino acid oxidase was determined to be an important component for the generation of HCN (84). In Anacystis nidulans another amino acid oxidase was identified which produced HCN from the basic amino acids L-arginine, L-lysine and L-ornithine and less effectively from L-histidine. EDTA completely inhibited an unidentified co-factor of the oxidase (85).

An alternative mechanism for the synthesis of cyanide was proposed by Solomonson and Spehar (86). According to this scheme, cyanide is produced from glyoxylate oxime (HON = CH - COOH), which is synthesized by condensation of glyoxylate with hydroxylamine. This scheme could account for the increased inactivation of Chlorella nitrate reductase under conditions of high 0_2 because a high proportion of 0_2 to CO_2 would increase photorespiration and therefore increase the production of glyoxylate. It would also account for the promotion of

inactivation of nitrate reductase by ammonium because according to Loussaert and Hageman (87), addition of ammonium led to the synthesis *in vitro* of hydroxylamine from nitrite by nitrite reductase. As further evidence for the existence of this mechanism, the formation of HCN could be stimulated in extracts of Chlorella by addition of glyoxylate plus hydroxylamine (88).

Metabolism of Cyanide

Cyanide appears to be metabolized primarily by the action of the enzyme, ß-cyanoalanine synthase which catalyzes the conversion of cysteine and cyanide into ß-cyanoalanine plus sulfide (89, 90, 91). This enzyme could be detected in each of 13 plant genera which were tested (89). In subsequent reactions ß-cyanoalanine can be metabolized into asparagine and the assimilation of cyanide into asparagine has been observed in corn (92), sorghum, blue lupine, vetch and trefoil (93).

Effects of Metals Upon the Abundance of Cyanide

Gewitz *et al*. (82) reported that Mn^{+2} stimulated cyanide production in Chlorella. Other metals or metal complexes such as vanadium or the o-phenanthroline complex of iron also had a stimulatory effect upon cyanide production (83, 94). During the extraction of nitrate reductase from the cyanogenic plant, sorghum, inclusion of Ni^{+2} in the extraction buffer increased the enzyme activity which could be recovered (95). Presumably Ni^{+2} bound to cyanide and prevented it from inactivating nitrate reductase (77).

Protein Factors Which Inactivated or Inhibited Nitrate Reductase

In addition to the chemical inactivation of nitrate reductase observed in algae, several proteinaceous effectors of the enzyme have been identified in higher plants. In rice, a 150,000 dalton factor was extracted (69). This factor bound reversibly to nitrate reductase (96) and could be released from the enzyme by reduction of the enzyme with NADH (70). The factor inactivated the component activities of NADH nitrate reductase, $FMNH_2$ nitrate reductase and NADH cytochrome c reductase but not the reduced methyl viologen nitrate reductase activity (97). Metal chelators inhibited the activity of the effector (97). Furthermore, the detectable quantity of the effector was highest during the early and late stages of growth (98) or when nitrate was withheld from the medium (99).

From the root tips of corn seedlings (100), a heat labile (101), 44,000 dalton (102) inactivating factor was extracted. This factor possessed proteolytic activity (96, 103) and affected the NADH cytochrome c reductase activity of the enzyme (68).

There are several other reports of inactivation of nitrate reductase. Behrend and Mateles (11) reported that an inhibitor of tobacco nitrate reductase could be removed by gel filtration. In tobacco (104) and cucumber (105), ammonium sulfate precipitation removed an inhibitor of nitrate reductase activity. The inhibitor in cucumber was additionally characterized as being a NADH-oxidase activity. In soybean, an inhibitor was identified the activity of which seemed to respond to light intensity; more activity was present in the dark than in the light. Furthermore, this inhibitor appeared to possess a protease activity (106). Finally, Jordan and Fletcher

(107) obtained evidence of a nitrate reductase inhibitor in tissue cultures of Paul's Scarlet Rose. This inhibitor was present in greater quantity in older cultures than in younger cultures and its inhibitory activity could be diminished by inclusion of casein in the extraction or assay medium.

Spontaneous Activation of Nitrate Reductase

An inactive nitrate reductase was first detected by the observation of spontaneous activation of the enzyme from Chlorella after addition of ammonium to nitrate-grown cells (57, 75). A similar spontaneous activation was also observed in Chlamydomonas (56). The spontaneous activation of nitrate reductase in algae is believed to be due to oxidation of the enzyme by oxygen which results in the release of cyanide from the enzyme-cyanide complex (57, 75).

In higher plants, the only reported case of spontaneous activation is for the nitrate reductase of Lemna minor. After addition of ammonium to the culture medium, an inactive enzyme was formed which could be partially reactivated upon storage of the enzyme at 1-2°C (61). The authors suggested that this spontaneous activation was due to the release of an inactivating protein during storage (108).

Chemical Activation of Nitrate Reductase

Nitrate reductase which had been inactivated either in vitro or in vivo could also be activated by chemical methods. The preferred method of activation of nitrate reductase which had been inactivated by NADH or NADH plus cyanide was by incubation with the oxidant ferricyanide (56, 64, 68, 75, 79, 109). Other oxidants such as nitrate

(56, 75), cytochrome c or DCPIP (75) were also reported to cause activation. These oxidizers are believed to function by oxidizing the molybdenum of the enzyme's molybdenum-containing complex from the +4 valence to the +6 valence. Because the cyanide complex of Mo (+6) is unstable, spontaneous dissociation would occur and activation of the enzyme would result (110).

The cyanide-inactivated nitrate reductase of spinach could also be oxidized using trivalent manganese (110). The trivalent manganese which was used was in the form of the manganipyrophosphate complex or it was generated by oxidation of Mn^{+2} in the present of illuminated chloroplasts.

Nitrate also activated an inactive form of nitrate reductase from barley (111). This activation was proposed to be due to activation of the molybdenum-containing complex of nitrate reductase which had been released when cyanide bound to the enzyme (D. Kaplan - personal communication).

Heating of extracts containing nitrate reductase enzyme could also activate the enzyme in some cases. This effect was observed in the algae Cyanidium caldarium (112, 113), Porphyridium aerugineum (114) and in Dunaliella purva (115).

Activating Factor of Nitrate Reductase

Yamaya and Oaks (116) reported the isolation of a nitrate reductase activating factor from the primary leaves and root tips of corn seedlings. The factor was stable to heat treatment and trypsin digestion which suggested that it was not a protein. The cationic factor acted upon the NADH nitrate reductase and NADH cytochrome c reductase

activities of the enzyme complex which prompted a proposal that the physiological role of the activator might be to counteract the nitrate reductase inactivating protein of corn (116).

Coordinated Regulation Between the Nitrate and Sulfate Assimilatory Pathways

ATP sulfurylase is a derepressible enzyme in tobacco XD cells with an increase in detectable activity when sulfur nutrition is inadequate (6). However, when the nitrogen nutrition of tobacco XD cells was inadequate, ATP sulfurylase could not be derepressed even if the cells were starved for sulfur (6). Under these conditions, assimilation of sulfate would be retarded, and therefore, nitrogen deprivation should result in the accumulation of sulfate. As predicted this situation was observed in corn (5) and in tobacco (117). Smith (117) also observed that other enzyme activities besides ATP sulfurylase were affected during nitrogen deprivation. Specifically, the activities of O-acetylserine sulfhydrylase and sulfate transport in tobacco XD cells were diminished when the cells were starved for nitrogen.

Conversely, inadequate sulfur nutrition caused nitrate reductase activity to be less than fully induced even though nitrate availability might be adequate. In sulfur-starved tobacco XD cells, nitrate reductase could not be induced by nitrate (P. Filner - personal communication). When corn was deprived of sulfur, nitrate reductase activity was 50% less than the activity in the control plants with adequate sulfur (118). Likewise, in Burley tobacco, with decreasing sulfur supplies there was a decrease in the activity of nitrate reductase (119). Furthermore, because the synthesis of sulfur compounds was

inadequate, certain amino acids accumulated during sulfur inadequacy. In tobacco, arginine accumulated over 180-fold above the level in control samples with adequate sulfur (120), whereas in perennial rye grass (121) and barley (122) asparagine preferentially accumulated.

These biochemical differences were also manifested in field experiments. Using corn (123, 124) increased application of ammonium nitrate increased the sulfur content of the plant. Correspondingly, when the application of sulfate was increased, the nitrogen content also increased. Eppendorfer (122) reported that for barley, increased applications of nitrate required that sulfate applications also be increased for there to be an improvement of yield. The coordination between the nitrate and sulfate assimilatory pathways appeared to be functioning to permit the most efficient assimilation of the available nutrients.

MATERIALS AND METHODS

Cultures of Tobacco XD Cells

The tobacco XD cell line was initiated by P. Filner in 1961 from stem sections of Nicotiana tabacum L. cv. Xanthi (125). The tissue cultures were maintained in liquid suspension cultures of 500 ml of M-1D medium in 1 liter flasks. Subculturing of stationary phase cells was performed by making a 1:20 dilution of 14 day-old cultures into fresh medium. The size of the inoculum ranged from 1.4 to 1.8 g/l. Agitation of the cultures was accomplished using a horizontally reciprocating shaker with a displacement of 7 to 8 cm at a frequency of 72 cycles/min. The cultures were grown at 28°C.

Culture Medium

The basic culture medium was the M-1D medium as described by Filner (125). This medium contained 2.5 mM nitrate and 3 mM sulfate as the sole nitrogen and sulfur sources respectively. Nitrate-less M-1D medium (N^-M-1D) was formulated by substituting chloride salts for the nitrate salts (53). Sulfate-less M-1D medium (S^- M-1D) was formulated by substituting chloride salts for the sulfate salts and by using a purified sucrose from which sulfur contaminants had been removed by ion exchange using Dowex 2 (22). Nitrate-less, sulfate-less

M-1D (N^-S^- M-1D) was prepared by substituting chloride salts for nitrate and sulfate salts and by using purified sucrose (6). Ammonium succinate M-1D was formulated by adding 3 mM ammonium chloride and 1.5 mM succinic acid and adjusting the pH of the medium to the usual value of 6.2 using NaOH. Medium was sterilized by autoclaving.

Harvesting of Tissue

Tissue cultures were harvested by vacuum filtration through two layers of Whatman 4 filter paper. The cells were rinsed once with deionized water and the tissue was transferred onto weighing paper for determination of fresh weight. The dry weight of the tissue was determined after heating at 80°C overnight.

NADH Nitrate Reductase Assay

To the harvested tissue was added buffer composed of 0.1 M potassium phosphate, 1 mM L-cysteine, 1 mM EGTA, 1 µM FAD pH 7.5 (modified Zielke buffer) at a ratio of 5 ml per gram of fresh weight of tissue. The mixture was homogenized by 30 strokes of a Teflon-glass tissue grinder while being maintained at ice temperature. The homogenate was centifuged at 12000 x g for 20 minutes at 4°C. The supernatant fraction was designated as the crude extract. Unless otherwise indicated saturated ammonium sulfate pH 7.5 was added to a portion of the crude extract to make a 50% saturated ammonium sulfate solution. This was incubated for 60 minutes at 4°C. Following this incubation, a second centrifugation was performed at 12000 x g for 20 minutes at 4°C. The resulting pellet was dissolved in a volume of the modified

Zielke buffer equal to the volume which was precipitated by ammonium sulfate and this fraction designated as the ammonium sulfate resuspension.

The NADH nitrate reductase activity was assayed by the method of Wray and Filner (39) by mixing 0.5 ml of 0.1 M potassium phosphate, pH 7.5 buffer, 0.1 ml of 0.1 M KNO_3, 0.1 ml of 1 mM NADH plus 0.3 ml of extract plus water. The reaction was started by adding the extract to the reagent mixture and transferring the reaction tube to a water bath maintained at 25°C. After 30 minutes, the reaction was stopped by adding 1 ml of 1% sulfanilamide in 3 N HCl. One milliliter of 0.02% N-1-naphthyl ethylene diamine dihydrochloride was subsequently added. Color development was allowed to proceed for at least 15 minutes. The solution was clarified by centrifugation at 3000 x g for 5 minutes and the absorbance at 540 nm was determined. The absorbance was converted to nmoles of nitrate based on the results obtained using a standard nitrite solution.

Assay of ATP Sulfurylase Activity

ATP sulfurylase activity was extracted and assayed essentially by the method of Reuveny and Filner (126). Tissue was homogenized at 4°C in 0.1 M glycine plus 5 mM ascorbate pH 9.0 buffer (5 ml/gram fresh weight) with 20 strokes of a Teflon-glass tissue grinder. The homogenate was centrifuged at 35,000 x g for 10 minutes at 4°C. The supernatant fraction was used for the assay of enzyme activity.

To a 15 ml Corex centrifuge tube was added:

0.05 ml of 0.1 M glycine pH 9

0.05 ml of 0.1 M $MgCl_2$

0.05 ml of 0.2 M Na_2SO_4

0.01 ml of inorganic pyrophosphatase (6-7 units/0.01 ml)

0.05 ml of 0.1 M ATP pH 7

0.05 ml of $Na_2\ ^{35}SO_4$ (1 mCi/ml)

0.1 ml to 0.2 ml of enzyme extract plus water to give a final volume of 0.5 ml

The reaction mixtures were incubated at 30°C for 0, 5, 10 and 15 minutes. Ice cold absolute ethanol (2.5 ml) was added dropwise to the mixture with vortexing to stop the reaction and to precipitate $Na_2\ ^{35}SO_4$. The reaction tube was centrifuged at 35,000 x g for 10 minutes at 4°C. The supernatant fraction was carefully removed and saved. To the supernatant fraction 0.2 ml of 1.0 M Na_2SO_4 (stored at room temperature) was added dropwise with vortexing. The mixture was again centrifuged for 10 minutes at 35,000 x g at 4°C. The precipitation with Na_2SO_4 was repeated twice more. One milliliter of the final supernatant was used for the determination of radioactive sulfur using the scintillation fluid described by Bray (127). The quantity of adenosine 5'-phosphosulfate formed could be calculated based on the measured specific radioactivity of sulfate in an aliquot of the reaction mixture.

Protein Determination

Protein was determined by the method of Lowry *et al.* (128). A 0.5 ml sample was precipitated by adding an equal volume of 20% tri-

chloroacetic acid (TCA) and boiling for 5 minutes. Four milliliters of 10% TCA was added prior to centrifugation at 1800 x g for 10 minutes at 4°C. The supernatant fraction was decanted and the pellet was washed with 5 ml of cold 95% ethanol. After centrifugation at 1800 x g at 4°C for 10 minutes, the supernatant fraction was decanted and discarded. The pellet was dried with a stream of nitrogen. Depending upon the size of the pellet, 0.2 to 1.0 ml of 1 N NaOH was added to dissolve the protein. This resuspension was assayed for protein using bovine serum albumin as a standard.

Assay for Nitrate

Nitrate was assayed by the method of Wray and Filner (39). A sample containing between 1 and 100 nmoles of nitrate was incubated with 0.5 ml of 0.1 M potassium succinate, pH 6.8 buffer, 0.1 ml of crude soya bean nodule extract plus water to yield a final volume of 1.0 ml. The solution was incubated for 30 minutes at 45°C. The reaction was stopped by addition of 1.0 ml of 1% sulfanilamide in 3 N HCL followed by 1.0 ml of 0.02% N-1-naphthyl ethylene diamine dihydrochloride. After allowing 15 minutes for color development, the mixture was clarified by centrifugation at 15,000 x G for 10 minutes. The absorbance was measured at 540 nm and compared to a standard curve.

Gel Filtration Apparatus

To obtain rapid and efficient separation of high and low molecular weight substances, a miniature Sephadex G-25 gel filtration column was used. The column consisted of a 5 ml disposable syringe with 4 ml (packed volume) of swelled Sephadex G-25 resin. The column could be

equilibrated with modified Zielke buffer by means of 7 cycles of adding 2 ml of buffer followed by centrifuging 15 seconds in a bench top clinical centrifuge. The first 6 centrifugations were at 3000 x g (setting #7) whereas the last centrifugation was at 2000 x g (setting #5). A sample could be filtered by applying 2 ml of that sample to a column equilibrated with modified Zielke buffer and centrifuging at 2000 x g for 15 seconds. The eluate contained approximately 60% of the high molecular weight components of the sample but only about 1% of the low molecular weight components as judged by the elution of blue dextran and riboflavin during preliminary tests. The column could be rinsed and reequilibrated by using the method described for preequilibration.

Ferricyanide Activation of Nitrate Reductase

To determine the quantity of ferricyanide activable nitrate reductase, 2.25 ml of sample plus 0.25 ml of 5 mM potassium ferricyanide (final concentration = 500 μM) were incubated for 30 minutes at 25°C. After incubation, 2.0 ml of the solution was applied to a Sephadex G-25 gel filtration column and centrifuged. The eluted volume was assayed for nitrate reductase activity and protein.

Partial Purification of Nitrate Reductase

Twenty-two grams of tobacco tissue were harvested and homogenized in modified Zielke buffer. The homogenate was centrifuged at 12,000 x g for 20 minutes at 4°C. To the supernatant fraction was added sufficient saturated ammonium sulfate solution, pH 7.5 to yield a 25% saturated solution. After a 30 minute incubation in an ice

bath, the mixture was centrifuged at 12,000 x g for 20 minutes at 4°C. To the supernatant fraction, additional saturated ammonium sulfate was added to yield a 40% saturated solution. After 30 minutes of incubation, the mixture was centrifuged as before. The resulting pellet was redissolved in modified Zielke buffer and designated the "25% - 40% saturated ammonium sulfate fraction".

The resuspension was dialyzed for 24 hours against 1.5 liters of 0.05 M potassium phosphate, 1 mM EGTA, 1 mM cysteine, 1 µM FAD pH 7.5, (MZ/2) buffer, in order to reduce the ionic strength of the solution. The buffer was changed once during the dialysis. This fraction was designated the "25% - 40% saturated ammonium sulfate fraction after dialysis".

This dialyzed fraction was applied to a DEAE Sephadex column (bed volume = 35 ml) which had been equilibrated with MZ/2 buffer. The column was rinsed with 50 ml of MZ/2 buffer. The nitrate reductase was eluted with 150 ml of a linear gradient of MZ/2 buffer and 0.2 M potassium phosphate, 1 mM cysteine, 1 mM EGTA, 1 µM FAD, pH 7.5, (2 x MZ) buffer. Finally the column was rinsed with 75 ml of 2 x MZ buffer. Four milliliter fractions were collected during the elution.

Peak fractions were combined and dialyzed overnight in 1.5 liters of MZ/2 buffer. This dialysate was termed the "DEAE Sephadex fraction" and was used to study the stability of partially purified nitrate reductase.

Preparation of Nitron Nitrate

Nitron nitrate was prepared by a method similar to that described by Cope and Barab (129). 0.25 g of nitron (8×10^{-4} moles) were

dissolved in 10 ml of 5% (v/v) acetic acid. Undissolved material was removed using a GS millipore filter. A nitrate solution was prepared by dissolving 0.27 g of $NaNO_3$ (3.2×10^{-3} moles) in 80 ml of water. Fifteen drops of an aqueous solution of H_2SO_4 (a 2:3 dilution of concentrated sulfuric acid) were added to acidify the solution. The nitrate solution was heated to a boil and then removed from the heat. Without stirring, the nitron solution was added dropwise to the hot nitrate solution. The resulting mixture was allowed to cool slowly. After 1 hour, the mixture was placed on ice for an additional 2 hours. The crystals which formed were harvested by vacuum filtration through a sintered glass filter. They were rinsed, first with a portion of the filtrate and then with 10 ml of ice cold water. The recovered material was dried by lyophilization. The yield of the white crystals was about 75% of the theoretical yield.

If necessary, this procedure could be performed under sterile conditions.

Nitron Assay

Nitron was assayed by the method of Babenko (130). To a large test tube were added 1 ml of nitron plus water, 2 ml of 2 mM methyl orange and 5 ml of dichloroethane. 0.1 M formic acid, pH 4 was added to produce a final volume of 10 ml. The solution was mixed and allowed to set until the phases had separated. The lower organic phase was carefully removed and the absorbance of this fraction was measured at 420 nm. The standard curve was fairly linear between approximately 75 and 400 nmoles of nitron.

Assay of Tetrazolium Violet

The assay of tetrazolium violet was based on the difference in the spectrum of the oxidized and reduced forms of the compound (131). The assay system consisted of 0.2 ml of 0.5 M ascorbic acid, 0.025 ml of 5 N NaOH plus tetrazolium violet and water in a final volume of 1.0 ml. After allowing 2 hours for complete reduction of the tetrazolium violet, the absorbance of the solution was determined at 583 nm. The standard curve was linear between 5 nmoles and 100 nmoles. Nitrate did interfere slightly with the assay.

Preequilibration and Recycling of the Ionac Ion Exchange Membranes

Ionac MA-3475 anion selective membranes were swelled and equilibrated by the following methods. All steps were performed with stirring unless otherwise noted.

1) Soak the membranes in 10% (w/v) NaCl overnight without stirring.
2) Rinse twice with deionized water.
3) Equilibrate the membranes in 0.5 N HCl for 30 minutes.
4) Rinse the membranes for 15 minutes in deionized water.
5) Equilibrate the membranes in 0.5 N NaOH for 30 minutes.
6) Rinse for 10 minutes in deionized water.
7) Equilibrate the membranes a second time in 0.5 N NaOH for 30 minutes.
8) Rinse twice for 10 minutes each with deionized water.
9) Rinse the membranes repeatedly for 10 minutes each cycle in 5 mM HCl until a constant pH is obtained.
10) Rinse the membranes with deionized water until a constant pH is obtained.

Membranes could be stored in water in the refrigerator until ready for use.

Previously used membranes could be recycled by the following protocol.

1) Soak the membranes overnight in 10% (w/v) NaCl without stirring.
2) Rinse the membranes four times in deionized water allowing 1 hour for each rinse.
3) Equilibrate in 5 mM HCl using 10 minute cycles until a constant pH is obtained.
4) Rinse with deionized water using 10 minute cycles until a constant pH is obtained.

Membranes may be sterilized by autoclaving while suspended in solution.

Preparation and Sterilization of Bio Fiber 20 Filters

A Bio Fiber 20 hollow fiber filter was prepared according to the manufacturer's instructions. The protective layer of plasticized glycerin which coated the fibers was removed by passing water through the fibers at a rate of about 100 ml/minute for 5 minutes. The filter was ready for use at this point.

The filter was stored and sterilized in a 1.5% formaldehyde solution. The formaldehyde was removed by passing 500 ml of water through the fibers and another 500 ml to 1000 ml through the container enclosing the fibers.

PART I: CONTROL OF NUTRIENT CONCENTRATIONS IN LIQUID SUSPENSION CULTURE

Introduction

Few reports have described the biochemical and physiological responses of plants to sustained and suboptimal nutrient availability. In most cases when using liquid suspension cultures the common practice has been to study the response of the tissue to either no nutrient or to an optimal nutrient concentration. When intermediate, suboptimal concentrations have been used, the nutrient conditions often changed markedly during the course of the experiment due to depletion of the nutrient from the medium by the plant tissue. In field experiments, though, investigations have routinely been conducted using suboptimal and sustained nutrient availability. However, the actual nutrient concentrations available to the plant in the field were poorly defined because of the complicating factors of ion exchange, leaching and water availability. In order to develop a better understanding of the response of plants to sustained and suboptimal nutrient availability, such as that which a plant experiences in the field, the nutritional status of the plant must be strictly defined or monitored.

Some plant biochemists and physiologists, attempting to imitate, *in vitro*, the environment of plants in their natural state, have tried many techniques designed to produce relatively constant availability of nutrients. These techniques can be categorized into three classes;

a) Batch cultures

b) Chemostat cultures

c) Equilibrium chemostat cultures

The first two classes can be described as kinetic methods of maintaining nutrient concentration because the concentration of nutrient is determined by the kinetic parameters of rate of supply and rate of consumption. The third method can be classified as an equilibrium method in which the concentration of nutrient depends on the rate of supply, the rate of consumption and a chemical equilibrium which exists between the nutrient solution and a nutrient reservoir.

Batch Cultures

A batch culture consists of a fixed volume of nutrient which is not replenished during the course of the growth of the tissue. Consequently, the nutrients may be depleted from the medium. As a result of this depletion, physiological or biochemical observations may reflect a response to transient environments rather than a constant, well-defined environment. To overcome this limitation, it is often possible to use a large volume of nutrient. The removal of a given quantity of nutrient from a large volume decreases the concentration of the nutrient less than the removal of the same quantity of nutrient from a smaller volume. Table (1) illustrates an example of the volume of nutrient which would be required to maintain a nitrate concentration within 20% of the initial concentration assuming that the growth of tobacco tissue would require about 85 μmoles of nitrate/g.f.w.

Several investigators have recognized this problem of nutrient depletion and have employed large volumes of medium in attempts to maintain relatively constant nutrient concentrations. Friedrich and Schrader (5) studied the development of hydroponically grown corn using 20 liter tubs. Kato et al. (132) utilized a 1500 liter fermenter to study the growth characteristics of tobacco.

Chemostat Cultures

Chemostat cultures involve the continuous replacement of nutrient at a fixed rate or at a rate periodically adjusted in response to estimated needs. As the tissue grows, the excess tissue may be removed, the rate of nutrient addition may be increased or the ambient concentration of limiting nutrient may decline but at a rate of decline which is less than that observed in batch cultures.

Table 1. Liters of growth medium required to grow tobacco XD tissue with only a 20% decrease in the initial concentration of nitrate in the medium.

Initial Nitrate Concentration	Quantity of new tissue formed 4g	3g	2g	1g
1 mM	1.70	1.28	0.85	0.42
500 µM	3.40	2.55	1.70	0.85
200 µM	8.50	6.38	4.25	2.12
100 µM	17.0	12.8	8.50	4.25

Calculations are based on the assumption that 85 µmoles of nitrate are required to grow 1 gram fresh weight of cells.

Unfortunately, this technique has characteristic difficulties. Often it requires large volumes of nutrient, elaborate plumbing, or expensive equipment. Additionally, maintaining sterile conditions can be difficult.

May investigators have chosen to utilize this method in their research. Asher et al. (133) sustained nutrient concentrations as low as 10 nM but required up to 1300 liters of nutrients per day per pot. Datko et al. (134) studied the sulfate nutrition of Lemna minor over the range of 1000 μM to 0.26 μM using up to 10 liters of nutrient per day. Reisenauer (135) used a technique which pumped 0-25 liters of nutrient per day through vessels containing wheat seedlings. Using this system, he observed that plants growing in dilute nutrient solution developed more extensive root systems than plants growing in more concentrated nutrient solution.

Equilibrium Chemostat Culture

The method of equilibrium chemostasis is based on a principle of equilibrium rather than on kinetics. An equilibrium is established between the nutrient solution and a reservoir of the nutrient. As nutrient is consumed from the growth solution, it is replaced from the reservoir in order to maintain the equilibrium. The reservoir may consist of an ion exchanger, an insoluble salt of the nutrient or a substance into which the nutrient may partition. The concentration of nutrient in the growth medium is determined by three parameters:

1) The concentration of nutrient in the reservoir.
2) The concentration of counterions of the insoluble salt of the nutrient, the quantity of ion exchange sites or the volume of partitioning solvent.
3) The affinity of the nutrient for the binding or partitioning substance.

By judicious choice of the quantity and properties of the reservoir material, comparatively large quantities of nutrient can be supplied to the culture with only minimal changes in the concentration of the nutrient. This technique was successfully used by Graham and Albrecht (136) to supply nitrate to corn using an Amberlite ion exchange resin. Similarly, Converse et al. (137) used ion exchangers to supply potassium and phosphate to corn.

Proposed Method to Maintain Constant Nitrate Concentrations in Cultures of Tobacco Cells

In anticipation of studying the regulation of nitrate and sulfate assimilation at limiting but sustained concentrations of these two nutrients, methods were investigated to sustain the concentration of nitrate and sulfate at concentrations below 1mM and 100 μM, respectively. Because several grams of plant material would be required for the anticipated biochemical studies, the technique of batch culture was eliminated because of the cumbersome quantity of medium that would be required (see Table 1). The system of chemostat culture was likewise eliminated because of the expense of available instruments. Therefore, the method of equilibrium chemostasis was chosen as the most promising technique.

Since only nitrate and sulfate were to be controlled and limited, the use of ion exchange resins was rejected. The available resins were relatively non-selective for these two ions and, as a result, would alter the concentration of other nutrients in the medium as well. The method of partioning between solvents likewise did not seem feasible because of poor selectivity.

To meet the stringent requirement of selectivity, an alternative method was proposed for development. This would involve the use of an insoluble salt of the nutrient and a counterion selective for that nutrient. The system was envisioned as consisting of a culture vessel containing a solution of the nutrient, the precipitating counterion and a reservoir of the precipitate of the two ions. As nutrient would be absorbed by the plant material, its concentration would tend to decrease. However, by the solubility product principle, some of the precipitated salt would dissolve in order to maintain the solubility product. Nutrient and counterion would be released into solution.

The dissolution of nutrient and precipitating counterion would occur in equal quantities. If the concentration of nutrient in solution is far less than the concentration of the precipitating counterion, the nutrient in solution could be consumed and totally replaced by dissolution of the precipitate without markedly altering the concentration of the counterion. Since the concentration of the counterion would remain nearly the same, the concentration of the nutrient would likewise remain nearly the same by virtue of the solubility product principle.

The solubility product of a nutrient-precipitating counterion salt could be used to evaluate the utility of that salt for the control of the nutrient concentration. For monovalent salts, the solubility product is the multiplicative product of the concentrations of the ions in a saturated solution. The square root of this solubility product would thus indicate the concentration at which both the nutrient and its precipitating counterion would be present at equal concentrations. Because it is desirable that the concentration of the precipitating counterion be far greater than the concentration of the nutrient, the square root of the solubility product should be greater than the concentration of the nutrient which would be desired for experimental purposes.

Ideally, the counterion of the nutrient should possess three characteristics:

1) The square root of the solubility product of the nutrient and the counterion should be greater than the anticipated range of nutrient concentrations to be investigated.
2) The counterion should be commercially available or easily synthesized.
3) The substance should be non-toxic or manipulatable so as to be non-toxic (e.g., by segregation from the living tissue).

Initially, a counterion for nitrate was sought. Several possible counterions were identified including nitron (129, 138, 139, 140), substituted naphthyl methyl amines (141, 142), α-phenyl-ß-diethyl amino ethyl-p-nitrobenzoate (140, 143) and the alkaloid cinchonamine (140, 144). Of these possible choices, nitron was chosen for more

extensive evaluation and experimentation because of its commercial availability.

Properties of Nitron

Nitron is the common name of 1, 4-diphenyl-3-anilino-1,2,4-triazole (145). The nitrate salt of nitron has a solubility product of 7×10^{-8} M^2 in slightly acidic water (139). Nitron could be obtained commercially from The Baker Chemical Company. Nitron could be solubilized by carefully dissolving the golden-yellow powder in water and slowly adding HCl until the pH stabilized. The pH was adjusted to 6.2, the acidity of M-1D medium. The spectrum of the solution showed no absorbance peak between 400-760 nm. However, in the ultraviolet, a peak was detectable at 246 nm with a molar extinction coefficient of 1.9×10^4 O.D. / mole / liter. The solubility of nitron was estimated to be 60 mM under these conditions.

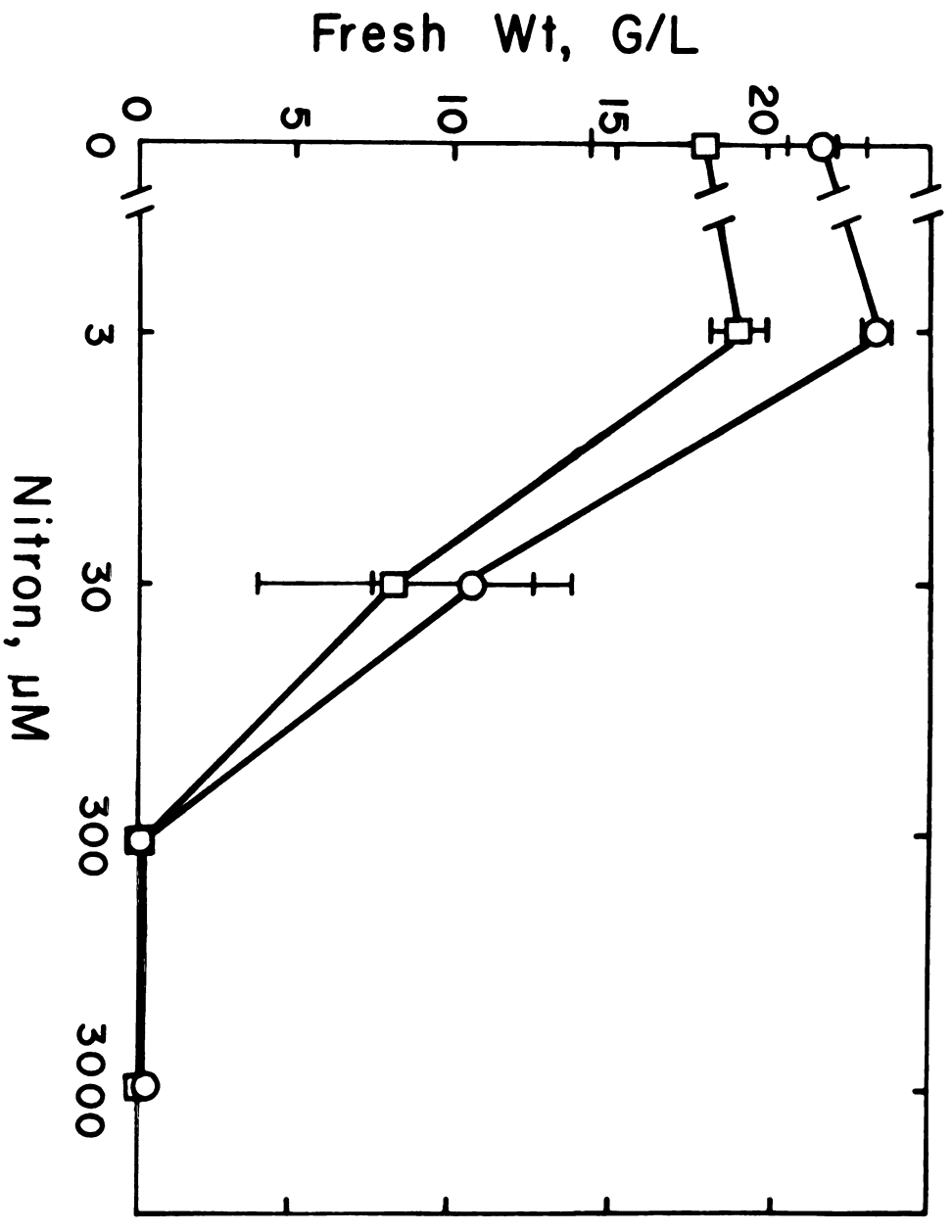
The only report of the physiological effects of nitron is that of Takatori *et al.* (146) which reported the effects of nitron on the enzyme, catalase, of mouse liver and showed an *in vivo* inhibition of activity of 65% when 100 mg / kg were injected abdominally. (CAUTION. Because of the structure of nitron, it is strongly advised that nitron be treated with extreme care and only be used under the assumption that it may be a potent carcinogen!)

Nitron Toxicity

The magnitude of the toxicity of nitron was of paramount importance. Complete inhibition of growth was observed at 300 μM nitron when either nitrate or ammonium was the nitrogen source (Figure 2).

Figure 2. Toxicity of nitron to tobacco XD cells with nitrate or ammonium as the nitrogen source.

Stationary phase tobacco XD cells were inoculated into 500 ml of M-1D (○) or N^{-}M-1D + 1.5 mM ammonium succinate (□) medium plus 0 µM, 3 µM, 30 µM or 3000 µM nitron. The average mass of the inoculum was 0.26 g fresh weight per 500 ml. After 14 days, the cells were harvested by vacuum filtration and weighed.


Fresh Wt, G/L
0
5
10
15
20
0
3
30
300
3000
Nitron, µM

Because inhibition of growth was similar when either ammonium or nitrate was the nitrogen source it appeared that the toxicity of nitron was not due to precipitation of nitrate by nitron. Figure 3 illustrates a more detailed dose-response curve for nitron. Fifty percent inhibition of growth occurred at about 25 µM.

To avoid the toxic effect of nitron, the concentration would need to remain below approximately 10 µM. However, with a solubility product of $7 \times 10^{-8} M^2$, the concentration of nitrate would remain above 7mM throughout an experiment. For tobacco XD cells, nitrate concentrations above approximately 1 mM could be considered as being saturating levels for the nitrate uptake system. Therefore, in order to study the effects of nitrate below concentrations of 1 mM, the toxic effects would need to be overcome.

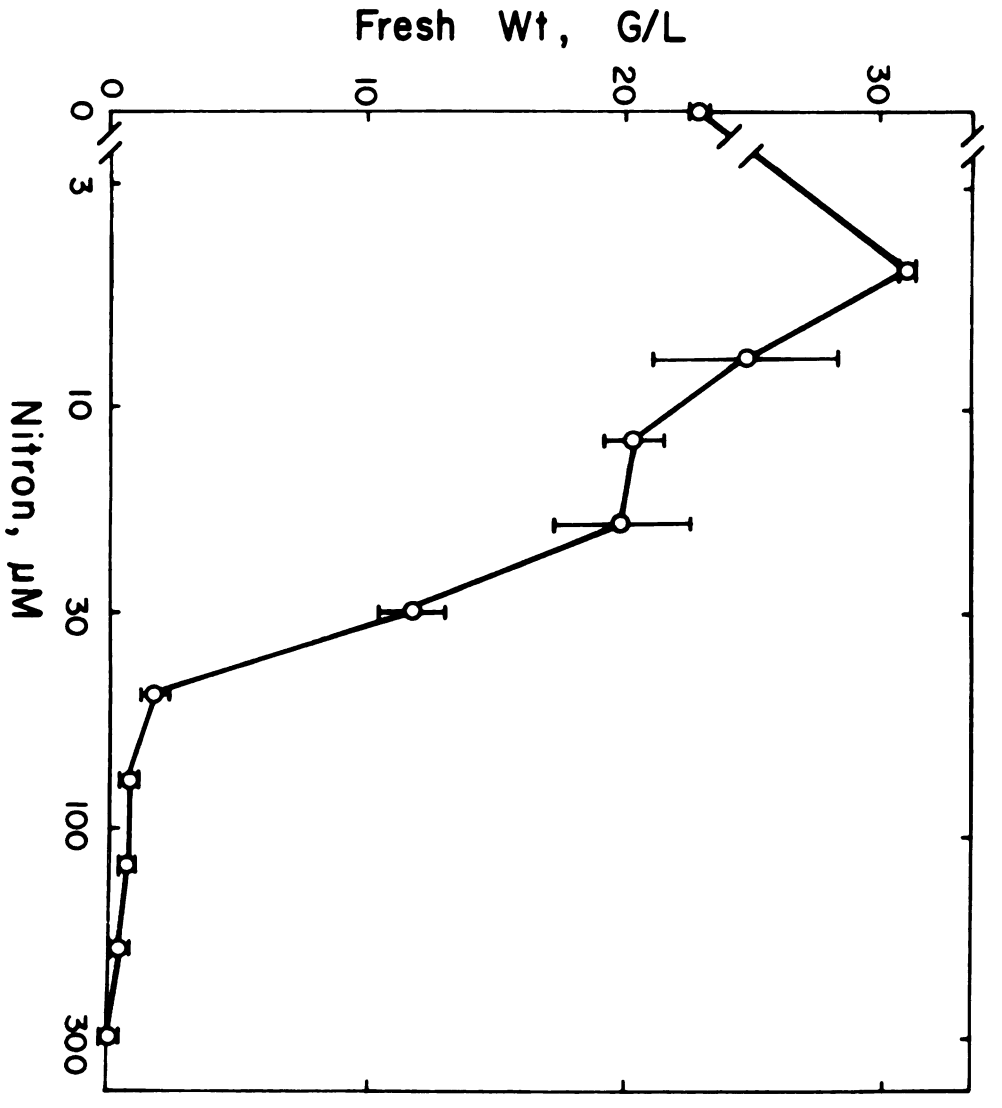
Attempts to Select Cell Lines Tolerant of Nitron

One possible approach to overcome the toxicity of nitron would be to develop strains of tobacco which would be tolerant to nitron. Heimer and Filner (147) had previously selected tobacco XD cells resistant to L-threonine which is a potent inhibitor of growth on nitrate. Using these same resistant cell lines, Flashman and Filner (148) developed strains of cells resistant to selenocystine and selenomethionine. The reported frequency of occurrence of resistant varieties was about 2×10^{-7}.

Two approaches were attempted in an effort to develop cell lines tolerant to nitron. The first involved a straight-forward selection in which 1×10^{8} cells were inoculated into a medium of M-1D + 200 µM nitron. After several months, no growth was evident in the cultures.

Figure 3. Toxicity of nitron to tobacco XD cells growing on M-1D medium.

Stationary phase cells were inoculated into replicates containing 500 ml of M-1D medium plus nitron at the indicated concentrations. The mass of the inoculum was about 0.4 g fresh weight per 500 ml. After 14 days, the tissue was harvested and weighed.


Fresh Wt, G/L
0
10
20
30
Nitron, μM
0
3
10
30
100
300

The second approach involved an enrichment program in which the cells would be exposed to progressively higher concentrations of nitron in an effort to increase their tolerance. The first stage was to inoculate tobacco cells into M-1D + 45 μM nitron, (a concentration sufficient to result in 90% inhibition of growth). The second and third stages increased the nitron concentration to 75 μM (98% inhibition) and 125 μM (greater than 99% inhibition), respectively. Because no growth was observed when the higher concentrations were used, the enrichment program was discontinued.

Segregation of Nitron from Living Tissue

In addition to attempts to develop lines of tobacco which would be tolerant to the effects of nitron, other methods to overcome the problem of toxicity were pursued. These were methods to selectively segregate nitron from the plant tissue using a perm-selective barrier. Two methods were studied; the first involved the use of an ion exchange membrane while the second employed the principle of ultrafiltration.

Ion Exchange Membranes

In solution, nitrate is a negatively charged species. Nitron, although possessing both positive and negative character, possesses regions of distinct positive charge, localized predominantly around the ring and anilino nitrogens (145). It was anticipated that discrimination between nitrate and nitron could be achieved based on differences in their ionic character.

The selective exclusion and passage of charged ions has been a routine practice in certain types of water desalination programs.

This technology uses ion exchange membranes which were considered to be possible means to discriminate between nitrate and nitron.

Ion exchange membranes are ionic polymers formed into a flat sheet with minute channels penetrating that sheet. If the membrane is of the anion exchange type, anions bind to positively charged functional groups interspersed along the walls of the channel. Migration of ions from site to site results in a net migration across the membrane. The driving force for the movement of anions is the chemical potential gradient which would be imposed across the membrane. Cations would, however, be excluded from the membrane due to repulsion by the cationic functional groups lining the channel. By this principle, nitrate could traverse an anion exchange membrane while nitron would ideally be excluded.

The membrane chosen for study was the MA-3475 anion exchange membrane manufactured by the Ionac Chemical Company. This product was chosen because of its high permselectivity (99.0%), considerable ion exchange capacity (0.70 milliequivalents per gram) and ability to withstand temperatures of up to 125°C which would permit autoclaving.

Flux of Nitrate Through MA-3475 Membranes

The flux of nitrate through the MA-3475 membranes and the ability of the membranes to discriminate between nitrate and nitron were estimated. At the time that this experiment was conducted, a chemical assay for nitron had no been identified. Therefore, spectrophotometric measurement of the absorbance at 246 nm was used as an assay. However, because the membranes themselves leached an ultraviolet absorbing chromogen into the medium, estimation of nitron using ultraviolet

absorbance was impractical. To overcome this limitation, an assayable analog of nitron was used. Tetrazolium violet, 2-(1-naphthyl)-3,5-diphenyl-1,2,3,4-tetrazole, closely approximated the structure and presumably the ionic character of nitron.

To measure the flux of nitrate and tetrazolium violet across the MA-3475 membrane, an apparatus was constructed to allow the membrane to separate two solutions. Across the membrane, concentration gradients of 0.1 M nitrate and 1 mM tetrazolium violet were established with KCl to act as a counterion.

The results indicated that if a gradient of 0.2 mM nitrate was established across the membrane, the nitrate flux would be 0.65 μmoles of NO_3^- per day per cm^2 of membrane. To grow 1 g of tobacco tissue per day, about 85 μmoles of nitrate would be required. In order to supply 85 μmoles of nitrate, 131 cm^2 of membrane would be required. Unfortunately, the flux of tetrazolium violet (or presumably nitron) across a 1 mM concentration gradient was 0.65 μmoles per day per cm^2 of membrane. With 131 cm^2 of membrane, the corresponding flux of tetrazolium violet (nitron) would be about 85 μmoles per day which would soon result in the accumulation of toxic levels of nitron (see Figure 3). Furthermore, 131 cm^2 of membrane would have an extremely inhibitory effect upon the growth of tissue because the membrane itself is toxic to the cells (Figure 4).

These results indicated that the MA-3475 ion exchange membrane would not be suitable for the proposed purpose. The flux of nitrate would be too meager, the corresponding flux of nitron would be too large and furthermore, the required quantity of membrane would be toxic. Reasonable methods to overcome these limitations were not

evident. Some other approach to segregating nitron from the plant tissue would be required.

Ultrafiltration of Nitrate and Nitron

An alternative method to discriminate between nitron and nitrate was based on the difference in their molecular sizes. The molecular weight of nitrate is 62 daltons while that of nitron is 312 daltons. In theory, the two species could be separated by ultrafiltration. [For a general discussion of ultrafiltration see Blatt (149) and Michaels (150)].

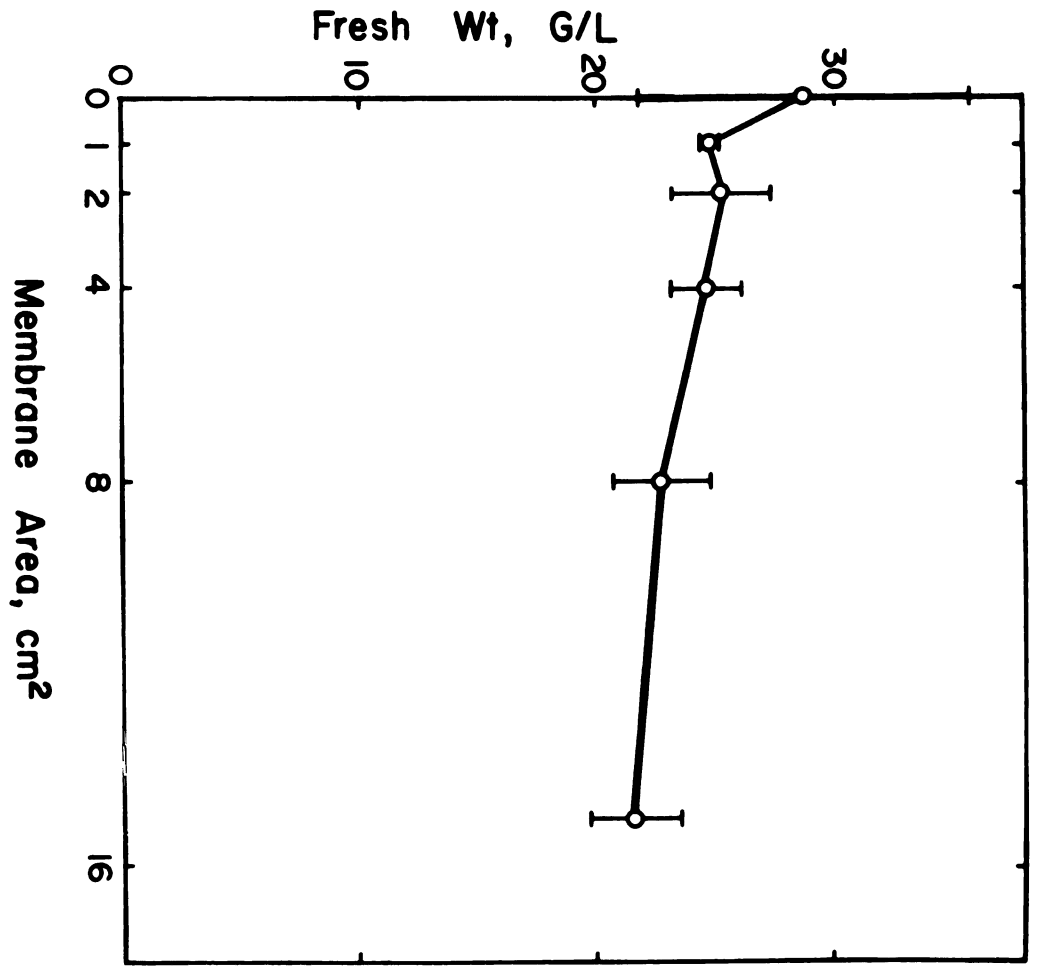
Only one commercial product appeared likely to accomplish the required separation - the Bio Fiber 20 Hollow Fiber Filter. This filter was manufactured by The Dow Chemical Company and distributed by Bio-Rad Laboratories. The Bio Fiber 20 consists of several dozen hollow fibers with minute channels penetrating cellulose acetate A walls. These channels would effectively discriminate against passage of molecules with molecular weights greater than 200 daltons. Large solute fluxes could be achieved as a result of the large surface area of the fibers - amounting to 1000 cm^2 according to the product information manual. However, these filters are no longer manufactured or distributed. We were fortunate to obtain the last remaining stocks of this product for study and evaluation.

Flux of Nitrate and Nitron Across the Bio Fiber 20 Filters

The presence of a large impermeable anion on the side of the filter containing the nitron nitrate enhanced the flux of nitrate across the filter. In initial experiments using citrate as the

Figure 4. Inhibition of growth of tobacco XD cells by Ionac MA-3475 membrane.

Stationary phase tobacco XD cells were inoculated into 500 ml of M-1D medium in 1 liter flasks containing the indicated quantity of Ionac MA-3475 membrane. After 14 days the cells were harvested and weighed. The mass of the inoculum was 0.5 g fresh weight per 500 ml.


Fresh Wt, G/L
0
10
20
30
Membrane Area, cm²
0
1
2
4
8
16

impermeable anion, a pronounced reaction occurred between nitron nitrate and citrate. As a consequence, a search was undertaken for a substitute for citrate. Several characteristics were important.

1) The molecular weight should be greater than 200 daltons in order for the anion to be retained by the filter.
2) The compound should be an anion or a zwitterion in order to reduce the boundary potential created when nitrate but not nitron passed through the filter.
3) It should be non-toxic and non-reactive.
4) The compound should be readily available and inexpensive.
5) Finally, it must be water soluble to a level of at least 20 mM to 50 mM in order to counteract the osmolarity of sucrose.

Hepes, a Good buffer (151) was chosen for experimental evaluation. This compound, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, has a molecular weight of 206 daltons and a solubility of 2.25 M. It has low reactivity, low metal binding and may exist as a zwitterion. Hepes is frequently used as a buffer in biological media and is therefore readily available.

In a preliminary experiment two flasks were prepared, one containing $\bar{N}$M-1D (the "sink" flask) and the other containing nitron nitrate, 58.4 mM Hepes plus $\bar{N}$M-1D minus sucrose (the "source" flask). Note that sucrose was withheld from the "source" solution in order to supply sufficient Hepes to overcome a membrane potential which might develop between nitrate and nitron; Hepes was present at a concentration which would provide equal osmolarity with the sucrose across the membrane.

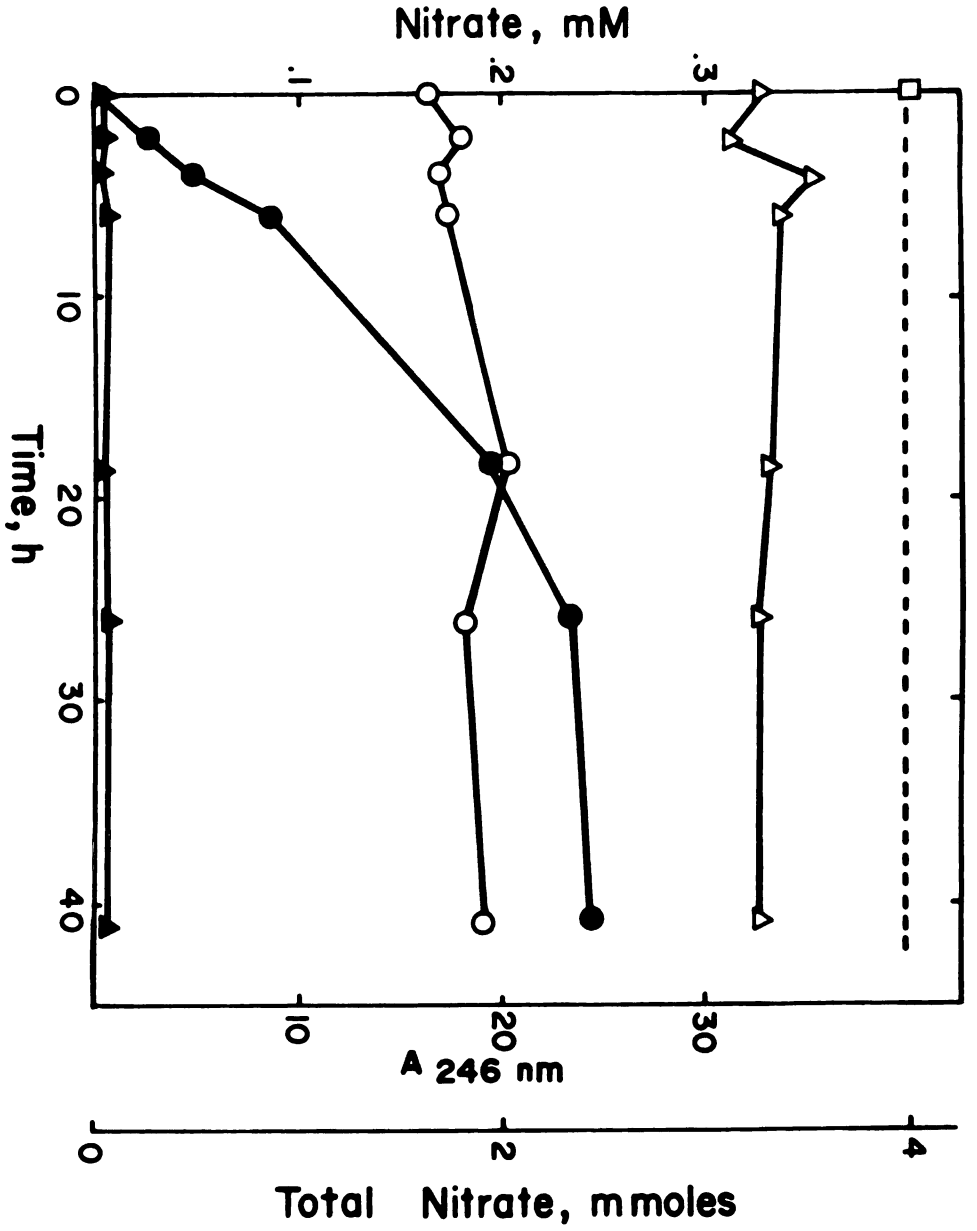
Nitrate rapidly migrated across the filter as illustrated in Figure 5 and equilibrium was established within 24 hours. The calculated flux of nitrate was 45 μmoles/day across a concentration gradient of 0.1 mM nitrate. Observe that the concentration of nitrate in the "sink" flask actually exceeded the concentration of nitrate in the "source" flask suggesting that two factors influenced the flux of nitrate. The primary factor was the concentration gradient of nitrate across the filter. The second was probably an electropotential gradient which in effect electrophoresed nitrate through the filter. This electropotential gradient may have developed from the migration of the sodium ion of the sodium Hepes across the filter while the negative Hepes molecule remained behind.

The concept of nitron nitrate acting as a "buffer" of nitrate was supported by this experiment. The two flasks contained equal volumes of solution. If nitron nitrate had not dissociated, the final concentration in both flasks would simply have been one-half the concentration in the "source" solution. To the contrary, even after supplying a significant quantity of nitrate to the "sink" solution, the concentration of nitrate in the "source" flask remained undiminished!

Whereas the nitrate flux was rapid, the flux of nitron was much slower. The absorbance at 246 nm increased to a constant value of about 0.6 O.D. in the "sink" flask while in the "source" flask, the absorbance was 32 O.D. Less than 2% of the chromogenic material migrated across the filter. Assuming that nitron was equimolar with the 0.2 mM nitrate, then the concentration of nitron in the "sink" flask was only 4 μM, a non-toxic level (see Figure 3). The results

Figure 5. Equilibration of nitrate and nitron across a Bio Fiber 20 hollow fiber filter.

The assembly consisted of two 1 liter flasks plus a Bio Fiber 20 hollow fiber filter. One flask, the "source" (open symbols) contained 600 ml of N^{-}M-1D (minus sucrose), 58.4 mM Hepes buffer, pH 6.2 plus 1.5 g of nitron nitrate. The second flask, the "sink" (filled symbols), contained 600 ml of N^{-}M-1D. The contents of each flask were pumped through the hollow fiber filter at 6-7 ml/minute to allow equilibration to occur across the filter membrane. During the course of the experiment, samples were withdrawn for analysis of nitrate (○,●) and nitron (△,▲). Open square (□) indicates the initial quantity of total nitrate. Nitron was estimated by the absorbance of the solution at 246 nm. All manipulations of the assembley were performed sterilely.


Nitrate, mM
.1
.2
.3
0
10
20
30
40
Time, h
10
20
30
A 246 nm
0
2
4
Total Nitrate, mmoles

suggested that the chromogenic material was not only nitron. The absorbance of the material in the "source" flask was much greater than expected based on the absorptivity of nitron, and secondly, the absorbance in the "sink" flask reached a plateau early in the experiment. These two observations suggested that some decomposition of the nitron occurred either during preparation or storage and that the material which migrated across the filter was a low molecular weight product of this decomposition. With the development of a more refined method for preparing nitron nitrate, decomposition may be reduced or eliminated.

In conclusion, hollow fiber filters might allow a moderate but adequate flux of nitrate. Additionally, segregation of nitron from the living tissue is possible by means of an appropriate hollow fiber filter.

Toxicity of the Hollow Fiber Filter Assembly

The next stage was to investigate whether the assembly just described could support growth of tobacco cells. Tobacco tissue was inoculated into a flask containing $\bar{N}$M-1D + 0.2 mM KNO_3, the equilibrium concentration of nitrate as defined in the previous experiment. This flask was connected to a hollow fiber filter which was also connected to a second flask which would act as a reservoir of nitrate. The flasks and filters were connected using latex tubing and silicone peristalic tubing. Peristalic pumps were used to pump the media.

After 7 days, the tobacco cells had turned dark brown and had the consistency of a paste when collected by filtration. Some component

of the assembly was toxic to the tissue. Because the toxicity was again observed when the hollow fiber filter, Hepes and the nitron nitrate were excluded from the assembly, it was concluded that a toxic substance was released from the connecting tubing.

Effects of Various Types of Tubing Upon Growth of Tobacco Cells

To confirm that the tubing released a growth inhibiting substance and to attempt to identify types of tubing and methods of sterilization which would not affect the growth of tobacco cells, two experiments were conducted. The combined results are presented in Table 2. Obviously, the tubing used in the previous experiment, the latex amber and the silicone peristalic tubing, were very detrimental to the tissue; harvested fresh weights were only 13.6% and 9.2% of the control culture, respectively. Only Teflon FEP tubing did not inhibit growth, showing a harvested fresh weight approximately that of the control. Silicone food peristalic tubing which had been sterlized using formaldehyde caused significant growth inhibition but because peristalic pumping was the method-of-choice for pumping the media, it was decided that very short segments of this tubing would be used in subsequent experiments.

Toxicity of the Bio Fiber 20 Filters

A test was performed to determine whether the hollow fiber filters were themselves toxic to the tissue. A flask containing $\bar{N}$M-1D + 0.2 mM KNO_3 was connected to the hollow fiber filter using only glass, Teflon FEP tubing and a short segment of silicone food peristalic tubing which had been sterilized using formaldehyde.

Table 2. Toxicity of various types of tubing to tobacco XD cells.

	Type of Tubing	Length of Tubing (cm)	Method of Sterilization	Harvested Fresh wt. (g)	Percent of Control
A	Control	-	-	2.56	100%
	Silicone food	100	Formaldehyde	1.76	69%
	Latex amber	100	Formaldehyde	0.25	9.8%
	Tygon	100	Formaldehyde	0.12	4.7%
	Teflon FEP	100	Autoclaving	3.09	121%
	Silicone food	100	Autoclaving	0.09	3.5%
	Tygon food	100	Autoclaving	0.02	0.8%
B	Control	-	-	5.3	100%
	Latex amber	190	Autoclaving	0.72	14%
	Silicone Peristalic	245	Autoclaving	0.49	9.2%
	Tygon	190	Autoclaving	0.02	0.4%

Two sets of experiments (A and B) are presented in this table. The indicated lengths of tubing were added to either 400 ml of N^-M-1D + 200 μM KNO_3 in 1 liter flasks (A) or 800 ml of N^-M-1D + 200 μM KNO_3 in 2 liter flasks (B). The tubing was sterilized either by autoclaving *in situ* or by immersion in a 3% formaldehyde solution followed by rinsing with sterile deionized water prior to addition to the sterile medium. Mid-exponential phase (A) or stationary phase (B) cultures were sterily harvested and 2g (A) or 3g (B) of tissue were inoculated into the test flasks. Tissue was harvested by vacuum filtration after 7 days (A) or 8 days (B) and weighed.

After 5 days, most of the tobacco tissue was dead or plasmolyzed. The fresh weight of the cells had decreased from 3.1g at inoculation to 0.53g. It appeared that the Bio Fiber 20 filter was itself toxic.

There are two components of the Bio Fiber 20 Filter which might have contributed to the toxicity: the cellulose acetate A fibers and traces of formaldehyde which adhered to the fibers after sterilization and rinsing. Because formaldehyde is a known antiseptic and presumably toxic to plant tissue, a simple experiment was conducted to test if the toxicity was due to formaldehyde adhering to the fiber strands.

Judging from the topology of the intertwined fibers, it was estimated that 1 ml to 3 ml of 1.5% formaldehyde might adhere to the surface of the fibers even after rinsing. To 500 ml cultures of tobacco XD cells was added either 1 ml or 3 ml of 1.5% formaldehyde which yielded final concentrations of 1 mM and 3 mM, respectively. After 8 days, the culture containing 1 mM formaldehyde contained tissue exhibiting some abnormal characteristics such as slightly expanded volumes with very refractile material near the nuclei. The cells in the culture containing 3 mM formaldehyde were all plasmolyzed. It is quite conceivable that 3 ml of formaldehyde could adhere to the fibers after rinsing. It can be calculated that to retain 3 cm^3 of liquid over an area of 1000 cm^2 (the surface area of the fibers) it would only require that the liquid have a depth of 3 μm!

Formaldehyde adhering to the fibers may have been responsible for the observed toxicity of the sterilized fibers. However, it has not been proven that the filter assembly itself is not a toxic component.

Conclusion

The concept of using nitron nitrate and a semipermeable membrane to maintain a suboptimal concentration of nitrate seems valid. However, the toxicity of the only available semipermeable membrane with the required selectivity remains an obstacle. Alternative approaches are numerous. More intensive research might identify an insoluble salt which would be less toxic than nitron nitrate. A device could be developed which could successfully allow the nutrient to pass while excluding the toxic substances from the cells. An organism other than tobacco XD cells could be studied which would be less sensitive to the chemicals contained in plastic tubing.

The potential uses of insoluble, dissociable salts of nitrate or other nutrients are likewise numerous. In the field of biochemistry and physiology, this method of controlling nutrient concentration would provide a convenient method to study the response of organisms to suboptimal but sustained concentrations of chemicals. In fermentations, it could provide a technique to elicit the production of certain chemicals from organisms which produce those chemicals only under the stress of suboptimal nutrition. This method might also provide a strategy for selecting variants which could grow more vigorously under conditions of suboptimal nutrient concentrations. Finally, for the agronomist, the concept of an insoluble salt may provide a method to supply crops with nutrient over extended periods of time and to supply the nutrients in direct response to the nutritional needs of the crop.

PART II: REGULATION OF NADH NITRATE REDUCTASE

Coupled Regulation of Nitrate Reductase and ATP Sulfurylase

There have been a few reports which suggested that the activity of nitrate reductase was modulated by the adequacy of the sulfur nutrition of the plant. Early observations in the 1930's and 1940's using tomato, soybean, sunflower and cotton (Tabulated in reference 3) showed that during sulfur deficiency, nitrate accumulated in the tissue. Nitrate reductase of tobacco XD cells cannot be fully induced in cells starved for sulfur (P. Filner - personal communication). Nitrate reductase was lowered in maize (118) and in Burley tobacco (119) by sulfur deficiency.

ATP sulfurylase becomes derepressed in tobacco XD cells deprived of sulfur, provided that an adequate supply of nitrogen is available (6). However, derepression of ATP sulfurylase in response to sulfur starvation does not occur if nitrogen nutrition is also inadequate (6). Sulfate accumulated during nitrogen deficiency in corn (5) and in tobacco (117) which suggests that nitrate deprivation has less effect on sulfate uptake than on ATP sulfurylase.

It is not clear from the above results whether the decreased enzyme activities reflect a general decrease in protein synthesis or specific regulatory modulations. Furthermore, the dependence of the modulation on nitrogen concentration has not been determined.

The previous results concerning the coordinated operation of the nitrate and sulfate assimilatory pathways had been obtained by completely withholding either nitrate or sulfate from the tissue which created a condition that ultimately would be fatal. This approach is inadequate to answer the more importrant quantitative and mechanistic questions concerning the regulatory coupling between the two pathways. To answer such questions adequately, plant tissues should be studied while exposed to sustained conditions of nutrient inadequacy. This was the reason for the study of the nitron nitrate system.

While I was attempting to develop the nitron nitrate system, Dr. Ziva Reuveny and Dr. Donald Dougall performed a single experiment designed to show that in tobacco XD cells the activity of nitrate reductase was proportional to the adequacy of the sulfate nutrition and that ATP sulfurylase activity was proportional to the adequacy of the nitrate nutrition. In their experiment, the initial sulfate concentration was varied while the initial concentration of nitrate was uniform at a concentration that was adequate to meet the nitrogen requirements of the tissue. They observed that the activity of nitrate reductase was proportional to the initial sulfate concentration. Additionally, they varied the initial nitrate concentration while the initial sulfate concentration was uniformly inadequate such that ATP sulfurylase activity should be derepressed. They observed that the magnitude of derepression of ATP sulfurylase activity was proportional to the initial nitrate concentration (152,153). It is important to note that the nutritional status of the tissue was not maintained throughout the duration of the experiment, but varied markedly as the

tissue rapidly depleted the nutrients from the medium. In addition, critical control segments of the experiment failed.

Because a major objective of my thesis research was to investigate the coordination between the nitrate and sulfate assimilatory pathways, I was approached by Dr. Filner and Dr. Reuveny and asked if I would independently complete the investigation started by Dr. Reuveny and Dr. Dougall.

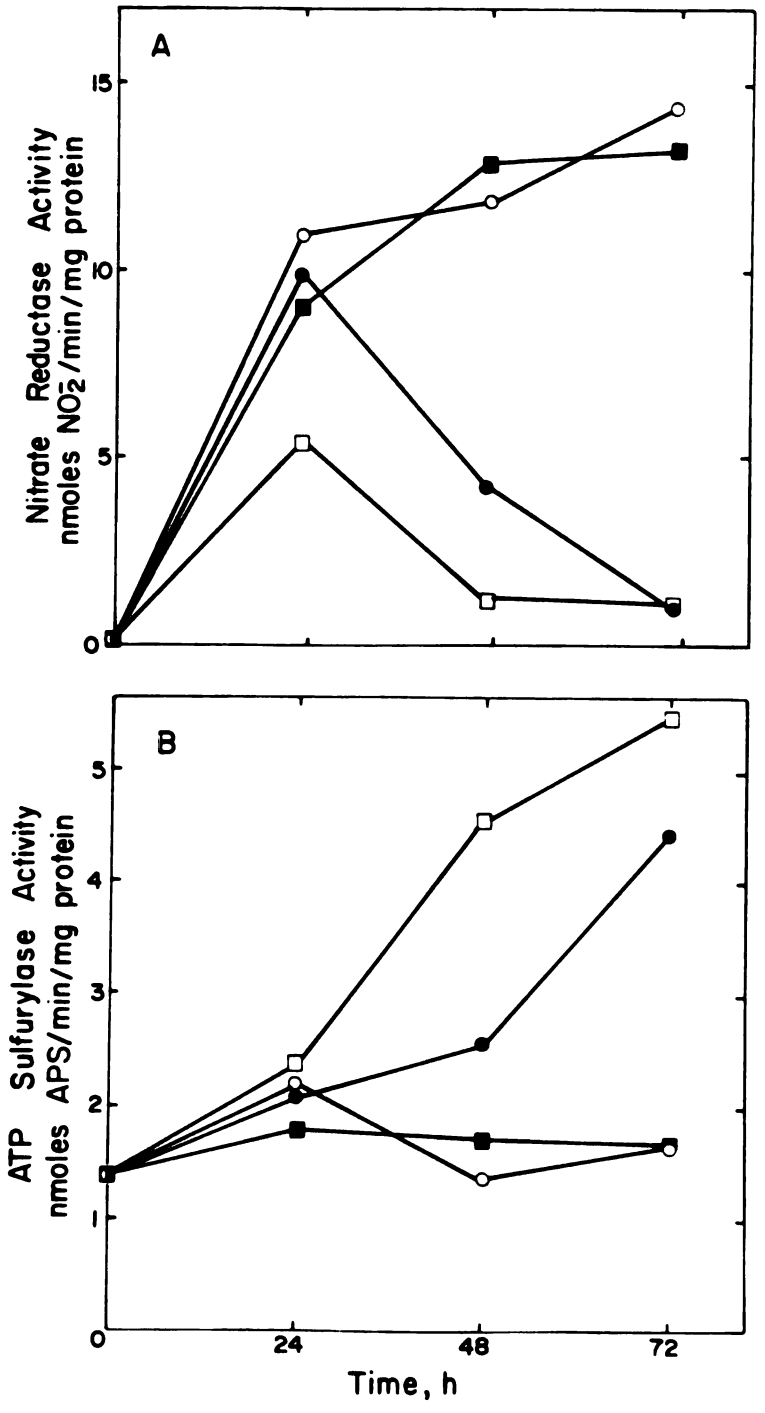
Dependence of Nitrate Reductase Activity Upon Sulfate Nutrition

Half of my initial investigation was to determine the activity of nitrate reductase in relation to the initial sulfate concentration. Tobacco XD cells were inoculated into $N^{-}S^{-}$M-1D media containing 2.5 mM KNO_3 plus 0 μM, 10 μm, 33 μM or 100 μM K_2SO_4. With 2.5 mM nitrate and 100 μM sulfate, the cells grew exponentially for about 10 days and both nutrients became depleted from the medium by the end of the exponential phase of growth (22). Cells in the other media would be expected to experience sulfur deficiency. [It should be noted that the culture to which no sulfate had been added contained trace amounts of sulfur, (about 1-2 μM) due to the trace of sulfur in the reagent grade sucrose used to prepare the medium (22)].

In the cultures with 100 μM and 33 μM sulfate, nitrate reductase activity appeared to be fully induced (Figure 6A). Apparently, sulfur nutrition of the cells was adequate as evidenced by the lack of derepression of ATP sulfurylase activity (Figure 6B). In the cultures with 0 or 10 μM sulfate, the rate of increase and the maximum induced level of nitrate reductase activity was markedly lower than in the cells grown on 33 μM or 100 μM sulfate (Figure 6A). At the 48 hour

Figure 6. Dependence of nitrate reductase and ATP sulfurylase activities upon the initial sulfate concentration of the medium.

Stationary phase tobacco XD cells grown on N^- S^- M-1D + 2.5 mM KNO_3 + 100 μM K_2SO_4 were inoculated into 6 liter flasks containing 3 liters of N^-S^- M-1D + 2.5 mM KNO_3 plus K_2SO_4 at concentrations of none (□), 10 μM (●), 33 μM (■) or 100 μM (○). The quantity of the inoculum was 4 g/l. Daily, portions of each culture were harvested and the activities of nitrate reductase (Frame A) or ATP sulfurylase (Frame B) were assayed by the methods described in MATERIALS AND METHODS except that 0.1 M Tris-HCl + 1 mM cysteine, pH 7.5 buffer instead of the modified Zielke buffer was used for the extraction of the nitrate reductase activity and the dissolving of the 50% saturated ammonium sulfate precipitate.


A
Nitrate Reductase Activity
nmoles NO_2^-/min/mg protein
0
5
10
15
B
ATP Sulfurylase Activity
nmoles APS/min/mg protein
0
1
2
3
4
5
24
48
72
Time, h

time point, the enzyme activity was negligible in the culture with no added sulfate while in the culture with 10 μM sulfate, the nitrate reductase activity was only about 40% of the control. By the 72 hour time point, even the activity in the culture with 10 μM sulfate had declined to negligible levels. The two cultures given 0 and 10 μM sulfate experienced sulfur limitation, based on the derepression of their ATP sulfurylase activity (Figure 6B). Clearly, the magnitude of induction of nitrate reductase activity was strongly dependent upon the adequacy of the sulfate nutrition of the cells.

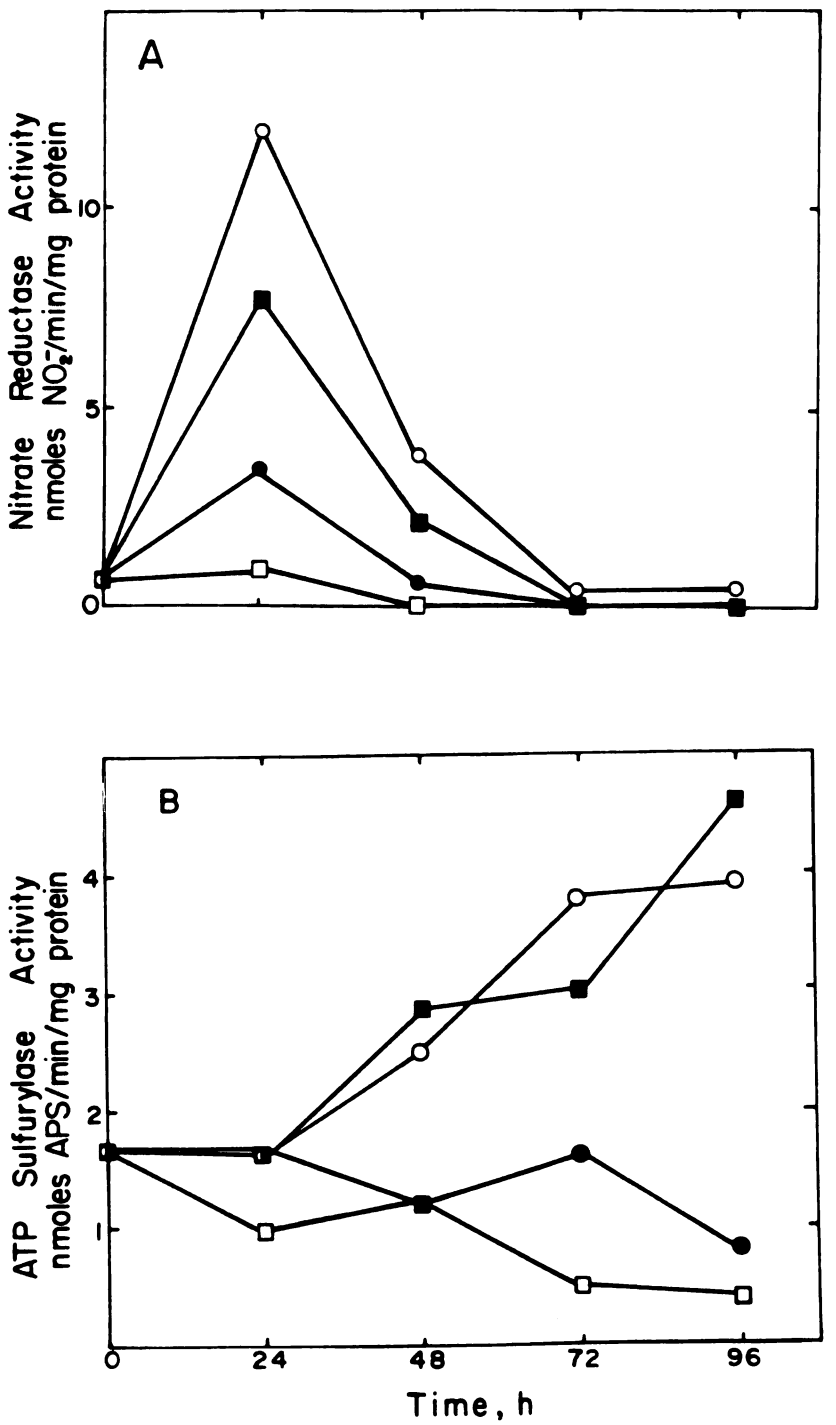
Dependence of ATP Sulfurylase Activity Upon Nitrate Nutrition

The second half of this study was designed to determine how the derepression of ATP sulfurylase activity depended upon nitrate nutrition. The initial concentration of nitrate in the medium was varied while the initial concentration of sulfate was held constant. The initial sulfate concentration was 10 μM, a concentration which would be expected to result in the derepression of ATP sulfurylase activity if nitrogen nutrition was not limiting. The initial concentrations of nitrate were 0 mM, 0.25 mM, 0.8 mM and 2.5 mM. Derepression of ATP sulfurylase activity depended upon the initial concentration of nitrate (Figure 7A); no derepression occurred when the cells were starved for nitrate (0 mM nitrate), an intermediate level of derepression occurred with 0.25 mM nitrate while full derepression occurred at 0.8 mM and 2.5 mM nitrate.

The induction of nitrate reductase activity likewise showed a dependence upon the initial nitrate concentration (Figure 7B). The

Figure 7. Dependence of ATP sulfurylase and nitrate reductase activities upon initial nitrate concentration.

Stationary phase tobacco XD cells grown on S^- M-1D + 100 μM K_2SO_4 were inoculated into 2 liter flasks containing 1 liter of N^-S^- M-1D + 10 μM K_2SO_4 plus KNO_3 at concentrations of none (□), 0.25 mM (●), 0.8 mM (■) or 2.5 mM (○). The inoculum was 6.4 g/l. Daily, portions of each culture were harvested and the activity of ATP sulfurylase (Frame A) was assayed by the method described in the MATERIALS AND METHODS except that the activity was measured after storage of the extract at -20°C for about 1 week. Additionally, portions of each culture were harvested and the activity of nitrate reductase (Frame B) was assayed by the method described in MATERIALS AND METHODS except that 0.1 M Tris-HCl + 1 mM cysteine, pH 7.5 buffer instead of the modified Zielke buffer was used for the extraction of the enzyme activity and for dissolving the 50% saturated ammonium sulfate precipitate.


A
Nitrate Reductase Activity
nmoles NO_2^-/min/mg protein
0
5
10
B
ATP Sulfurylase Activity
nmoles APS/min/mg protein
1
2
3
4
0
24
48
72
96
Time, h

magnitude of induction increased with an increase in the initial concentration of nitrate.

Stabilization of Nitrate Reductase Activity

In the course of the study of the regulation of nitrate reductase and ATP sulfurylase activities, it became desirable to store one of the enzymes for at least 24 hours prior to assaying without loss of activity. If this were possible, two different assays would not have to be performed simultaneously. Although nitrate reductase is notoriously unstable, Zielke and Filner (51) had devised a buffer in which the nitrate reductase from tobacco XD cells could retain activity even after a CsCl density gradient centrifugation lasting 60 hours. Wray and Filner (39) used a similar buffer to preserve the nitrate reductase of barley during sucrose gradient sedimentation. In contrast, very little was known about the stability of ATP sulfurylase. A study of the stability of nitrate reductase in various buffers was therefore undertaken.

Stability of Nitrate Reductase Activity in Various Buffers

When Tris was the principle component of the storage buffer, (Figure 8) rapid inactivation occurred (Buffers 1 and 2). Using 0.1 M potassium phosphate, pH 7.5 there was an apparent 3.5-fold activation of the enzyme activity within 5 days along with considerable subsequent stability of the activity (Buffer 3). Addition of 1 mM cysteine to the phosphate buffer enhanced the activity but stability was not improved (Buffer 4). With the addition of 1 mM EDTA (Buffer 6) activation increased but the stability declined somewhat. The highest degree of activation and stability was obtained with 1 μM FAD added to the

Figure 8. Stability of nitrate reductase in various buffers.

Seven day old cultures of tobacco XD cells (late exponential phase) grown on M-1D medium were harvested and homogenized in 0.1 M Tris-HCl + 1 mM cysteine pH 7.5 buffer. After centrifugation to remove the debris and precipitation by ammonium sulfate as described in MATERIALS AND METHODS the pellet was dissolved in one of the following buffers.

(1) 0.1 M Tris-HCl + 1 mM cysteine, pH 7.5

(2) 0.1 M Tris-HCl + 5 mM cysteine, pH 7.5

(3) 0.1 M potassium phosphate, pH 7.5

(4) 0.1 M potassium phosphate + 1 mM cysteine, pH 7.5

(5) The pellet was not dissolved but stored as the dry pellet in a vial with an atmosphere of nitrogen. Just prior to assay, the pellet was dissolved in 0.1 M Tris-HCl + 1 mM cysteine, pH 7.5.

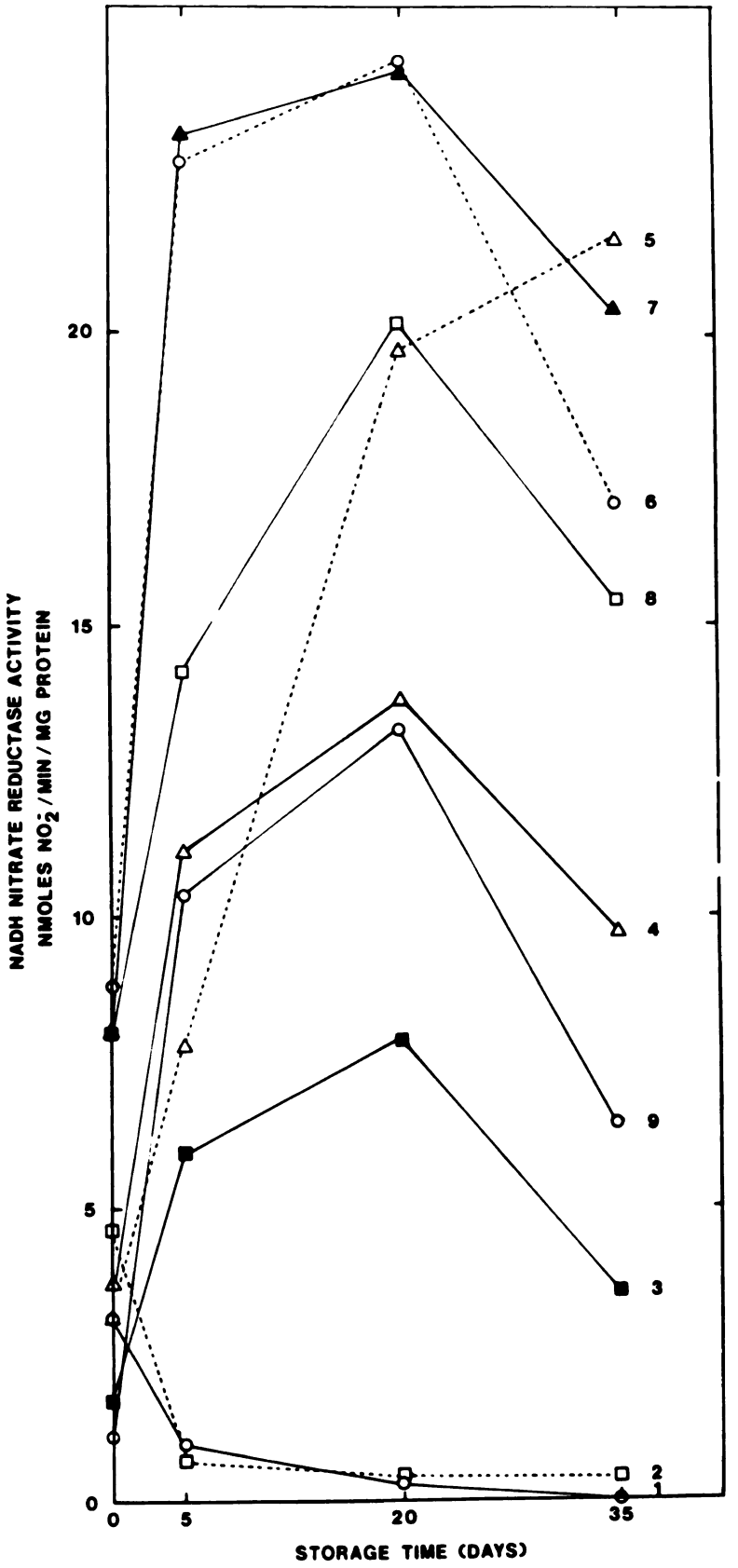
(6) 0.1 M potassium phosphate + 1 mM cysteine + 1 mM EDTA, pH 7.5.

(7) 0.1 M potassium phosphate + 1 mM cysteine + 1 mM EDTA + 1 μM FAD, pH 7.5 (Zielke buffer)

(8) 0.1 M potassium phosphate + 1 mM cysteine + 1 μM FAD, pH 7.5

(9) 0.1 M potassium phosphate + 1 mM EDTA, pH 7.5

Samples were distributed to vials, quick frozen in liquid nitrogen and stored at -20°C in an atmosphere of air. At the indicated times, samples were thawed and assayed for NADH nitrate reductase activity.


NADH NITRATE REDUCTASE ACTIVITY
NMOLES NO_2^- / MIN / MG PROTEIN
0
5
10
15
20
0
5
20
35
STORAGE TIME (DAYS)
1
2
3
4
5
6
7
8
9

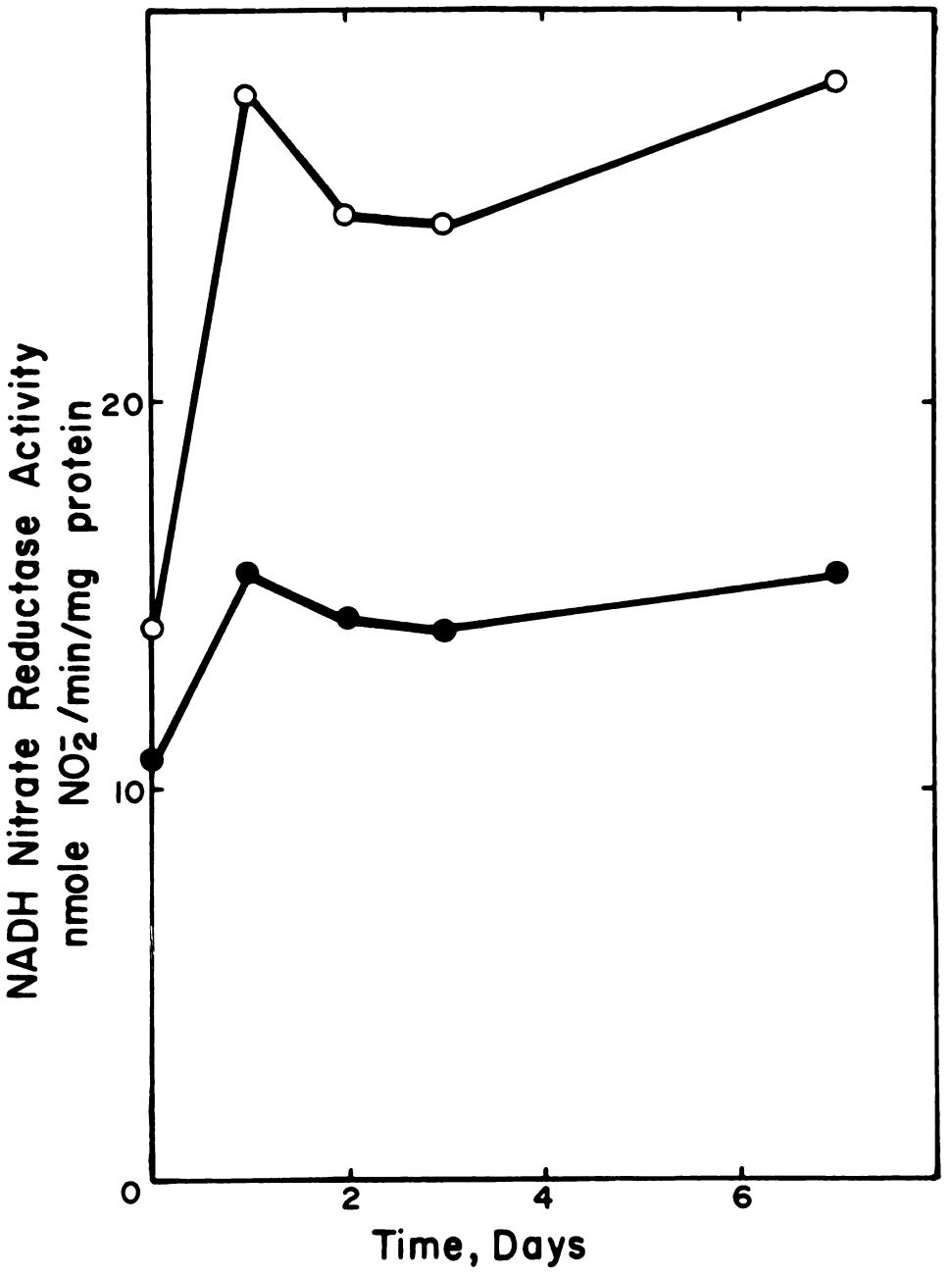
0.1 M phosphate, 1 mM cysteine and 1 mM EDTA, pH 7.5 (Buffer 7). (This buffer, the Zielke buffer, had originally been developed by Zielke and Filner (51) to study the synthesis and turnover of nitrate reductase. They never observed spontaneous activation, however.) Elimination of any of these components reduced the activation or stability of the extract. When the 50% saturated ammonium sulfate precipitate was stored as the protein pellet, and then assayed after resuspension in Tris-cysteine buffer (condition 5), the assayable activity rose steadily during the 35 days of the experiment. This suggested that activation was a result of cold storage and not merely a result of storage in a particular buffer.

Yamaya and Ohira (97) had partially characterized an inhibitor of the nitrate reductase of rice and showed that EDTA decreased the inactivating properties of this factor. Addition of Ca^{+2}, in turn, partially enhanced the inactivating ability of this factor. Speculating that the activation observed in Figure 8 may have been due to the release of an inactivating factor, the possible role of Ca^{+2} upon activation and stability was examined.

EGTA, ethylene glycol bis(ß-aminoethylether)-N,N'-tetraacetic acid, is a potent and relatively specific chelator of Ca^{+2} (154). In order to examine the influence of Ca^{+2}, EGTA was substituted for the EDTA of the Zielke buffer to produce a buffer with the composition 0.1M potassium phosphate, 1 mM EGTA, 1 mM cysteine, and 1 µM FAD, pH 7.5 (the modified Zielke buffer). EGTA did not improve the stability of the stored nitrate reductase activity but did enhance activation compared to EDTA (Figure 9). With EGTA there was greater

Figure 9. Effects of EDTA and EGTA upon spontaneous activation and stability of nitrate reductase.

Seven day old tobacco XD cells (late exponential phase), grown on M-1D medium, were harvested and homogenized in 0.1 M Tris-HCl + 1 mM cysteine, pH 7.5 buffer. After centrifugation to remove debris and precipitation by ammonium sulfate as described in MATERIALS AND METHODS, the pellet was dissolved in 0.1 M potassium phosphate + 1 mM EDTA + 1 mM cysteine + 1 μM FAD, pH 7.5 (●) (Zielke buffer) or 0.1 M potassium phosphate + 1 mM EGTA + 1 mM cysteine + 1 μM FAD, pH 7.5 (○), (modified Zielke buffer). Aliquots were stored at -20°C and assayed after the indicated lengths of storage.


NADH Nitrate Reductase Activity
nmole NO_2^-/min/mg protein
0
10
20
2
4
6
Time, Days

activation than with EDTA. This suggested that Ca^{+2} or possibly some related divalent cation might have a role in the activation of nitrate reductase.

To determine whether Ca^{+2} would inhibit or reverse the activation of nitrate reductase in extracts using the modified Zielke buffer, Ca^{+2} was incubated with these extracts. Table 3 shows that Ca^{+2} had no effect when compared to a control extract with no metal nor when compared to a sample with Mg^{+2}.

Stability of Nitrate Reductase at Various Temperatures

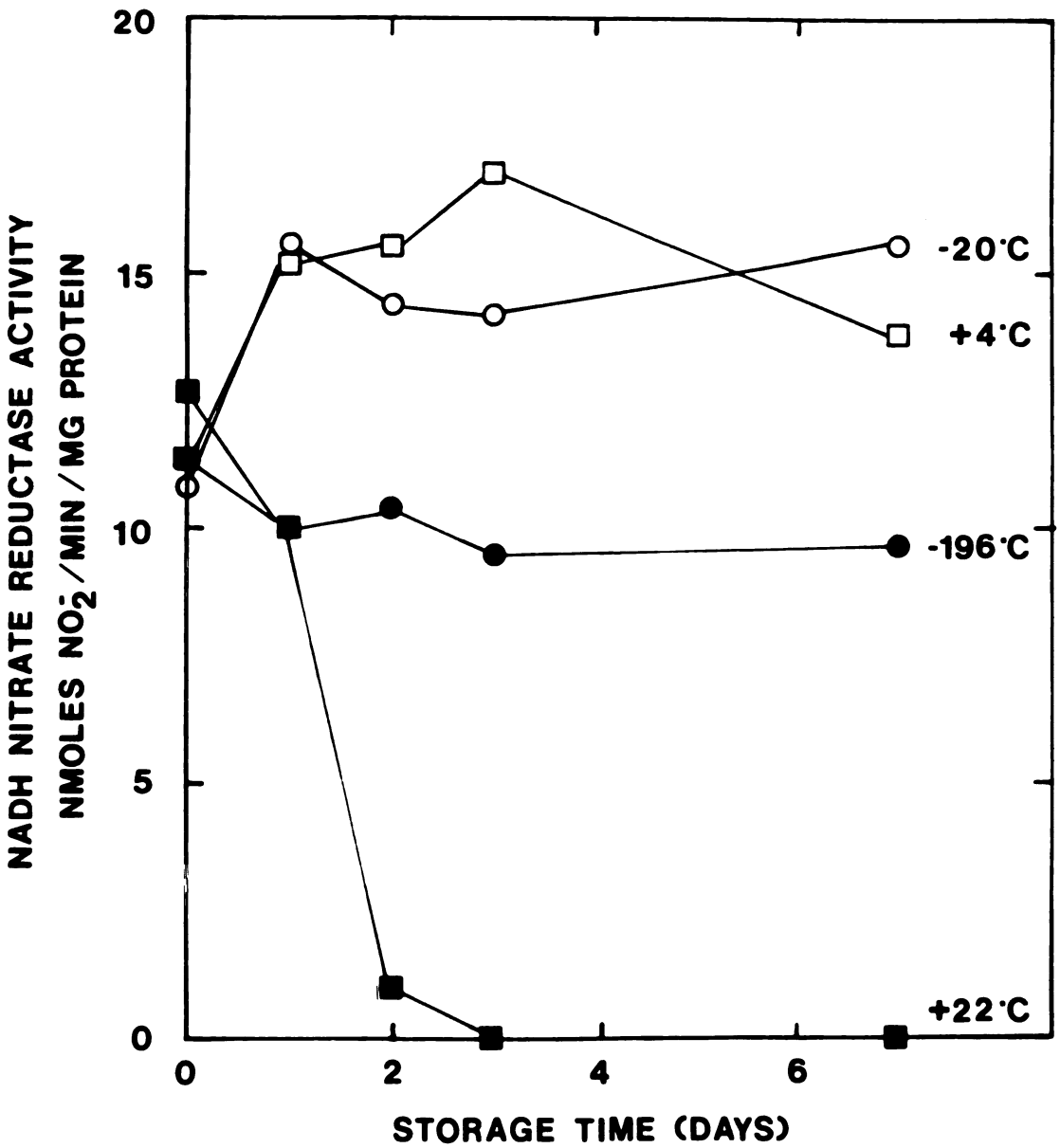
The effect of storage temperature upon nitrate reductase activity was examined. After extraction in the modified Zielke buffer, storage at room temperature resulted in complete loss of activity within 3 days (Figure 10). Activation of the nitrate reductase activity occurred when extracts were stored at -20°C or +4°C. However, stability without activation was observed when the extracts were stored in liquid nitrogen (-196°C). (In situations which would require that the freshly prepared extracts be stored for future analysis, the ammonium sulfate precipitate of the extract should be resuspended in the modified Zielke buffer. This solution should then be stored at the temperature of liquid nitrogen until assayed.)

Partial Purification of Nitrate Reductase

The major barrier to purification of nitrate reductase has been its instability. The establishment of conditions under which the enzyme was stable raised the possibility that the problems of instability during purification might be overcome. With a purified

Figure 10. Stability of nitrate reductase activity at various temperatures.

Seven day old tobacco XD cells (late exponential phase) grown on M-1D medium were harvested and homogenized in 0.1 M Tris-HCl + 1 mM cysteine, pH 7.5. After centrifugation to remove debris and precipitation by ammonium sulfate as described in MATERIALS AND METHODS, the pellet was dissolved in 0.1 M potassium phosphate + 1 mM cysteine + 1 mM EDTA + 1 μM FAD, pH 7.5. Aliquots were stored at +22°C (■), +4°C (□), -20°C (○) and -196°C (●). After the indicated lengths of storage, nitrate reductase activity was assayed.


NADH NITRATE REDUCTASE ACTIVITY
NMOLES NO_2^-/MIN/MG PROTEIN
20
15
10
5
0
0
2
4
6
STORAGE TIME (DAYS)
-20°C
+4°C
-196°C
+22°C

enzyme, the mechanism of activation and inactivation could be investigated in more detail. Therefore a purification program was undertaken. The first step involved partial purification by ammonium sulfate fractionation. The second step involved DEAE Sephadex chromatography followed by affinity chromatography using Blue Sepharose (33). Blue Sepharose functions as an affinity agent for nitrate reductase because the blue chromophore (identical to the chromophore of blue dextran) possesses structural dimensions similar to NAD^+ for which nitrate reductase has a binding site (33,155).

Table 3. Effect of Ca^{+2} and Mg^{+2} upon nitrate reductase activity in modified Zielke buffer.

Treatment	Nitrate Reductase Specific Activity (nmoles NO_2^- /min-mg protein) Incubation time (min)		
	0	30	60
Control	10.2	12.6	14.9
0.5 mM Ca^{+2}		13.7	15.6
1.25 mM Ca^{+2}		12.6	13.6
2.5 mM Ca^{+2}		10.3	14.7
1.25 mM Mg^{+2}		13.1	14.7

Six day old tobacco XD cells (late exponential phase), growing on M-1D medium were harvested and homogenized using the modified Zielke buffer. After precipitation with ammonium sulfate, the pellet was dissolved in the same buffer as described in the MATERIALS AND METHODS. Aliquots were incubated with the indicated concentrations of $CaCl_2$ or $MgCl_2$ for 30 or 60 minutes at 20°C. The activity of nitrate reductase was then assayed.

Ammonium Sulfate Fractionation of Nitrate Reductase

The partial purification of nitrate reductase was accomplished using ammonium sulfate fractionation. The highest specific activity was recovered in the 25% - 40% saturated ammonium sulfate fraction with an 8-fold purification. This 8-fold purification was not due solely to a decrease in the protein content because the protein content of this particular fraction only decreased by 2.8-fold. As previously observed (104), ammonium sulfate fractionation removed an inhibitor of the nitrate reductase activity.

Long Term Stability of Nitrate Reductase

The long term stability of the partially purified enzyme was determined (Figure 11). The crude fraction and the 25% - 40% saturated ammonium sulfate fractions were stored in the modified Zielke buffer at -20°C and assayed periodically. Even after a year of storage, over 20% of the original activity remained in both fractions. The half-time for decay was 185 days for the crude extract and 191 days for the 25% - 40% saturated ammonium sulfate fraction.

Purification by DEAE - Sephadex Chromatography and Stability of Nitrate Reductase

DEAE-Sephadex chromatography resulted in considerable purification of nitrate reductase (Table 4 and Figure 12). Over 37-fold purification was obtained with 10% recovery of the original activity. The resulting specific activity was 356 nmoles of NO_2^- formed/min/mg protein. However, this fraction was very unstable with a half-life of less than two days. In an effort to stabilize the activity in this

Figure 11. Long term stability of nitrate reductase activity in modified Zielke buffer.

Stationary phase tobacco XD cells were inoculated into M-1D medium. After 3 days, the tissue was harvested and homogenized in 0.1 M potassium phosphate + 1 mM cysteine + 1 mM EGTA + 1 μM FAD, pH 7.5 (modified Zielke buffer). The homogenate was centrifuged at 12,000 x g for 20 minutes at 4°C. The supernatant fraction was used as the crude fraction. To a portion of the crude fraction was added saturated ammonium sulfate, pH 7.5 to give a 25% saturated solution. After a 30 minute incubation at 4°C, the sample was centrifuged at 12,000 x g for 20 minutes at 4°C. To the recovered supernatant fraction was added additional saturated ammonium sulfate, pH 7.5 to give 40% saturation. After incubation and centrifugation as before, the pellet was dissolved in modified Zielke buffer. This 25% - 40% saturated ammonium sulfate fraction (□) and the crude fraction (○) were stored at -20°C. After the indicated length of storage, nitrate reductase activity was assayed to determine the percentage of the original activity remaining. The original specific activities were 3.86 and 30.5 nmoles NO_2^- formed/minute/mg protein for the crude and the 25% - 40% saturated ammonium sulfate fraction, respectively.

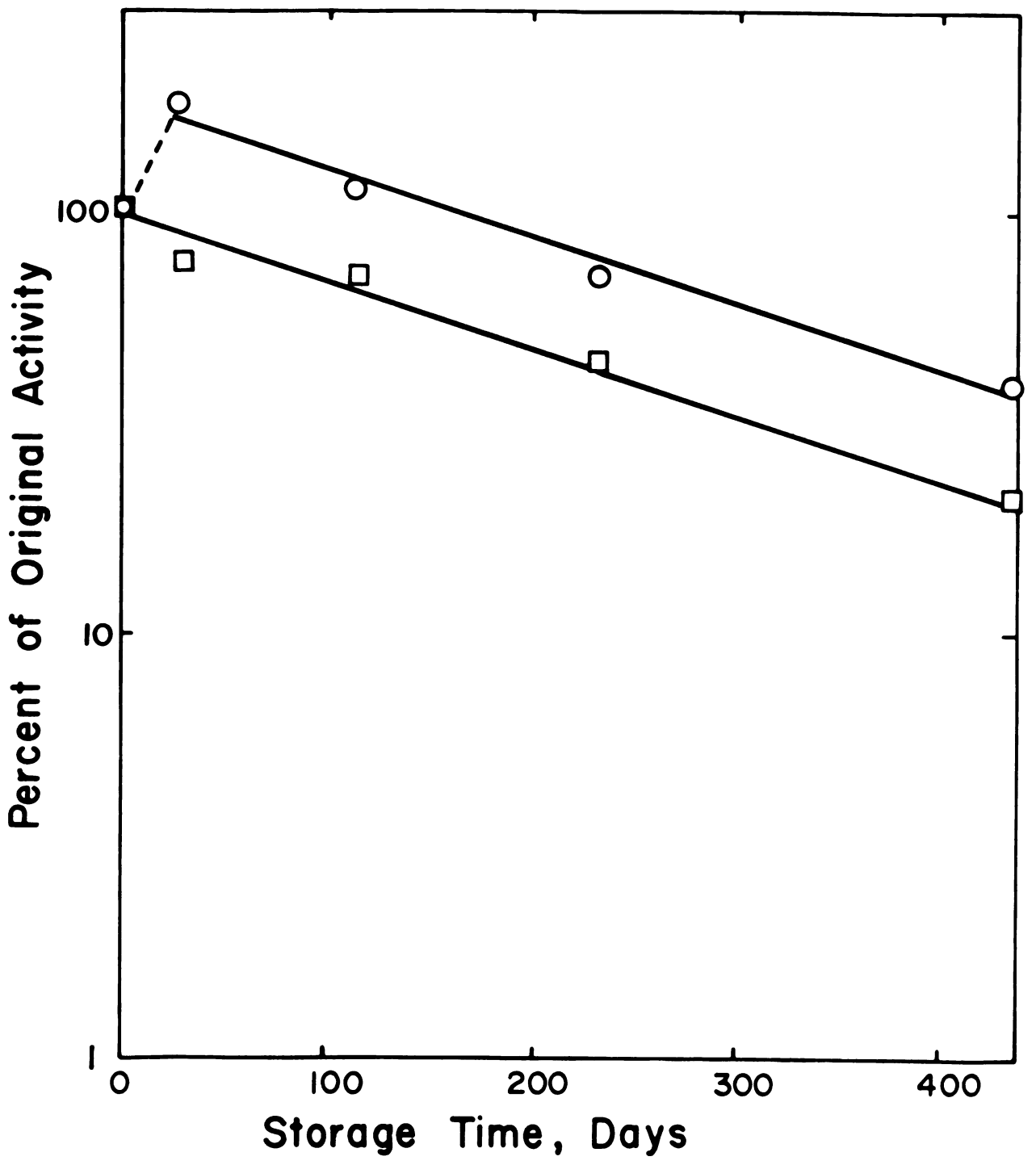

Percent of Original Activity
100
10
1
0
100
200
300
400
Storage Time, Days

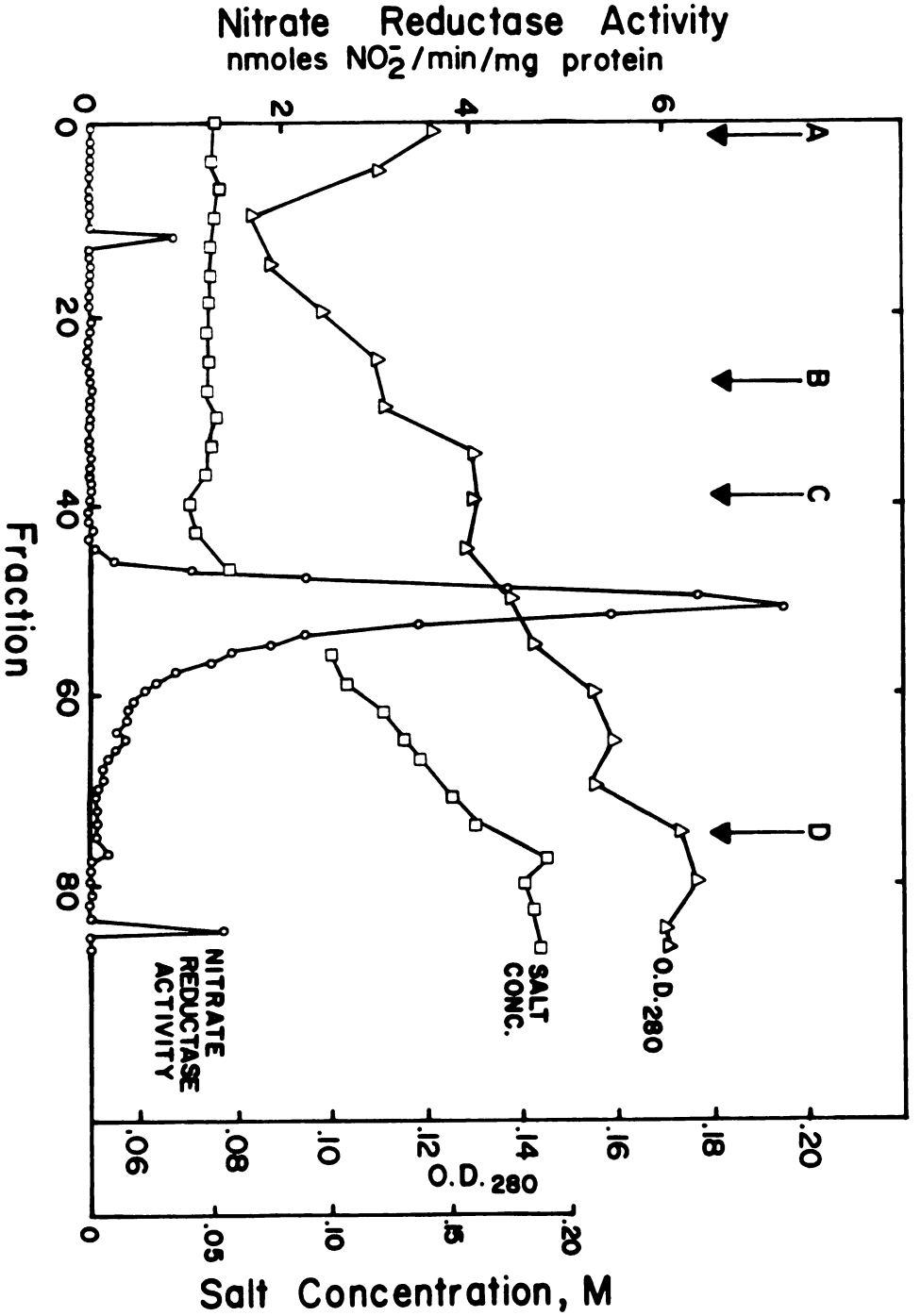
Table 4. Partial purification of nitrate reductase.

Step	Volume (ml)	Total protein (mg)	Specific activity nmoles NO formed minute-mg protein	Fold Purification	Percent Recovery
Crude	118	74.8	9.6	1.0	100
25%-40% saturated ammonium sulfate fraction before dialysis	114	19.2	17.8	1.9	48.1
25%-40% saturated ammonium sulfate fraction after dialysis	108	12.3	22.7	2.4	39.0
DEAE Sephadex fraction	30	0.2	356.	37.2	10.4

See MATERIALS AND METHODS for details of purification.

Figure 12. Elution profile of nitrate reductase activity from DEAE Sephadex column.

Nitrate reductase activity was eluted from the DEAE Sephadex column as described in MATERIALS AND METHODS. Collected fractions were assayed for nitrate reductase activity (○), absorbance at 280 nm (△) and conductivity (□). Conductivity measurements were made using a Thomas Serfass Conductance Bridge with dilutions of the modified Zielke buffer as standards.


Nitrate Reductase Activity
nmoles NO_2^-/min/mg protein
Fraction
Salt Concentration, M
O.D. 280
NITRATE REDUCTASE ACTIVITY
SALT CONC.
O.D. 280
A
B
C
D

fraction, several methods were tested. Glycerol, a stabilizing agent for the nitrate reductase of Chlorella (34), was added to a level of 50% (v/v) to the nitrate reductase obtained from DEAE-Sephadex chromatography. BSA another stabilizer of the nitrate reductase of Chlorella (96) was added to another sample to a level of 0.1%. NADH was also tested to determine whether the reduced form of the enzyme might possess greater stability. Finally, a portion of the eluate from the DEAE Sephadex column was stored at room temperature to test whether a portion of the nitrate reductase protein might be hydrophobic and therefore be destabilized by freezing.

The greatest stability was obtained when 0.1% BSA was added to the sample (Table 5). With BSA present, the half-life of nitrate reductase activity after DEAE Sephadex chromatography was greater than 22 days - far longer than in any of the other buffers. Glycerol stabilized the activity slightly more than did NADH or storage at room temperature. Because of the lack of success in stabilizing the partially purified nitrate reductase with non-protein additives, the purification of the enzyme was discontinued.

Activation and Inactivation of Nitrate Reductase

As stated previously while investigating the stability of nitrate reductase activity, spontaneous increases in activity were observed (Figures 8, 9, 10). This was similar to the reported activation of nitrate reductase from Chlorella (57) and Lemna minor (61) which had been fed ammonium. This prompted a search for conditions under which tobacco nitrate reductase could be inactivated and activated in

Table 5. Stability of partially purified nitrate reductase after DEAE-Sephadex chromatography.

Storage conditions	Estimated half-life
MZ/2 buffer. Stored at -20°C	2 days
MZ/2 buffer. Stored at +23°C	2 days
MZ/2 buffer + 0.1% BSA. Stored at -20°C	Greater than 22 days
0.025 M potassium phosphate + 0.5 mM EGTA + 0.5 mM cysteine + 0.5 uM FAD, pH 7.5 + 50% (v/v) glycerol. Stored at -20°C	2 days
MZ/2 buffer + 100 uM NADH. Stored at -20°C	1.5 days

The combined active fractions obtained from DEAE-Sephadex chromatography (see MATERIALS AND METHODS) were stored as indicated and periodically assayed for nitrate reductase activity. The half-lives of the activity were estimated. The composition of the MZ/2 buffer was 0.05 M potassium phosphate + 1 mM EGTA + 1 mM cysteine + 1 μM FAD, pH 7.5.

vitro. Conditions were also sought during which nitrate reductase might be inactivated and activated in vivo.

Inactivation of Nitrate Reductase by NADH

As stated in the literature review, NADH could inactivate nitrate reductase from several organisms. Incubation of tobacco nitrate reductase with NADH also produced limited inactivation (Figure 13). The inactivation occurred within minutes and the magnitude of inactivation increased with increasing concentrations of NADH. However, the magnitude of inactivation was highly variable.

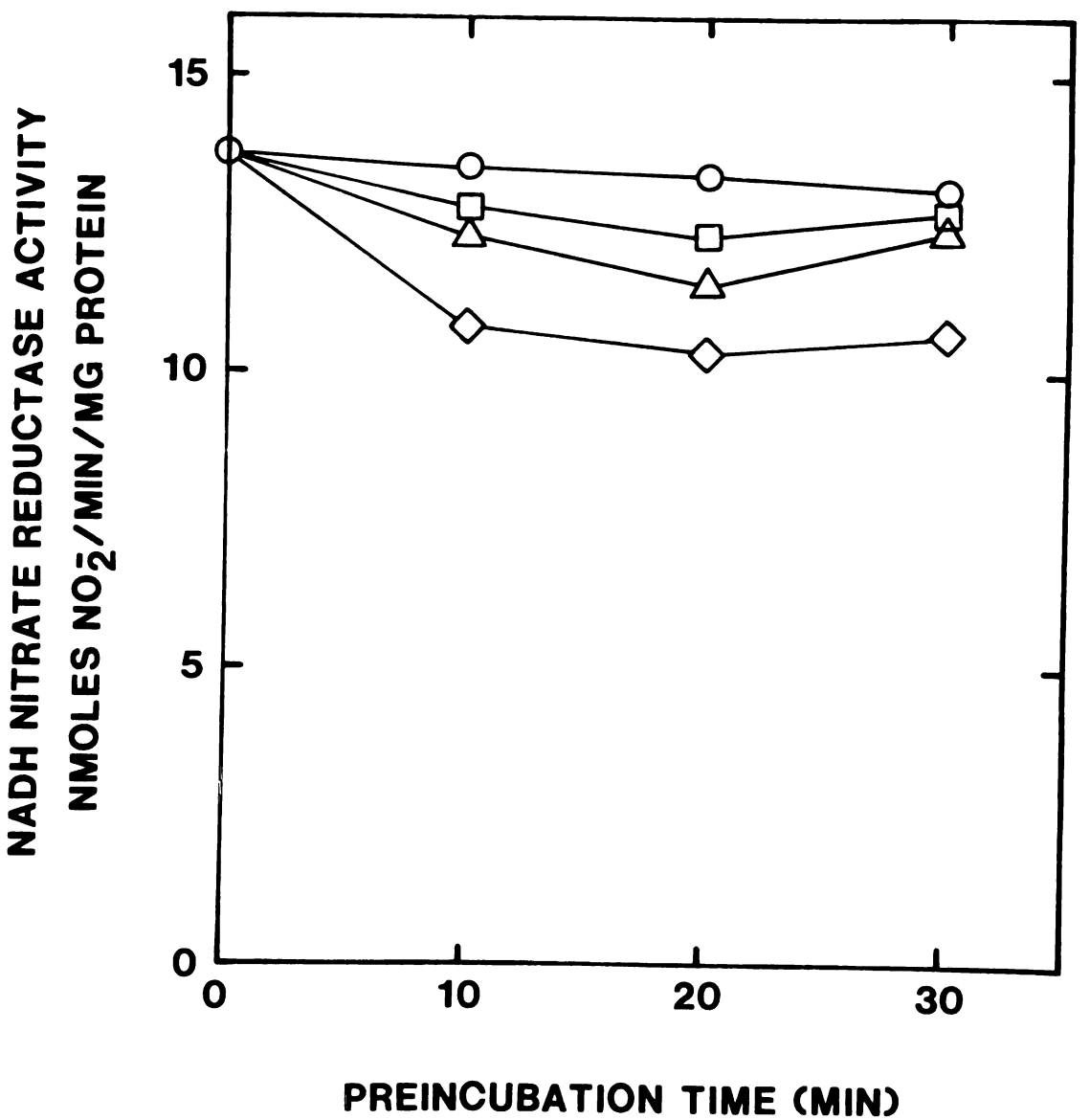
In a personal communication with Dr. Elfrieda Pistorius, she related that she had been unable to observe inactivation of algal nitrate reductase by NADH when the experimental glassware had been acid washed; soap residue plus NADH were the presumptive cause of the observed inactivation. Suspecting that this might be the source of the variability of NADH inactivation of tobacco nitrate reductase, glassware was soaked in 0.5N HCl and then rinsed thoroughly. However, considerable inactivation still occurred. Inactivation by NADH appeared to be a genuine property of the enzyme.

Effects of Various Reagents Upon Nitrate Reductase

Various reagents either inactivate or activate nitrate reductase extracted from a wide range of organisms (please see LITERATURE REVIEW). Table 6 lists the relative activities of tobacco nitrate reductase following a 30 minute incubation with the indicated reagents. Whereas 200 μM NADH alone resulted in over 50% inactivation, addition of micromolar concentrations of cyanide plus NADH resulted in

Figure 13. Inactivation of nitrate reductase by NADH.

Stationary phase tobacco XD cells were inoculated into M-1D medium. After 3 days, the tissue was harvested, homogenized and precipitated with ammonium sulfate as described in MATERIALS AND METHODS. However, the quantity of modified Zielke buffer used to dissolve the precipitated pellet was only one-half of the volume of sample precipitated by ammonium sulfate. Aliquots of this dissolved sample were incubated with 50 μM (□), 100 μM (△), 250 μM (◇) NADH or water (○) at 25°C. After the indicated length of time, samples were assayed for nitrate reductase activity.


NADH NITRATE REDUCTASE ACTIVITY
NMOLES NO_2^-/MIN/MG PROTEIN
15
10
5
0
0
10
20
30
PREINCUBATION TIME (MIN)

nearly complete inactivation. NADPH also produced 30% inactivation; however, it was not discounted that NADH might have been formed by the action of a phosphatase as suggested by Wells and Hageman (44). NAD^+, $NADP^+$, ADP and ATP had little effect upon the assayed activity.

Ferricyanide reversed the inactivation and even activated the nitrate reductase activity after incubation with NADH plus cyanide. Ferricyanide alone caused minor activation at 200 μM but caused inactivation at 50 μM. The other electron acceptors, cytochrome c and DCPIP, also caused inactivation at a concentration of 50 μM.

Ferricyanide Activation of Nitrate Reductase

Because nitrate reductase was activated by 200 μM ferricyanide but inactivated by a 50 μM concentration of the oxidant, it was desirable to determine the concentration of the electron acceptor which would produce the greatest activity. Tobacco extracts were incubated with ferricyanide at concentrations ranging over two orders of magnitude (Figure 14). Between 0 μM and 100 μM ferricyanide, an inhibition of activity was observed. At ferricyanide concentrations greater than 100 μM, activation of the nitrate reductase activity occurred, with the maximum activation being 3.6-fold at 300 μM. Above 500 μM ferricyanide, a gradual decrease in activity was observed, probably due to inactivation of the enzyme or interference with color development during the assay.

There was an abrupt increase in activity above 100 μM ferricyanide. This strongly resembled a titration effect in which substances more readily oxidized than nitrate reductase were reduced by the ferricyanide prior to the activation of nitrate reductase. (For

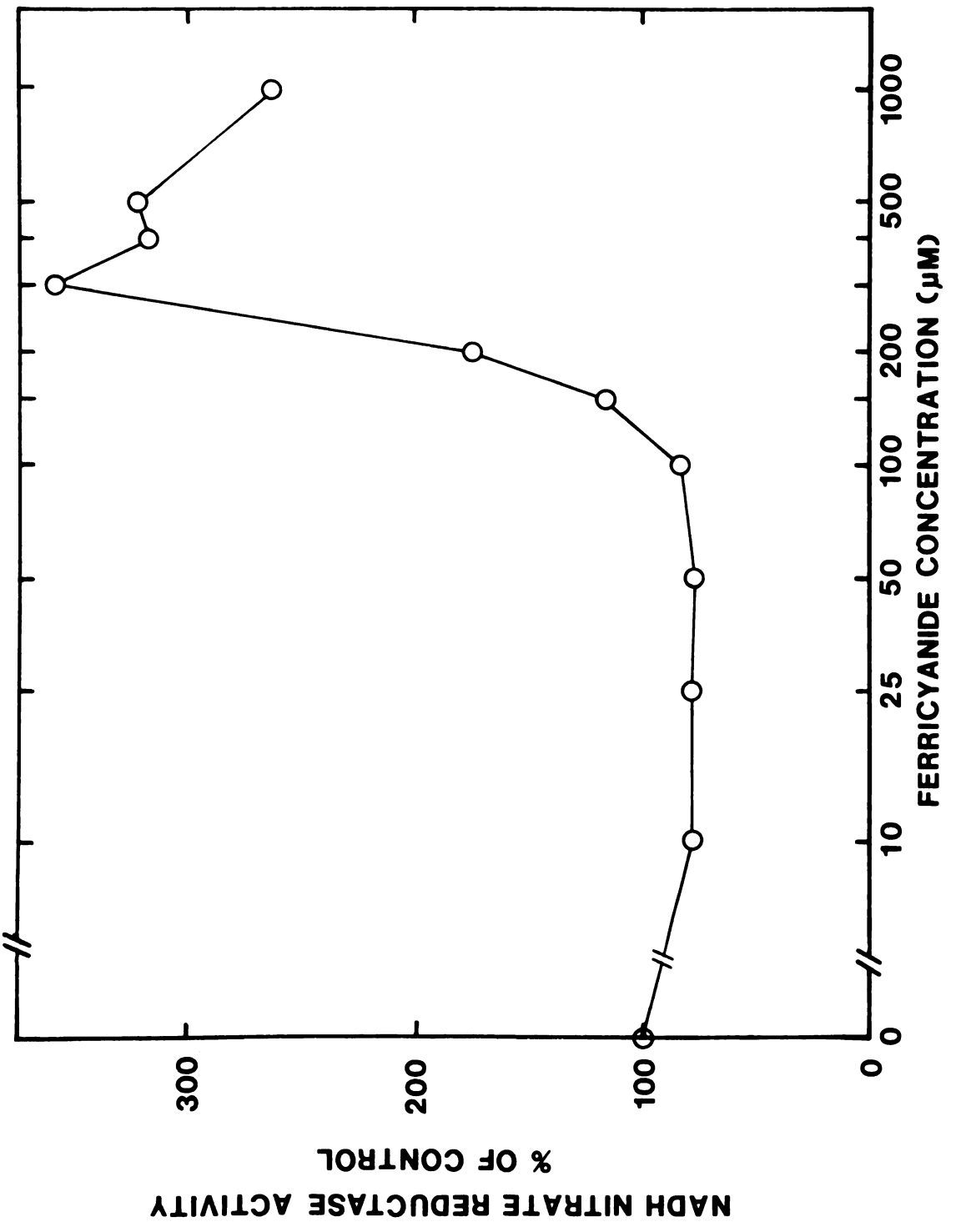
Table 6. Relative effects of various reagents upon nitrate reductase activity.

First 30 min Incubation	Second 30 min Incubation	Relative Activity (%)
H_2O - Control		100
200 μM NADH		46
200 μM $K_3Fe(CN)_6$		134
200 μM NADH	200 μM $K_3Fe(CN)_6$	254
200 μM NADH + 5 μM KCN		4
200 μM NADH + 5 μM KCN	200 μM $K_3Fe(CN)_6$	128
200 μM NADH + 0.5 μM KCN		35
200 μM NADH + 0.5 μM KCN	200 μM $K_3Fe(CN)_6$	252
5.0 μM KCN		86
5.0 μM KCN	200 μM $K_3Fe(CN)_6$	235
50 μM NADH		75
50 μM NADH	50 μM $K_3Fe(CN)_6$	136
50 μM NADH	50 μM Cytochrome c	77
50 μM NADH	50 μM DCPIP	93
50 μM $K_3Fe(CN)_6$		67
50 μM Cytochrome c		68
50 μM DCPIP		67
200 μM NADPH		70
200 μM NAD^+		93
200 μM $NADP^+$		101
200 μM ADP		97
200 μM ATP		96

Stationary phase tobacco XD cells were inoculated into M-1D medium. After 6 days the late exponential phase tissue was harvested and nitrate reductase activity extracted as described in MATERIALS AND METHODS. Aliquots were incubated for 30 minutes with the indicated reagents at 25°C. In some cases, a second reagent was added after the initial 30 minute incubation. This second incubation was also for 30 minutes at 25°C. Following the incubations, nitrate reductase activity was assayed. The specific activity of the control was 9.9 nmoles NO_2^- formed/minute/mg protein (100% relative activity).

Figure 14. Activation of nitrate reductase by ferricyanide.

Stationary phase tobacco XD cells were inoculated into M-1D medium. After 5 days (mid-exponential phase) the tissue was harvested and the nitrate reductase activity was extracted as described in MATERIALS AND METHODS. Aliquots of the extract were incubated with the indicated concentrations of potassium ferricyanide for 30 minutes at 25°C and then passed through a Sephadex G-25 column as described in MATERIALS AND METHODS. The eluted fractions were assayed for nitrate reductase activity. The specific activity of the control sample which was incubated with water was 9.97 nmoles NO_2^- formed/min/mg protein (100% activity).


NADH NITRATE REDUCTASE ACTIVITY
% OF CONTROL
300
200
100
0
0
10
25
50
100
200
500
1000
FERRICYANIDE CONCENTRATION (μM)

example, cysteine in the buffer might reduce ferricyanide.) Using ferricyanide concentrations below the "endpoint" resulted in no observable activation. Additionally, using concentrations of ferricyanide in the region of the sharp increase in activity (100 μM to 300 μM) might give an erroneous indication of the magnitude of activation of nitrate reductase due to variable quantities of oxidizable materials in different extracts. Therefore, a ferricyanide concentration of 500 μM was established as the standard concentration for determination of the ferricyanide-dependent activation of nitrate reductase.

Inactivation of Nitrate Reductase by Cyanide plus NADH and Reversal of Inactivation by Ferricyanide

The cyanide-dependent inactivation of nitrate reductase of tobacco extracts was determined by incubation with cyanide plus 200 μM NADH. Subsequently, 500 μM ferricyanide was added to the incubation mixture to determine the magnitude of reactivation. The magnitude of inactivation increased as the cyanide concentration increased (Figure 15). Almost complete inactivation was observed at 1 μM CN^-. Ferricyanide reversed the inactivation at all concentrations of cyanide. In a parallel test, neither 500 μM nitrate nor 500 μM nitrite could reverse the inactivation by 5 μM cyanide plus 200 μM NADH. This was contrary to the results of Herrera *et al.* (56) using Chlorella and Kaplan (personal communication) using barley nitrate reductase but was in agreement with the results of Maldonado *et al.* (110) using spinach. It is important to note that the cyanide-inactivated tobacco nitrate reductase remained inactive after passage through a Sephadex G-25

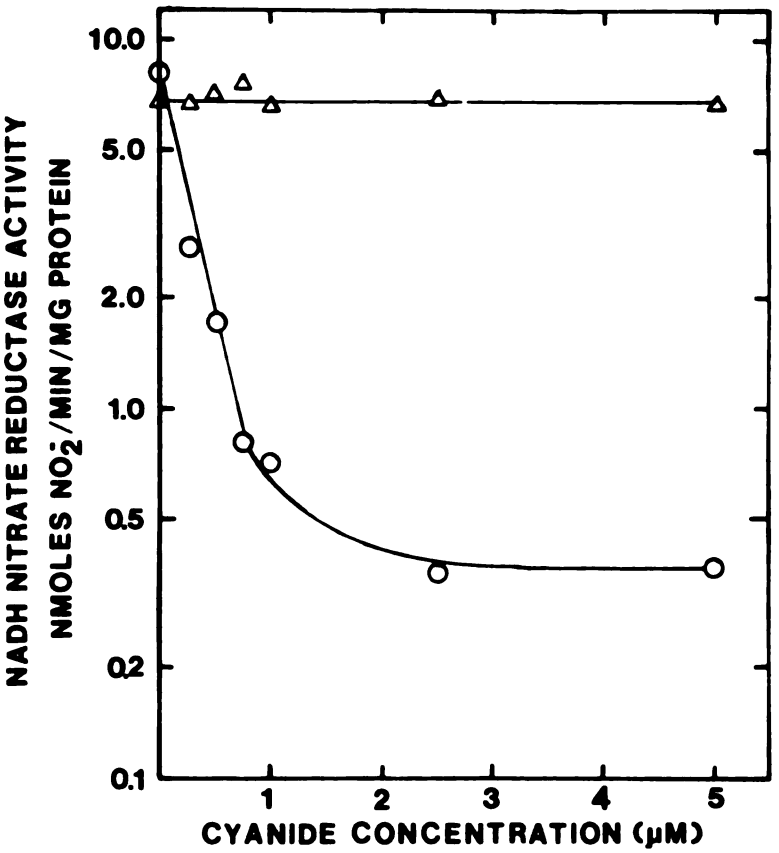


Figure 15. Inactivation on nitrate reductase by cyanide plus NADH and reactivation by ferricyanide.

Stationary phase tobacco XD cells were inoculated into M-1D medium. After 5 days, the tissue was harvested and the nitrate reductase activity was extracted as described in MATERIALS AND METHODS. Aliquots were incubated with the indicated concentrations of cyanide plus 200 μM NADH for 35 minutes at 25°C. A portion of the incubated samples was eluted through a Sephadex G-25 column and the eluate was assayed for nitrate reductase activity (○). To a portion of the sample not eluted through the column was added potassium ferricyanide to yield a final concentration of 500 μM ferricyanide. After incubation for 30 minutes at 25°C, this portion was passed through a Sephadex G-25 column and assayed for nitrate reductase activity (△).

column. This was in contrast with the considerable activation which occurred upon gel filtration of the cyanide-inactivated nitrate reductase of rice (79).

Relationships Among Culture Age, Nitrate Reductase Activity and the Activable Nitrate Reductase Activity

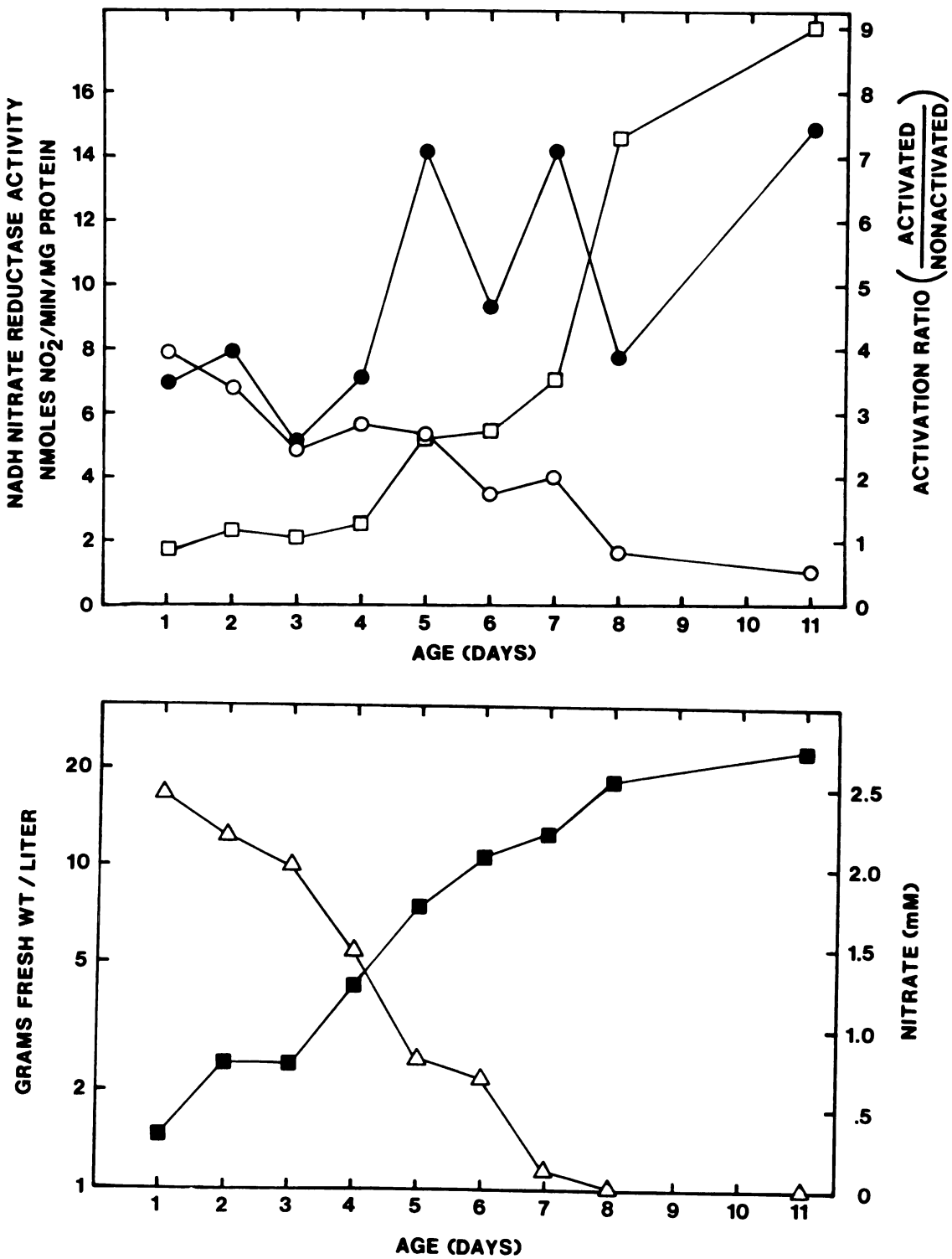
Preliminary experiments indicated that the magnitude of the ferricyanide-dependent activation was a function of the age of the tissue culture. In order to examine this relationship more closely, the overt and activable nitrate reductase activity were measured during the growth cycle of tobacco cultures. Additionally, the magnitude of spontaneous activation upon storage was examined to determine whether spontaneous activation and ferricyanide-dependent activation might be manifestations of the same phenomenon.

Growth of tobacco tissue (closed squares), development of overt nitrate reductase activity (open circles) and depletion of nitrate from the medium (open triangles) proceeded as anticipated (53), (Figure 16). The activation ratio is the ratio of nitrate reductase activity after incubation with 500 μM ferricyanide divided by the activity after incubation with water and gives an indication of the quantity of latent enzyme activity. This ratio remained near unity for the first few days of growth (open squares). By day 5, the activation ratio began to rise and was particularly sharp as the cultures entered stationary phase (observe days 7 and 8 in particular) until by day 11, the ratio was 9.

An indication of the sum of the nitrate reductase activity (both overt and latent) in the cell can be calculated by multiplying the

Figure 16. Variations in overt and latent nitrate reductase activity as a function of the age of tobacco XD tissue cultures.

Stationary phase tobacco XD cells were inoculated into M-1D medium. At the indicated times, cultures were harvested for the determination of fresh weight (■), nitrate remaining in the medium (△), overt nitrate reductase activity (○), activation ratio - the ratio of the nitrate reductase activities with and without activation by 500 μM ferricyanide (□), and the sum of both overt and latent nitrate reductase activity (●).


NADH NITRATE REDUCTASE ACTIVITY
NMOLES NO_2^-/MIN/MG PROTEIN
ACTIVATION RATIO (ACTIVATED / NONACTIVATED)
AGE (DAYS)
GRAMS FRESH WT / LITER
NITRATE (mM)
AGE (DAYS)

overt activity by the activation ratio. This total activity (closed circles) remained relatively constant throughout the growth cycle of the culture! The observed fluctuations were most likely due to the accumulation of errors during the numerous mathematical manipulations of the data. The persistently high value of the total activity in the cells was in sharp contrast to the nearly complete loss of overt activity as the cultures entered stationary phase. In fact, when the total nitrate reductase activity (overt plus latent) in the culture is plotted along with the total soluble protein of the culture which is precipitable by 50% saturated ammonium sulfate (Figure 17), the two lines nearly coincide. This suggests that the nitrate reductase enzyme is present as a constant proportion of the protein of the cell. Consequently, modulation of the activity of the enzyme appears to be accomplished by activation/inactivation processes during the course of the culture cycle.

When the total reducing activity (sum of overt plus latent activities) was integrated over the first eight days of the growth of the culture, the total nitrate reductase activity was sufficient to account for the reduction of 105% of the nitrate initially present in the medium. Integration of only overt activity could account for the reduction of only 38% of the nitrate initially present.

There was a strong correlation ($r = 0.97$) between the magnitude of spontaneous activation upon storage and the magnitude of activation by ferricyanide (Figure 18). This strongly suggested that the activations observed by these two methods were manifestations of the same phenomenon. The correlation declined to $r = 0.95$ when the activation values for days 3 and 4 were included in the comparison.

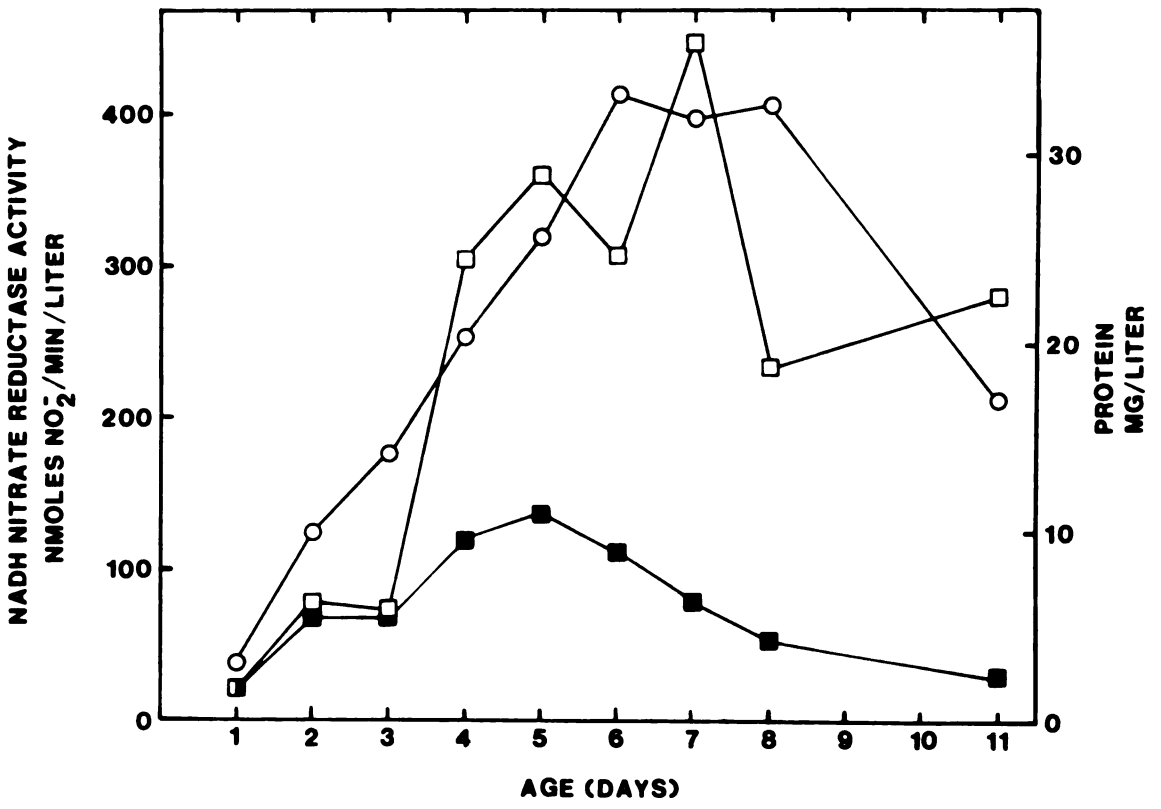


Figure 17. Total nitrate reductase activity and protein precipitable by 50% saturated ammonium sulfate per liter of tobacco XD cultures as a function of culture age.

The data illustrated is from the same experiment as in Figure 16. The total latent plus overt nitrate reductase activity per liter of culture (□) and the total protein precipitable by 50% saturated ammonium sulfate per liter of culture (○) were calculated and plotted along with the total overt nitrate reductase activity per liter of culture (■).

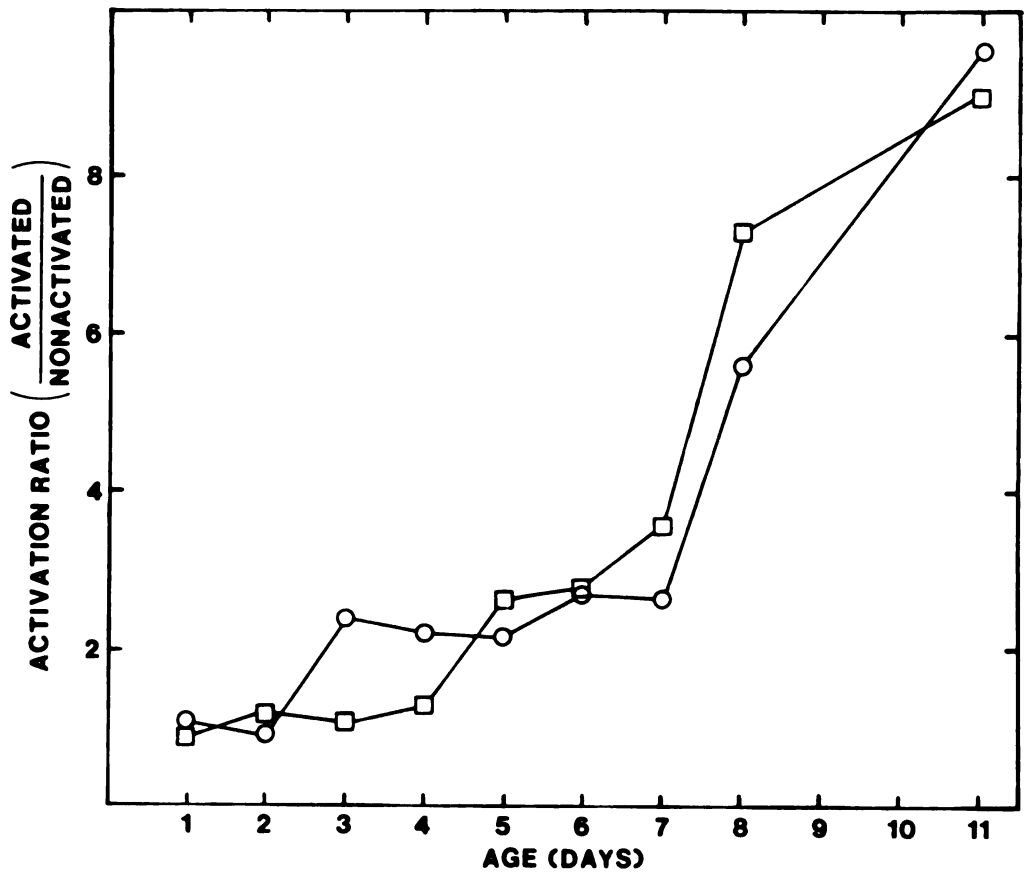


Figure 18. Comparison of spontaneous activation and ferricyanide dependent activation of nitrate reductase as a function of culture age.

The data illustrated is from the same experiment as in Figure 16. The ratio of nitrate reductase activity after activation by ferricyanide divided by the activity without activation (□) and the ratio of nitrate reductase activity after storage for 11 to 22 days at -20°C divided by the fresh nitrate reductase activity (○) are illustrated.

This deduced correlation was due to an additional inhibitor of nitrate reductase activity during these two days. This inhibitor was deduced from the increase in activity observed following passage of the extracts through a Sephadex G-25 column. The inhibitor was not further characterized.

Induction of Nitrate Reductase

On day 11 of the growth cycle, the total nitrate reductase activity (overt plus latent) was similar to the total activity of the early exponential phase cultures (Figure 16). This raised the possibility that the "induction" of overt nitrate reductase activity upon subculturing actually represented the conversion of latent nitrate reductase to the overt form. To examine this possibility, both overt and latent nitrate reductase activities were examined in freshly subcultured tobacco cells.

Just prior to subculturing, time = 0 hr., the overt activity was low whereas the activation ratio was about 5 (Figure 19). Upon transfer to fresh medium, the overt activity rose about 9-fold while the activation ratio declined almost 60%. Judging from the 3- to 5-fold rise in total activity, there appears to have been considerable formation of new nitrate reductase activity as opposed to conversion of latent to overt activity.

An important point to observe is how the quantity of latent nitrate reductase activity rose during the induction period. This quantity was calculated from the difference between the overt activity and the total activity. The rise in the quantity of latent enzyme activity suggested that there was formation of both inactive along

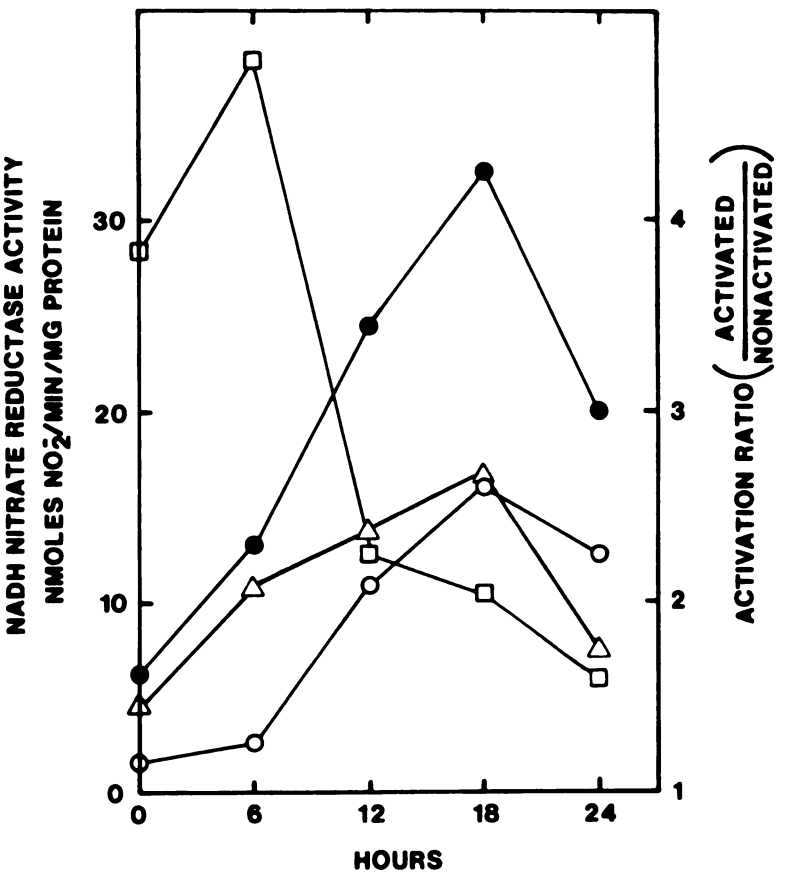


Figure 19. Changes in overt, latent and total activity of nitrate reductase in tobacco XD cells upon subculturing.

Stationary phase tobacco XD cells were subcultured into M-1D medium. At the times indicated, the overt nitrate reductase activity (○) and the activation ratio - the ratio of the nitrate reductase activities with and without activation by 500 μM ferricyanide (□) were determined. The total nitrate reductase activity (●) representing the sum of the overt and latent activities was the multiplicative product of the overt nitrate reductase activity and the activation ratio. The latent nitrate reductase activity (△) is the difference between the total and overt activities.

with active nitrate reductase enzyme during the induction period. Alternatively, an inactivator of the nitrate reductase was inactivating some of the newly formed activity. The quantity of the putative inactive or inhibited enzyme appeared to decline later in the induction period.

Influence of Nitrate Upon the Formation of Latent Nitrate Reductase Activity

As the cultures proceeded into late log phase, the quantity of latent nitrate reductase activity rose. The parameter which promoted the formation of an inactive nitrate reductase was of primary interest. Filner (156) had observed that as tobacco cultures proceeded into late log phase, nitrate, potassium and phosphate were rapidly depleted from the medium. Based on these observations, depletion of nitrate and phosphate were tested as possible promoters of the inactivation of nitrate reductase.

When stationary phase cells were subcultured into medium which lacked nitrate (but contained phosphate), there was no activation of the nitrate reductase (Figure 20). There was, in fact, a decline in the total nitrate reductase activity.

When phosphate was absent from the medium (but nitrate was present) there was a decrease in the activation ratio which was similar to that observed when subculturing into M-1D control. There was also a corresponding decline in the quantity of the inactive species such that by day 2, the overt activity essentially constituted the total activity.

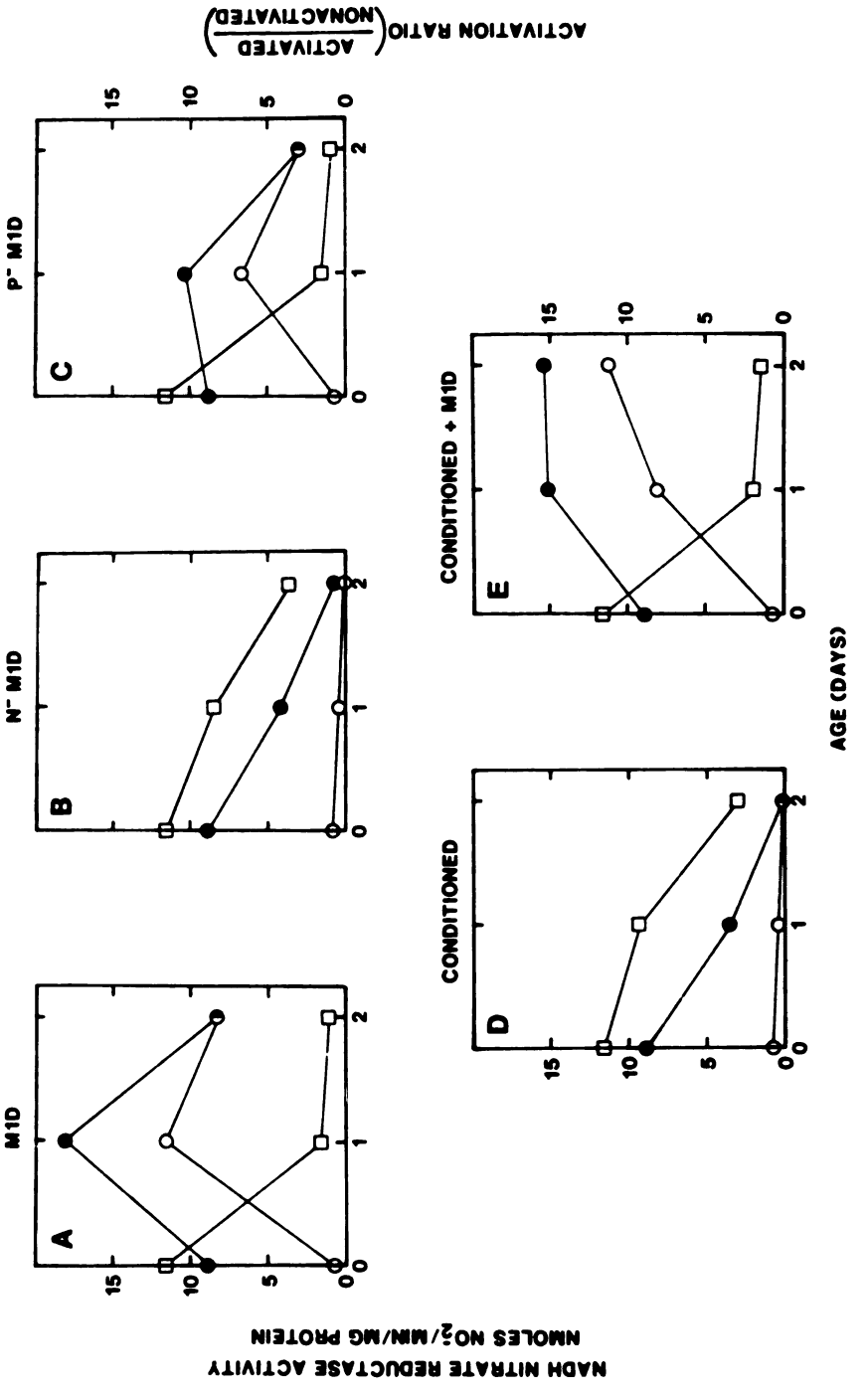
The results suggested that depletion of nitrate was the factor which promoted the formation of an inactive nitrate reductase. Replenishment of the nitrate (Figures 19, 20) resulted in a decrease in the quantity of latent activity.

Response of Nitrate Reductase Activity Upon Inoculation into Conditioned Medium

The presence of an inactivator was suggested by the effects of conditioned medium upon the induction of nitrate reductase. The conditioned media was M-1D media in which tobacco XD cells had been grown to stationary phase. There were differences in the kinetics of induction when tobacco cells were inoculated into fresh M-1D (Figure 20A) or into conditioned M-1D to which fresh M-1D nutrient stocks had been added (Figure 20E). In fresh M-1D, induction occurred rapidly with complete loss of the inactive form of the enzyme by day 2. However, in conditioned medium plus M-1D stocks, the induction was slower and latent nitrate reductase activity still accounted for about one-quarter of the total activity by day 2. These results suggested that the conditioned medium contained a factor which either stabilized or promoted the formation of inactive nitrate reductase. Upon subculturing into fresh M-1D medium, the quantity of the factor would be decreased so that an increase in overt nitrate reductase activity could occur more rapidly without retention of a considerable quantity of the inactive enzyme.

Figure 20. Effect upon nitrate reductase activity of subculturing tobacco XD cells into medium lacking nitrate or phosphate or into conditioned medium.

Stationary phase tobacco XD cells were inoculated into M-1D (A), nitrate-less M-1D (B) or phosphate-less M-1D (C) medium. Phosphateless M-1D medium was prepared by substituting chloride salts for phosphate salts during th formulation of the medium. Stationary phase tobacco XD cells were inoculated into M-1D medium. After two weeks, the conditioned medium was collected by sterilely removing the tobacco cells by filtration through two layers of Whatman 4 filter paper. For the portion of the experiment illustrated in frame (E), sterile M-1D stock solutions were added to the conditioned medium. Stationary phase cells were then inoculated into conditioned medium (D) or conditioned medium plus M-1D nutrients (E). At the indicated times, the overt nitrate reductase activity (○), the activation ratio - the ratio of the nitrate reductase activities with and without activation by 500 μM ferricyanide (□), and the total nitrate reductase activity - the multiplicative product of the overt activity and the activation ratio (●) were determined.


NADH NITRATE REDUCTASE ACTIVITY
NMOLES NO2/MIN/MG PROTEIN
ACTIVATION RATIO (ACTIVATED / NONACTIVATED)
A
M1D
B
N⁻ M1D
C
P⁻ M1D
D
CONDITIONED
E
CONDITIONED + M1D
AGE (DAYS)

Effects of Mixing Extracts from Old and Young Tissue Upon Nitrate Reductase Activity

Extracts containing entirely overt nitrate reductase (from 2 day old cultures) and extracts containing overt plus latent nitrate reductase (from 7 day old cultures) were mixed to test for the presence of a readily extractable inactivator or activator. Using modified Zielke buffer, mixed extracts from 2 and 7 day old cultures yielded activities which were simply the sums of the activities in the unmixed extracts (Table 7). Mixing of boiled and fresh extracts likewise gave results which simply reflected additivity. However, when cells of 2 day old and 7 day old cultures were homogenized together, the observed activity was greater than the sum of the components. This might reflect a stabilization of the activity in the 2-day old culture by the increased protein concentration contributed by the 7 day old culture as explained in the following section.

There does not appear to be an effector of nitrate reductase which can be detected by mixing experiments; no inactivator nor activator was observed. This might indicate that the effector was not solubilized, it was volatilized (HCN has a pK = 9.3 for example) or the homogenates contained an antagonist against inactivation (nitrate for example). Several parameters could thus have influenced the action of an effector such that its action would not have been observed.

Effects of Protein Upon Nitrate Reductase Activity

When either 1% BSA or a saturating quantity of casein (the quantity solubilized when the buffer was made 1% (w/v) casein) was included in the modified Zielke buffer, there was a pronounced effect upon the observed activity of nitrate reductase (Table 7). The

Table 7. Effects upon nitrate reductase activity of mixing extracts from young and old tissue and of addition of protein to the extraction buffer.

Sample	Nitrate reductase activity nmoles NO_2^- formed / min-mg protein		Percent of predicted value
	Observed	Predicted	
Crude 2 day old	1.96		
Crude 7 day old	1.02		
Crude 2 and 7 day old mixed	1.39	1.49	93
Crude 2 day old and boiled 7 day old mixed	0.98	0.98	100
Crude 7 day old and boiled 2 day old mixed	0.50	0.51	98
Crude 2 and 7 day old cells homogenized together	2.33	1.49	156
Am. Sulf. 2 day old	5.21		
Am. Sulf. 7 day old	4.99		
Am. Sulf. 2 and 7 day old mixed	5.30	5.10	104
Crude 2 day old + BSA	6.53	1.96	333
Crude 7 day old + BSA	1.02	1.02	100
Am. Sulf. 2 day old + BSA	11.3	5.21	217
Am. Sulf. 7 day old + BSA	4.39	4.99	88
Crude 2 day old + casein	7.09	1.96	362
Crude 7 day old + casein	1.33	1.02	130
Am. Sulf. 2 day old + casein	6.92	5.21	133
Am. Sulf. 7 day old + casein	5.94	4.99	119

Table 7. Effects upon nitrate reductase activity of mixing extracts from young and old tissue and of addition of protein to the extraction buffer.

Two day old tobacco XD cultures growing on M-1D were used as the source of extracts with fully active nitrate reductase (activation ratio = 1.06). Seven day old cultures were used as the source of extracts with predominantly inactive nitrate reductase (activation ratio = 3.68). Nitrate reductase activity was extracted using the modified Zielke buffer. Either the crude extract or the ammonium sulfate resuspension (Am. Sulf.) were used in the assays. In some cases, 1% BSA (listed as "+ BSA") or the concentration of casein which would saturate the modified Zielke buffer (listed as "+ casein") was included in the extraction buffer. All samples were incubated for 30 minutes at 25°C. When extracts were mixed, they were mixed in a 1:1 ratio. Extracts which had been boiled for 10 minutes were used in some cases. In one instance equal weights of cells from 2 day old and 7 day old cultures were mixed and homogenized together. This extract was then assayed for nitrate reductase activity.

activity of the enzyme from the 2 day-old culture was stimulated over 3-fold in the crude extract. The activity in the ammonium sulfate resuspension was likewise greater than expected. In contrast, the activity of the 7-day old culture did not exhibit a similar magnitude of activation.

The stimulation of activity in the 2-day old culture by BSA or casein may represent protection against protease activity. Alternatively, it may represent a stabilization of the nitrate reductase in the presence of an elevated concentration of protein. (See the section of this thesis concerning the stabilization of the partially purified nitrate reductase.) Jordan and Fletcher (107) had observed a similar increase in activity when extracts of Paul's Scarlet Rose cultures were prepared in the presence of casein. In contrast to the observation using tobacco cultures, they observed a much greater stimulation in old tissue than in younger tissue. The activation was attributed to the inhibition of an effector by the casein.

Kinetic Evaluation of the Binding of Cyanide to Nitrate Reductase

Three lines of evidence led to the suspicion that tobacco nitrate reductase might be regulated by cyanide.

1) Nitrate reductase of tobacco can be extracted from cultures in the inactive form, and be activated by ferricyanide.
2) Nitrate reductase of tobacco can be inactivated by cyanide *in vitro*.
3) Cyanide is known to inactivate algal nitrate reductase *in vivo* and in turn be reactivated *in vitro* by ferricyanide.

In algae, the *in vivo* regulation by cyanide was confirmed by purifying nitrate reductase which had been inactivated *in vivo*, releasing the bound effector and chemically identifying the released substance as cyanide.

However, this approach was not feasible for tobacco. The nitrate reductase of tobacco could not be adequately purified and stabilized. Furthermore, the quantity of nitrate reductase in algae is considerably greater than the quantity in tobacco. In tobacco XD cells, nitrate reductase accounts for only about 0.01% of the protein of the cell or 1.5 picomoles per gram fresh weight (P. Filner - personal communication). If it is assumed that only one cyanide molecule binds to each nitrate reductase molecule and that all of the nitrate reductase is present in the inactive state, then the nitrate reductase from 70g of tissue would need to be purified to yield the minimum quantity of cyanide (100 picomoles) which could be detectable by the colorimetric assay of Guilbault and Kramer (157). Therefore, another approach was required to determine whether cyanide was an *in vivo* effector of tobacco nitrate reductase.

The approach which was developed was based upon the affinity of nitrate reductase for cyanide. (See APPENDIX for a detailed derivation of the analysis which was used.) Extracts containing nitrate reductase were incubated with several concentrations of cyanide. After incubation with cyanide, the nitrate reductase activity which remained was determined. The activation ratio of the non-incubated extract was also determined in order to obtain a value for the total quantity of nitrate reductase activity, both overt and latent, which was present in the extract. The ratio of the total nitrate reductase activity

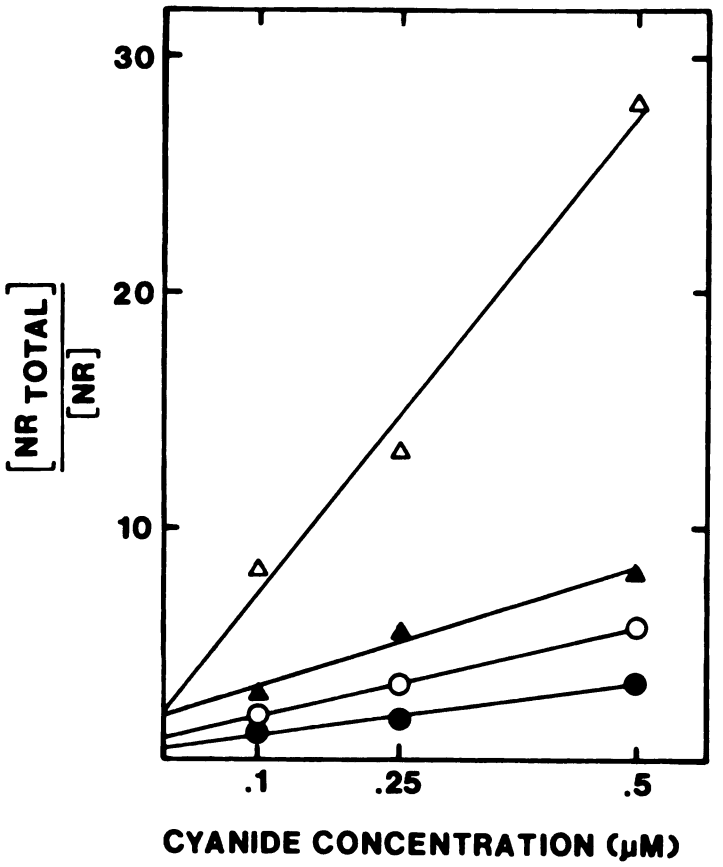
divided by the activity remaining after incubation with cyanide $\left[\frac{NR_{TOTAL}}{[NR]}\right]$ was calculated and according to the discussion found in the APPENDIX, the slope of the ratio as a function of cyanide concentration would be indicative of whether cyanide was the *in vivo* effector of nitrate reductase. If cyanide was the *in vivo* effector, the slope of ratio would be identical for different aged extracts whether or not an effector was bound to the enzyme. If cyanide was not the *in vivo* effector, the slopes would be dissimilar.

Extracts of 2 day old cultures were used as the source of nitrate reductase with no effector bound to it while extracts of 7 day old cultures were used as the source of partially latent nitrate reductase with a significant quantity of a bound effector. The results (Figure 21) indicated that the slope of the ratio $\left[\frac{NR_{TOTAL}}{[NR]}\right]$ vs. $[CN^-]$ using extracts of 7 day old cultures was over 5-fold greater than the slope using the extracts from 2 day old cultures. The analysis was repeated after the extracts had been stored for 33 days, a length of time presumably sufficient for an *in vivo* effector to have dissociated from the nitrate reductase. In this case, it was observed that the slope using extracts from the 7 day old cultures had decreased 75% to a value only 2.2-fold greater than the slope using the extracts from the 2 day old culture. The slope obtained using the extract from the 2 day old cultures had also decreased, but this decrease of 40% was not nearly as great as for the extract from the 7 day old culture.

From this evidence, it would appear that cyanide was not the *in vivo* effector. The slopes of the function $\left[\frac{NR_{TOTAL}}{[NR]}\right]$ vs. $[CN^-]$ were

Figure 21. Kinetic evaluation of the NADH plus cyanide induced inactivation of nitrate reductase.

Extract from 2 day old tobacco XD cultures (○ , ●) growing on M-1D medium was used as the source of nitrate reductase which was fully active (activation ratio = 1.0). Extract from 7 day old cultures (△ , ▲) was the source of nitrate reductase predominantly in the latent form (activation ratio = 3.0). Aliquots of each extract were incubated for 30 minutes at 25°C with 200 μM NADH plus 0.1 μM, 0.25 μM or 0.50 μM cyanide and the activity remaining [NR] was determined. The activation ratio of a portion of the extract which had not been incubated with cyanide was determined by comparing the nitrate reductase activity after ferricyanide-dependent activation with the activity without activation. The total nitrate reductase activity [NR_{TOTAL}] was calculated as the multiplicative product of the activation ratio and the overt activity (the activity without addition of cyanide or ferricyanide). The ratio of $\frac{[NR_{TOTAL}]}{[NR]}$ was calculated and plotted vs. the cyanide concentration used during the incubation. The analysis was conducted on fresh extracts (○ , △) or after storage for 33 days at -20°C (● , ▲).


30
20
10
[NR TOTAL] / [NR]
.1
.25
.5
CYANIDE CONCENTRATION (μM)

too dissimilar. The observation that the slopes became more similar after storage supported the proposal that an effector dissociated from the nitrate reductase during storage and further supported the deduction that cyanide was not the effector.

An alternative possibility is that cyanide is indeed the in vivo effector of nitrate reductase but that the observed differences in the slopes were due to different species of the enzyme. As the cultures aged, the different species of nitrate reductase would be present in different proportions. The changes in slopes which were observed upon storage could represent different rates of decay or modifications of the two species and would therefore be manifestations of changes in the average properties of each extract. This alternative possibility cannot be proved or disproved without more detailed characterization of the kinetic properties of the nitrate reductase from cultures of various ages.

Additional Factors Which Would Promote the Formation of Inactive Nitrate Reductase

It was previously demonstrated that inadequate nitrate nutrition would result in the formation of an inactive nitrate reductase whereas phosphate deprivation did not. It could be postulated that other perturbations of the normal metabolism of the cells might also result in the formation of an inactive species. The conditions tested were those which had previously been demonstrated to be associated with decreased activities of nitrate reductase.

Role of Threonine in the Regulation of Nitrate Reductase Activity

Filner (53) had demonstrated that in the presence of 100 μM threonine, induction of overt nitrate reductase activity was decreased. In light of the results just reported, it was uncertain whether the decreased overt activity was the result of a decrease in the quantity of overt plus latent nitrate reductase or if it was due to a shift in the proportion of latent vs. overt activity. Tobacco cells were inoculated into either M-1D medium or into M-1D plus 100 μM threonine medium. After 24 hours, the overt nitrate reductase activity and the activation ratio were determined (Table 8).

In the presence of 100 μM threonine, the quantity of overt activity was only 4.6% of the control and the total activity was only 13.7% of the control. The response to threonine was a decrease in the sum of latent plus overt activity rather than a shift from overt to latent activity.

Influence of Ammonium Upon Nitrate Reductase Activity

In green algae, the addition of ammonium to the nutrient solution promoted the formation of cyanide and NADH which in turn resulted in an inactivation of the nitrate reductase. Ammonium also caused a decrease in nitrate reductase activity in tobacco cells but it was not known whether this decrease was due to a diminution in the quantity of total nitrate reductase activity or was due to the formation of inactive enzyme. [Note: addition of an organic acid such as succinate along with ammonium, was required for ammonium to cause a decrease in the overt activity of nitrate reductase. (P. Filner - personal communication.)]

Table 8. Effect of threonine upon nitrate reductase activity.

Parameter	M-1D medium	M-1D + 100 μM Thr. medium	Percent of the value for the M-1D medium
Overt nitrate reductase activity	7.85	0.36	4.6%
Activation ratio	0.98	2.93	299.0%
Total nitrate reductase activity	7.70	1.06	13.7%

Stationary phase tobacco XD cells were inoculated into M-1D or M-1D + 100 μM threonine medium. After 24 hours, the tissue was harvested and the nitrate reductase activity was extracted. The overt activity was assayed and the activation ratio - the ratio of the activities with and without ferricyanide activation, was determined. The total activity is the product of the overt nitrate reductase activity multiplied by the activation ratio. The nitrate reductase activity is expressed in nmoles NO_2^- formed/minute/mg protein.

Eleven hours after the addition of ammonium succinate to nitrate-grown cells, the overt nitrate reductase activity had declined more than 60% relative to control cultures to which potassium succinate had been added (Table 9). This decline was <u>not</u> accompanied by an increase in the activation ratio of the nitrate reductase. It was concluded that the effect of ammonium succinate was a decrease in the total activity of nitrate reductase and not a reversible inactivation of the enzyme. The response of tobacco XD cells to ammonium appears to be different from the response of green algae.

Table 9. Effect of ammonium upon nitrate reductase activity.

Parameter		M-1D plus potassium succinate	M-1D plus ammonium succinate	Percent of the value for the M-1D plus potassium succinate
0 hr	Overt nitrate reductase activity	4.92 (± 3.37)	6.62 (± 1.06)	135
	Activation ratio	1.16 (± 0.03)	1.00 (± 0.02)	86%
	Total nitrate reductase activity	5.74 (± 2.89)	6.62 (± 0.98)	115
11 hr	Overt nitrate reductase activity	6.60 (± 1.61)	2.60 (± 0.91)	39
	Activation ratio	1.34 (± 0.03)	0.94 (± 0.12)	70
	Total nitrate reductase activity	8.76 (± 1.89)	2.40 (± 0.57)	27

Stationary phase tobacco XD cells were inoculated into M-1D medium. After two days, either potassium succinate or ammonium succinate (pH 6.2) was sterilely added to the cultures to yield a final concentration of 3.0 mM potassium or ammonium, respectively. At the indicated times the overt nitrate reductase activity and the activation ratio - the ratio of the activities with and without ferricyanide activation, were determined. The total activity is the product of the overt nitrate reductase activity multiplied by the activation ratio. The values in parentheses are the standard deviations. The nitrate reductase activity is expressed in nmoles NO_2^- formed/minute/mg protein.

Effects of Inadequate Sulfate Nutrition Upon Nitrate Reductase Activity

This thesis research program could now be brought full circle. The initial observations of this thesis (Figure 6A) were that the induction of nitrate reductase activity was diminished by suboptimal sulfate nutrition. At that time it was not clear whether this decrease was due to decreased synthesis of nitrate reductase enzyme or if it was due to inactivation of the enzyme. These alternatives could now be tested in light of the previously described results.

Exponentially growing tobacco XD cells were inoculated into either S^- M-1D plus 100 μM K_2SO_4 or S^- M-1D plus 200 μM KCl. After 24 hours, the overt activity and the activation ratio were determined (Table 10). In the cultures with no added sulfate, the overt activity declined sharply but the activation ratio rose correspondingly. The net result was that the average total activity remained approximately the same as in the control with added sulfate. It appeared that inactivation of nitrate reductase was the response of the cells to inadequate sulfate nutrition and was the method of coordination between the nitrate and sulfate assimilatory pathways.

Table 10. Effect of inadequate sulfate nutrition upon nitrate reductase activity.

Sample		Overt nitrate reductase activity	Activation ratio	Total nitrate reductase activity
S^- M-1D + 100 µM K_2SO_4	Replicate I	14.1	2.0	27.9
	Replicate II	13.8	2.0	28.1
	Average	14.0 (± 0.2)	2.0 (± 0.1)	28.0 (± 0.1)
S^- M-1D + 200 µM KCl	Replicate I	1.3	2.9	3.8
	Replicate II	6.0	6.7	39.8
	Average	3.6 (± 3.3)	4.8 (± 2.6)	21.8 (± 25.4)

Stationary phase tobacco XD cells grown on S^- M-1D + 100 µM K_2SO_4 were inoculated by a 1:10 dilution into S^- M-1D + 100 µM K_2SO_4 medium. After 4 days, the tissue of each individual flask was harvested by vacuum filtration and sterilely transferred quantitatively to flasks containing either S^- M-1D + 100 µM K_2SO_4 or S^- M-1D + 200 µM KCl. After 24 hours, the cultures were harvested and the nitrate reductase activity was extracted. The overt activity and the activation ratio - the ratio of the activities with and without ferricyanide activation, were determined. The total activity is the product of the overt nitrate reductase activity multiplied by the activation ratio. The values in parentheses are the standard deviations. The nitrate reductase activity is expressed in nmoles NO_2^- formed/minute/mg protein.

DISCUSSION

The results of this investigation have demonstrated several key points.

1. When the nitrate nutrition of the tobacco XD cells is inadequate, an inactive but activable form of the NADH nitrate reductase develops.
2. Under conditions of adequate nitrate nutrition, the activity of nitrate reductase increases with increased adequacy of the sulfur nutrition of the cell. Furthermore, this modulation involves the formation of an inactive but activable enzyme.
3. During conditions of sulfur inadequacy, the magnitude of derepression of ATP sulfurylase increases with the sufficiency of the nitrogen nutrition of the cell.

Considering that reduction of nitrate to ammonium can consume up to 30% of the energy available to the plant from photosynthesis (2), it would seem judicious that there be modulation of the flux of nitrate through the nitrate pathway when the sulfur nutrition of the cell is inadequate (Figure 6A). This modulation during periods of inadequate sulfur nutrition appears to be accomplished by the formation of an inactive nitrate reductase and not by alteration of the total quantity of the enzyme activity (Table 10). It is, however, not known whether

the mechanism of inactivation of nitrate reductase during sulfur inadequacy is identical to the mechanism of inactivation during nitrate inadequacy.

The modulation of ATP sulfurylase activity during nitrate inadequacy could be a response to conserve the synthetic capacity of the cell. It is, however, unknown whether the modulation of activity of ATP sulfurylase is due to decreased enzyme synthesis or to inactivation as with nitrate reductase.

Three fortuitous events were of critical importance for the observation of spontaneous activation of nitrate reductase. The first of these was the formulation of a buffer in which the nitrate reductase enzyme was stable (Figure 8). Of similar importance was the temperature chosen for storage of the extracts, -20°C. At room temperature, +22°C, the enzyme activity declined rapidly (Figure 10) whereas at -196°C, the activity was nearly identical to the fresh activity. The third event was perhaps the most critical of all. The experiment illustrated in Figure 8 required a considerable quantity of nitrate reductase activity. Due to serendipity and lack of experience, 7 day old cultures (late exponential phase cells) were chosen as the source of cells because of the higher quantity of tissue in these older cultures. Normally, 4 day old cultures (mid-exponential phase cells) would have been used because cultures of this age actually contained the maximum specific activity of nitrate reductase. By the fortunate choice of 7 day old cultures, an extract was obtained which contained a considerable quantity of inactive enzyme (Figures 8 and 16).

The observed spontaneous activation of tobacco nitrate reductase was strikingly similar to the spontaneous activation of nitrate

reductase of Chlorella which had been fed ammonium as reported by Losada et al. (57). This formation of inactive nitrate reductase in Chlorella was subsequently demonstrated to be the result of the binding of CN^- to the reduced form of the enzyme. Based on the suggestion that the tobacco nitrate reductase might be regulated in a manner similar to the algal enzyme, further characterization of the tobacco enzyme was undertaken.

NADH was an inhibitor of tobacco nitrate reductase but the magnitude of inhibition was extremely variable with no distinguishable pattern. Other chemicals also promoted inactivation, especially cyanide either alone or with NADH. NADPH caused some inactivation. No inactivation was observed using the oxidized forms of the dinucleotides, NAD^+ and $NADP^+$, or the phosphorylated nucleosides ATP and ADP (Table 6).

These results could be compared with previously reported results. NADH had frequently been reported to be an inactivator of nitrate reductase from the algae Chlamydomonas reinhardii (55), Chlorella vulgaris (63), Chlorella fusca (64) and Cyanidium caldarium (158) and from the higher plants rice (70), corn (68, 69) and spinach (66, 67). However, other reports suggested that NADH alone did not inactivate the enzyme from Chlorella fusca (71), Chlorella vulgaris (72) and Neurospora crassa (68). These variations might simply have reflected the purity of the nitrate reductase and the presence of trace amounts of effectors, such as cyanide, in the homogenates.

The observation that NADH plus cyanide inactivated tobacco nitrate reductase was very similar to the observations of other researchers. Solomonson (72) reported that NADH plus cyanide together

(but not individually) caused inactivation of the nitrate reductase from Chlorella. Wallace (68) also reported inactivation of nitrate reductase from corn and Neurospora when the enzyme was incubated with NADH plus cyanide. The same inactivating properties were reported for rice (79), barley (159) and spinach (80).

The inactivation of nitrate reductase from tobacco XD cells by cyanide plus NADH could be completely reversed by incubation with appropriate concentrations of ferricyanide (Figure 15). This property of reversible inactivation had been previously reported for Chlorella using concentrations of ferricyanide of about 500 μM (63, 72). Leong and Shen (79) using rice and A. Oaks (personal communication) using barley could also obtain complete or partial reactivation of cyanide-inactivated enzyme using ferricyanide.

Unlike other reports for higher plants, ferricyanide could activate extracts of tobacco nitrate reductase without prior inactivation (Figure 14 and Table 6). Additionally, this activation exhibited a pronounced concentration dependence for ferricyanide (Figure 14). At concentrations below 100 μM, no activation occurred whereas at concentrations above this, there was marked activation. The magnitude of activation plateaued and then declined above 300 μM ferricyanide. Only in a report by Leong and Shen (79) had a similar curve been reported. Jetschmann et al. (109) reported a concentration dependent activation by ferricyanide of extracts from Chlorella but there was no indication that a minimum concentration was required for activation.

The obvious lack of activation and even inactivation at ferricyanide concentrations below 100 μM brought into question the mechanism of activation. In algae, the activation was proposed to be

due to oxidation of the reduced form of nitrate reductase (109); cyanide would bind only to the reduced form of the enzyme. Although suspected, it was uncertain whether the ferricyanide activation of tobacco nitrate reductase also occurred as a result of oxidation of the enzyme.

This oxidation mechanism would explain the "titration" effect observed in Figure 14. Ferricyanide has a reported reduction potential of from + 0.48V (16) to + 0.360V (160). Any substance more easily oxidized than nitrate reductase would, of course, consume the oxidant and therefore prevent the oxidation of nitrate reductase. Cysteine with a reduction potential of - 0.34V (161) is a candidate as the reductant of ferricyanide.

Other oxidants should in theory perform a similar function as ferricyanide (Table 6). Fifty micromolar DCPIP ($E^{o'}$ = + 0.217V) (162) reversed the inactivation of 50 μM NADH. However, 50 μM cytochrome c ($E^{o'}$ = 0.235V) (163) did not reverse the inactivation. This suggested that the minimum reduction potential required for activation of nitrate reductase after NADH inactivation was between + 0.20V and + 0.25V. Unfortunately, data are not available to deduce similar parameters for the activation of nitrate reductase which had been extracted in the inactive state because neither ferricyanide, DCPIP nor cytochrome c at 50 μM activated the enzyme.

The similarities between the nitrate reductase enzymes of tobacco and algae are striking. Both systems contained an inactive enzyme <u>in vivo</u> which could be activated either by storage or by incubation with ferricyanide. Additionally, NADH partially inactivated the enzymes but with both NADH plus micromolar concentrations of cyanide, virtually

complete inactivation was obtained. The concentration of cyanide required to produce inactivation was similar for both systems. Furthermore, ferricyanide could reverse the inactivation by NADH plus cyanide.

These similarities led to the suggestion that the nitrate reductase of tobacco and algae are regulated similarly. To expand the comparison, the effect of ammonium upon the nitrate reductase of tobacco was examined. In algae, addition of ammonium to nitrate grown cells resulted in a rapid conversion of the active form of the enzyme to an inactive form. This inactive form could be reactivated by ferricyanide (52, 55, 56, 57). In tobacco, however, addition of ammonium to nitrate grown cells did not promote the formation of a ferricyanide-activable activity. The decrease in overt activity represented a decrease in the total activity, not a conversion of active to inactive enzyme. Nakagawa et al. (62) had also shown a decrease in overt nitrate reductase activity in spinach upon addition of ammonium; however, even though an inactive form of the enzyme was detected by immunochemical techniques, it was not demonstrated that the inactive species was activable. In Lemna minor, ammonium does cause partial inactivation with reactivation upon storage, but irreversible loss of activity was also observed (61, 108).

Another point of dissimilarity is illustrated in Figure 16. As the tobacco cultures entered late log or stationary phases, an inactive species of nitrate reductase developed which was activable by ferricyanide. Importantly, the quantity of total activity remained fairly constant throughout the growth cycle of the culture. Subsequent experiments (Figure 20) identified depletion of nitrate as being the

promoting factor for this inactivation. A similar effect had not been observed in algae. In other higher plants though, evidence for a similar regulation had been observed. Kaplan et al. (164) observed the development of a nitrite-activable species upon transfer of barley to nitrate-less medium.

Because depletion of nitrate from the medium apparently resulted in the formation of an inactive species of the enzyme, addition of nitrate should promote the formation of the fully active enzyme. When tobacco tissue was subcultured into fresh medium containing an adequate quantity of nitrate, the activation ratio of the enzyme declined (Figure 19). There was, however, no evidence for the conversion of inactive enzyme to the active form. In fact, the quantity of the inactive species appeared to increase. This result and the result showing a slower induction and decrease in the activation ratio when cells were inoculated into conditioned medium (Figure 20) could be considered suggestive of the presence of an inactivator in the cellular fluid or in the medium of the culture from which nitrate had been depleted.

In comparison, Pistorius et al. (60) had observed activation of nitrate reductase upon subculturing representing a conversion of inactive to active enzyme. They did not, however, observe an increase in the quantity of inactive species. Using density - labelling experiments, Johnson (52) confirmed the partial conversion of inactive to active enzyme but also concluded that there was new synthesis of enzyme.

The formation and accumulation of an inactive nitrate reductase as nitrate became depleted is noteworthy in several respects. The

first point is that the quantity of total enzyme activity (overt plus latent) per unit of protein remained relatively uniform throughout the growth of the culture. This was in sharp contrast to the decline in overt nitrate reductase activity which decreased about 8-fold during the same period.

When the enzyme activity of the culture was integrated over the first eight days, the overt activity could only be expected to reduce part of the nitrate initially present in the culture whereas the total activity (overt plus latent) could account for the reduction of all of the nitrate. This brings into question which value more accurately reflected the nitrate reductase activity *in vivo*, the measured overt activity or the total activity which took the ferricyanide activable species into consideration. A similar question has often been posed by other researchers. Does the activity of nitrate reductase which is measured *in vitro* accurately reflect the activity *in vivo*? Ferrari and Varner (165) measured the *in vivo* activity of barley nitrate reductase using $N^{18}O_3^-$ and observed that the *in vitro* assay indicated an activity 6-fold greater than that indicated by the *in vivo* assay. In contrast, purported mutants of barley with no detectable *in vitro* activity of nitrate reductase grew nearly as well on nitrate as the wild type plants (49).

Considering the differences in total reducing capacity of the tobacco nitrate reductase when overt or total activity are integrated, it could be suggested that the measurement of the inactive species merely represented an artifact. In the extracts from older tissue, there was obviously an inactive enzyme. It could be detected by ferricyanide activation *and* by spontaneous activation during storage

at -20°C. The magnitude of activation by these two methods of activation was highly correlated. The activation did not appear to be an artifact of the assay procedure since the activable species only developed during specific physiological conditions, that is, during nitrogen (Figure 16) or sulfur (Table 10) inadequacy. Under other physiological conditions, the inactive species was not detected.

It is also possible that *in vivo*, all of the enzyme existed in an active form but upon extraction, a portion of the enzyme activity became inactivated. This could have been the result of disruption and mixing of cellular compartments containing nitrate reductase enzyme and an inactivating agent. The quantity of this agent would vary according to the metabolic state of the cell. Thus the proportion of inactivate enzyme would appear to depend upon the physiological state of the tissue.

For the remainder of this discussion, it will be assumed that a naturally occurring inactive nitrate reductase does exist *in vivo*.

Two other activation processes for nitrate reductase were also detected in extracts of tobacco cells. Increased activity was observed following precipitation of the enzyme by ammonium sulfate (104). The magnitude of activation was constant throughout the growth of the culture and did not exhibit any physiologically related variability. There was also an increase in nitrate reductase activity following passage of an extract through a Sephadex G-25 gel filtration column. This activation was only observed in extracts of tissue from days 3 and 4 of the growth cycle (Figure 16) when the culture was just entering the exponential phase of growth. This may be similar to the phenomenon studied in tobacco by Behrend and Mateles (11) who could

separate an inactivator of nitrate reductase by gel filtration. Neither of these activations were characterized further nor were their relationships to other effectors in other organisms examined.

If it can be assumed that an inactive species of nitrate reductase exists *in vivo*, then the question of major concern is the mechanism of inactivation; is it due to binding of cyanide or some other effector or is it the result of some other process? Several experiments were undertaken in an attempt to characterize the mechanism.

The mixing of extracts from old and young tissue gave no indication of a readily solubilized effector (Table 7). The activities of the combined extracts exhibited additivity. This suggested that the effector may not be cyanide or some other easily bound effector. However, it could not be definitely concluded that the inactivation was not the result of an effector. In the modified Zielke buffer, an effector might not be functioning properly. For example, EGTA could chelate a metal which might be essential for an effector to exhibit its action (97). Furthermore, extracts from the younger tissue might contain an agent which would modify the action of an effector which might be present in the extracts of older tissue. For example, nitrate, which would probably accumulate in younger tissue, has been shown to competitively compete with cyanide for the active site of the nitrate reductase enzyme (13).

Because cyanide caused inactivation of the tobacco nitrate reductase *in vitro* and because of the many similarities between tobacco and algal nitrate reductases, it could be suggested that cyanide is the *in vivo* effector. Because of the poor sensitivity of

the chemical assay for cyanide (78), the kinetic analysis described in Figure 21 and in the APPENDIX was undertaken to test if cyanide was the *in vivo* effector. Based on the different apparent affinities of the nitrate reductase from young and old tissue for cyanide, it is suggested that the effector was *not* cyanide.

Therefore, what is the nature of the effector which inactivated nitrate reductase during conditions of nitrogen inadequacy? The inactivator appeared to be a stable entity; lingering in the cell and possibly in the medium even after the nitrate supply was replenished (Figures 19 and 20). The effector might require metal cations for full inactivation of nitrate reductase as judged by the effect of metal chelators upon enzyme stability and by the mixing experiment. The effector bound reversibly to the nitrate reductase molecule and probably bound to the reduced form of the enzyme as judged by the effects of the oxidizer ferricyanide. The available data are insufficient to determine whether the effector is a protein or some metabolic intermediate which accumulates when normal metabolism is perturbed.

Several researchers have characterized activation or inactivation phenomena involving nitrate reductase in higher plants. Comparison of the properties of the tobacco system with some of the better characterized systems could be informative. Yamaya and several other authors have extensively characterized an inactivating factor from rice. This factor was a protein with a molecular weight of about 150,000 daltons (69). The factor bound reversibly (70, 96) to the oxidized form of the enzyme (70) and could be dissociated from the enzyme by addition of NADH (70). The action of the effector was

inhibited by the metal chelators EDTA and o-phenanthroline (97) and was enhanced by addition of the metal ions cobalt, calcium and nickel (97). The factor was quite stable at -20°C and +4°C with the pH of maximum stability being pH 7.5 (97). Physiologically, the factor was more abundant during the early and late stages of growth of the rice tissue cultures (98) and during deprivation of nitrate (99).

Wallace has isolated and partially characterized a nitrate reductase inactivating factor from maize roots. This seemingly specific factor exhibited proteolytic activity (102) and is presumed to function by degrading the nitrate reductase protein (69, 96). Furthermore, the inactivation by this factor was not reversed by treatment with 300 μM ferricyanide although NADH inactivation could be reversed by ferricyanide (68). As with the factor from rice, the action of the corn inactivating factor was also inhibited by the metal chelators EDTA and o-phenanthroline (102). This factor was also inhibited 16% by 0.1 M phosphate whereas the same concentration of NaCl had no effect (102). The effector was stable at room temperature, 0°C and at -15°C (102). Wallace also reported that the 44,000 dalton (102) factor was present in greater quantity in the older sections of the root than in the younger segments (102).

In barley, a nitrite activable nitrate reductase activity was formed during nitrate deprivation (111). Fifty micromolar ferricyanide did not reactivate the inactive species (111). The nitrite-activable species had different kinetic and pH optimum properties from those of the overt nitrate reductase; the two eluted separately from a DEAE - cellulose column (111) and when eluted from a Sephadex G-25 column, the overt activity eluted in the void volume whereas the nitrite-

activable species eluted subsequently. Binding of cyanide to the fully active overt enzyme caused the release of a component which exhibited nitrite-activable activity. This nitrite-activable species was speculated to be the molybdenum containing component of the nitrate reductase complex (Kaplan, personal communication).

Of the various reported inactivators of nitrate reductase of higher plants, that from rice appears most similar to the tobacco system. But differences exist even between these two systems. Most likely, the process functioning in the tobacco cultures is distinct from those characterized in other plants.

The accumulated results suggest that two effectors may be functioning to modify the activity of nitrate reductase in tobacco XD cells. The first effector, designated as "E", develops in response to nitrate deprivation. It causes reversible inactivation. Cyanide may also play a regulatory role in tobacco. Cyanide was shown to be an effector of tobacco nitrate reductase in vitro (Figure 15) and the presence of cyanide in tobacco is almost certain considering that cyanide is a common component of plants (90).

With two possible effectors of nitrate reductase, E and CN^-, the scheme illustrated in Figure 22 could be proposed. In this model, the nitrate reductase enzyme possesses two distinct binding sites, one for E and one for CN^-. Presumably, NR-CN and NR_E -CN would possess little or no assayable activity whereas the activity of NR_E is unknown.

The role of the effector, E, might be to regulate the activity of nitrate reductase in response to the availability of nitrate for reduction. Considering the nearly constant relationship between the total nitrate reductase activity of the culture in relation to the

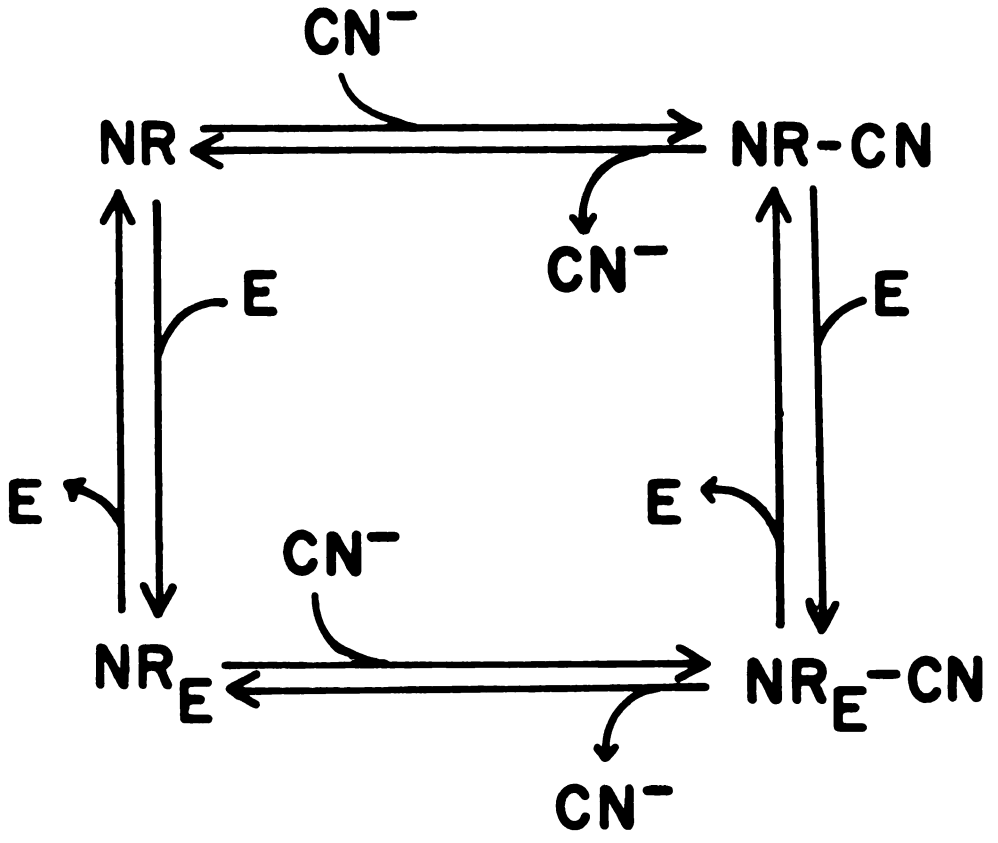


Figure 22. Proposed model for the binding of an effector and cyanide to nitrate reductase.

The explanations of the symbols are:

- NR - nitrate reductase with no bound effector
- E - unbound effector of nitrate reductase
- CN^- - unbound cyanide
- NR_E - nitrate reductase with bound effector
- NR-CN - nitrate reductase with bound cyanide
- NR_E-CN - nitrate reductase with both bound effector and cyanide

See text for discussion of model.

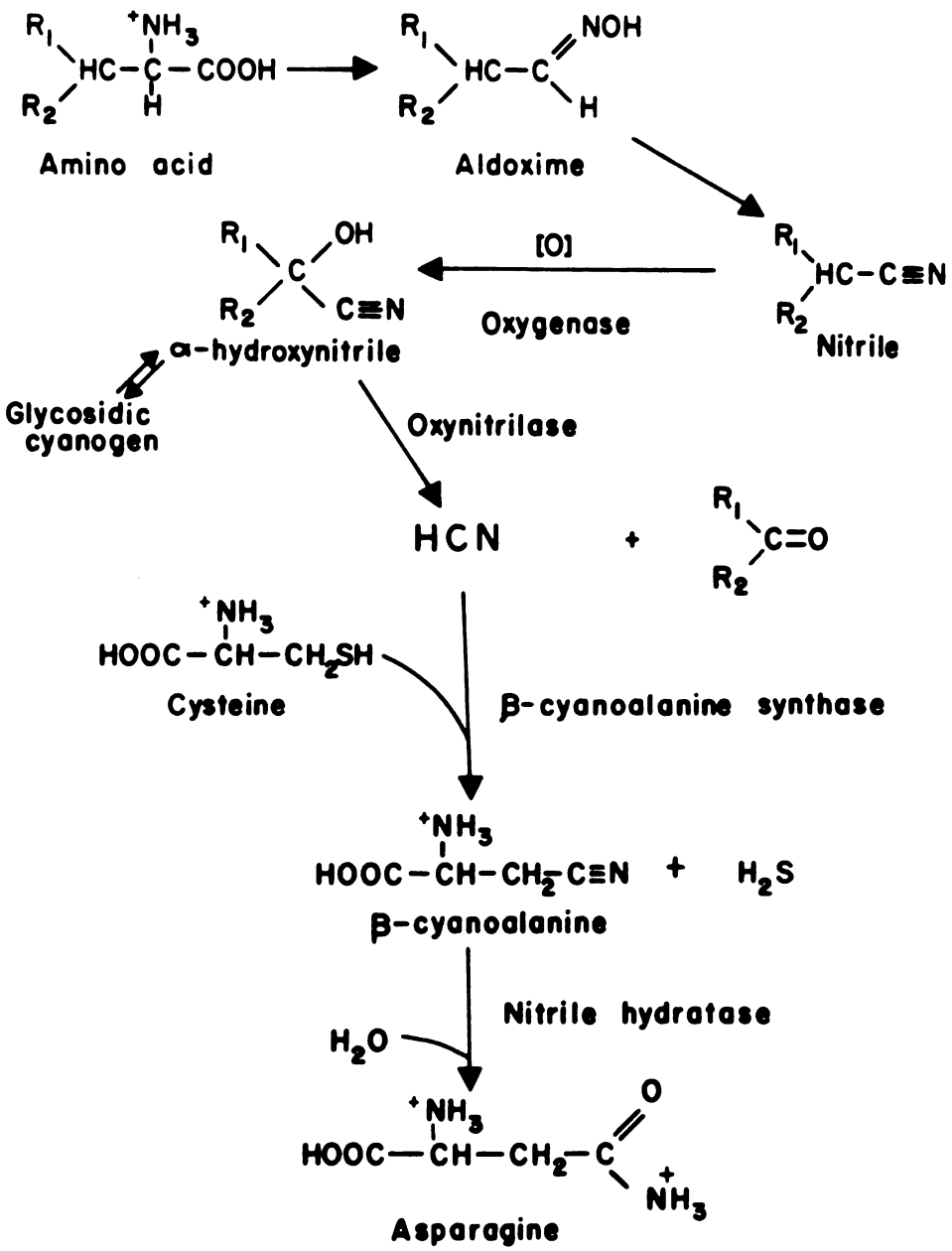
total protein of the culture (Figure 17), it could be speculated that the nitrate reductase enzyme would be synthesized at a constant rate as long as sufficient nitrate was available for induction. The actual activity of the enzyme could, in turn, be modulated in response to nitrate supplies through the action of the effector E, possibly to conserve the reducing potential of the cell. In addition to induction, the activity of nitrate reductase appears to be regulated by the process of activation/inactivation. Furthermore, this process of activation/inactivation appears to be the major determinant of the functional activity of the enzyme.

The possible role of cyanide is even more speculative. In algae, of course, cyanide is considered to be an effector which causes inactivation when ammonium is supplied to the tissue. However, ammonium did not result in the formation of a ferricyanide-reversible enzyme activity. Other physiological events may, nevertheless, promote the formation of excess cyanide _in vivo_ which might inactivate the nitrate reductase.

Cyanide is generally believed to be produced _in vivo_ by the action of amino acid oxidases upon particular amino acids (Figure 23 and reference 90). In algae, the primary sources of cyanide were histidine, tyrosine and basic amino acids (82, 83, 85). Accumulation of key amino acids in tobacco cells might hypothetically promote inactivation of nitrate reductase mediated by cyanide. Despite the observation that no appreciable quantity of inactive nitrate reductase was detected when threonine was added to tobacco cultures (Table 8) other amino acids may promote inactivation.

Figure 23. Biosynthesis and metabolism of cyanide.

Cyanide is known to be synthesized by the action of amino acid oxidases and metabolized by the reaction with cysteine to form ß - cyanoalanine (see reference 90).


Amino acid
Aldoxime
Nitrile
[O]
Oxygenase
α-hydroxynitrile
Glycosidic cyanogen
Oxynitrilase
HCN
Cysteine
β-cyanoalanine synthase
β-cyanoalanine
Nitrile hydratase
Asparagine

The formation of an inactive nitrate reductase also occurred during sulfur inadequacy (Table 10). From this observation, cyanide could be proposed as an important coupling factor between the nitrate and sulfate assimilatory pathways. The proposed regulation might involve the scavenging of cyanide by cysteine (Figure 23). When the sulfur nutrition of the cells is adequate, cysteine should be in adequate supply. By the action of ß - cyanoalanine synthase identified in each of 13 plant genera surveyed by Miller and Conn (87), cyanide would displace the sulfhydryl group of cysteine to produce ß - cyanoalanine plus sulfide. The ß - cyanoalanine could in turn be used as a substrate for the synthesis of asparagine (89, 90). This reaction would keep the concentration of cyanide to a minimum as long as the supply of cysteine would be adequate. When the cells become sulfur starved, the concentration of cysteine would decline and cyanide would be scavenged less effectively, accumulating to a level which results in inactivation of nitrate reductase.

If cyanide and cysteine are the effectors signaling the inadequacy of the sulfur supply, then the coupling between the nitrate and sulfate assimilatory pathways would be a prime example of metabolic interlock as described by Jensen (166). He described the coordinated coupling of converging pathways by means of small molecules. These signal molecules would indicate the status of coupled pathways so that converging pathways would only provide sufficient quantities of a metabolite to allow optimum metabolism of the cell. Thus, according to this model cysteine, an end product of the sulfate assimilatory pathway, would scavenge some of the inactivator, cyanide, so that the nitrate pathway would function optimally.

There is uncertainty concerning the identity of the coupling factor "NY" (167) which signals the sulfate assimilatory pathway that the nitrogen nutrition of the cell is sufficient. Several authors have suggested that O-acetylserine might act as this signal (117, 167). Supporting evidence for this choice is that this compound is required for the derepression of the sulfate assimilatory pathway in Salmonella (31). Alternatively, the effector E (see Figure 24) might act as a negative effector for the sulfate assimilatory pathway as well as for the nitrate assimilatory pathway. E might therefore act as a coupling factor signaling that the nitrate pathway is operating at a reduced capacity indicating that the sulfate pathway should be modulated correspondingly.

In conclusion, one or more regulatory mechanisms have been proposed for the nitrate reductase of tobacco XD cells. One may be an effector, E, which develops during nitrogen inadequacy and the other might be cyanide.

Of primary concern in future investigations should be the identification and characterization of the effector, E, observed during nitrogen limitation. Is it a protein or a metabolic intermediate? The physiological role, if any, of cyanide is uncertain. It is unknown whether nitrate reductase is the only enzyme modified by these proposed effectors or whether other activities such as nitrate uptake, nitrite reductase or possibly glutamine synthetase might also be affected.

Other physiological conditions should be sought which would promote the formation of an inactive nitrate reductase. In addition to nitrate and sulfate limitation, one could examine the role of perturbations such as amino acid imbalance, carbon inadequacy, water

or salt stress, or even the effect of light using photoautotrophic tissue. Nitrate reductase is a highly regulated enzyme but its mechanisms of regulation are still poorly understood.

Of practical significance, what could be gained by further study of the inactivation of nitrate reductase? One possible use could be to use the magnitude of activation of nitrate reductase by ferricyanide as an indication of the adequacy of the nitrate nutrition of a plant tissue. Greater activation could indicate a greater magnitude of nitrate inadequacy and readily signal the need for more nitrogen fertilizer. Methods of reversing or diminishing the quantity of inactive enzyme in vivo might permit more effective utilization of available nitrate supplies and reserves. Additionally, a better understanding of the complex coordination among the nitrate pathway and several other vital metabolic pathways (sulfate assimilation, carbon assimilation, etc.) could provide parameters by which more efficient formulations of fertilizers could be developed.

The massive agricultural requirement for nitrate demands that the metabolism of this vital nutrient be understood as thoroughly as possible. It is my hope that the research presented in this thesis has made a contribution to this understanding.

APPENDIX

APPENDIX

Theory for the Determination of Whether Cyanide Is An In Vivo Effector of Nitrate Reductase

The test of whether cyanide was bound *in vivo* to inactive nitrate reductase was based on probable differences in affinity of the different forms of the enzyme for cyanide. Prior to discussion of the theory, some general definitions are presented.

$[CN^-]$ = concentration of free cyanide in solution. This concentration will be assumed to be equal to the concentration of added cyanide since the concentration of cyanide will greatly exceed the concentration of nitrate reductase enzyme.

[NR] = concentration of nitrate reductase to which neither cyanide nor other effectors is bound. This quantity will be assumed to be equal to the assayed activity of nitrate reductase after incubation with cyanide.

[NR-CN] = concentration of nitrate reductase to which cyanide is bound. This species is assumed to exhibit no assayable activity unless it is activated by ferricyanide.

$[NR_{TOTAL}]$ = the concentration of the total nitrate reductase. This quantity is the sum of both the overt activity plus the activity detectable after ferricyanide activation.

$[NR_{TOTAL}] = [NR] + [NR\text{-}CN]$

In practice, $[NR_{TOTAL}]$ was calculated as the product of the overt activity multiplied by the activation ratio.

CASE I

Cyanide is the only observed effector of nitrate reductase activity and only cyanide is bound to the nitrate reductase in vivo.

The association constant, K_A, of nitrate reductase for cyanide is

$$K_A = \frac{[NR\text{-}CN]}{[NR]\ [CN^-]}$$

From the definition of $[NR_{TOTAL}]$, rearrangement yields

$$[NR\text{-}CN] = [NR_{TOTAL}] - [NR]$$

Substituting this value of [NR-CN] into the expression for K_A yields

$$K_A = \frac{[NR_{TOTAL}] - [NR]}{[NR]\ [CN^-]}$$

Separating terms and eliminating expressions of unity

$$K_A = \frac{[NR_{TOTAL}]}{[NR]\ [CN^-]} - \frac{1}{[CN^-]}$$

Rearrangement yields

$$K_A + \frac{1}{[CN^-]} = \frac{[NR_{TOTAL}]}{[NR]} \times \frac{1}{[CN^-]}$$

Combining terms on the left yields

$$\frac{K_A \times [CN^-] + 1}{[CN^-]} = \frac{[NR_{TOTAL}]}{[NR]} \times \frac{1}{[CN^-]}$$

Multiplying both sides by $[CN^-]$ produces

$$K_A \times [CN^-] + 1 = \frac{[NR_{TOTAL}]}{[NR]}$$

This indicates that after *in vivo* incubation with cyanide, the ratio of total nitrate reductase activity to overt activity will depend only upon the concentration of cyanide. Thus, extracts which contain only overt activity (from young tissue) and extracts which contain both overt plus inactive nitrate reductase (from older cultures) should yield identical ratios for a given concentration of cyanide. Additionally, the slope, K_A of the relationship $\frac{[NR_{TOTAL}]}{[NR]}$ vs. $[CN^-]$ should be identical for extracts from cultures of any age.

Note: This derivation assumes that the cyanide concentration in which the extracts are incubated is greater than the *in vivo* concentration of CN^- or that NR-CN molecules will dissociate to satisfy the thermodynamic requirement of the association constant.

CASE II

An effector which is not cyanide binds to the nitrate reductase molecule *in vivo* and alters the affinity of the enzyme for cyanide.

Some additional definitions are required.

$[NR_E]$ = the concentration of nitrate reductase to which an effector is bound. Ferricyanide causes release of the effector yielding NR.

$[NR_E\text{-}CN]$ = the concentration of nitrate reductase to which both cyanide and the effector are bound. This species exhibits no assayable activity unless it is activated by ferricyanide treatment.

Let K_A be the association constant of nitrate reductase for cyanide when the nitrate reductase has no effector bound to it.

$$K_A = \frac{[NR\text{-}CN]}{[NR]\ [CN^-]}$$

Let K_{AE} equal the association constant of nitrate reductase for cyanide when the nitrate reductase has an effector bound to it.

$$K_{AE} = \frac{[NR_E\text{-}CN]}{[NR_E]\ [CN^-]}$$

Assume that NR_E is present at a concentration which is a multiple "B" of NR.

$$[NR_E] = B \times [NR]$$

Substituting this into the expression for K_{AE} yields

$$K_{AE} = \frac{[NR_E\text{-}CN]}{B \times [NR]\ [CN^-]}$$

Rearranging

$$B \times [NR] = \frac{[NR_E\text{-}CN]}{K_{AE}\,[CN^-]}$$

A similar rearrangement of the expression for K_A yields

$$[NR] = \frac{[NR\text{-}CN]}{K_A\,[CN^-]}$$

Let the assayable activity of the nitrate reductase with no bound effectors $(NR)_A$ be represented by its concentration [NR].

$$(NR)_A = [NR]$$

Assume that a molecule of nitrate reductase with an effector bound to it does not reduce nitrate at the same rate as does a molecule of nitrate reductase without a bound effector. Therefore, the activity of this species with the bound effector, $(NR_E)_A$, will be a multiple "F" of the concentration of that species.

$$(NR_E)_A = F \times [NR_E]$$

In a given extract, the activity of the nitrate reductase with an effector bound to it will be a multiple "C" of the activity of the nitrate reductase without a bound effector.

$$(NR_E)_A = C \times (NR)_A$$

Substituting the expression $(NR_E)_A = F \times [NR_E]$ yields

$$F \times [NR_E] = C \times (NR)_A$$

But $(NR)_A = [NR]$, so this gives

$$F \times [NR_E] = C \times [NR]$$

$$[NR_E] = \frac{C}{F} \times [NR]$$

But by a previous definition

$$[NR_E] = B \times [NR]$$

or

$$B = C/F$$

Therefore, the multiple "B" represents a quantity determined by the relative turnover rate "F" and the relative overt activity "C" of nitrate reductases with and without bound effector.

In an extract, the observed activity would be the sum of

$$(NR)_A + (NR_E)_A.$$

But $(NR_E)_A = F \times [NR_E]$ and $(NR)_A = [NR]$ so

$$(NR)_A + (NR_E)_A = [NR] + F \times [NR_E]$$

Expressing the right hand side in terms of the association constants

$$[NR] + F \times [NR_E] = \frac{[NR\text{-}CN]}{(K_A)\,[CN^-]} + \frac{F \times [NR_E\text{-}CN]}{(K_{AE})\,[CN^-]}$$

But $[NR_E] = B \times [NR]$ and $B = C/F$ so

$$F \times [NR_E] = C \times [NR]$$

Substituting into the above yields

$$[NR] + C \times [NR] = \frac{[NR\text{-}CN]}{(K_A)\ [CN^-]} + \frac{F \times [NR_E\text{-}CN]}{(K_{AE})\ [CN^-]}$$

Let K_{AE} be a multiple "D" of K_A

$$K_{AE} = D \times K_A$$

Substituting and combining terms produces

$$(1 + C)\ [NR] = \left[\frac{D \times [NR\text{-}CN] + F \times [NR_E\text{-}CN]}{D \times K_A}\right] \times \frac{1}{[CN^-]}$$

Multiplying by $\frac{1}{(1+C)}$ produces

$$[NR] = \left[\frac{D \times [NR\text{-}CN] + F \times [NR_E\text{-}CN]}{D \times K_A}\right] \times \frac{1}{[CN^-] \times (1+C)}$$

But $[NR_{TOTAL}] = [NR] + [NR\text{-}CN] + [NR_E] + [NR_E\text{-}CN]$

Rearranging

$$[NR_E\text{-}CN] = [NR_{TOTAL}] - [NR] - [NR\text{-}CN] - [NR_E]$$

Substituting the value for $[NR_E\text{-}CN]$ yields

$$[NR] = \left[\frac{D \times [NR\text{-}CN] + F \times [NR_{TOTAL}] - F \times [NR] - F \times [NR\text{-}CN] - F\ [NR_E]}{D \times K_A}\right] \times \frac{1}{[CN^-] \times (1+C)}$$

Combining terms

$$[NR] = \left[\frac{(D-F) \times [NR-CN] + F \times [NR_{TOTAL}] - F \times [NR] - F \times [NR_E]}{D \times K_A} \right] \times \frac{1}{[CN^-] \times (1+C)}$$

But $[NR_E] = B\ [NR]$

Substituting and combining terms

$$[NR] = \left[\frac{(D-F) \times [NR-CN] + F \times [NR_{TOTAL}] - (F+FB) \times [NR]}{D \times K_A} \right] \times \frac{1}{[CN^-] \times (1+C)}$$

From the association constant K_A

$$[NR-CN] = (K_A) \times [NR] \times [CN^-]$$

Substituting produces

$$[NR] = \left[\frac{(D-F) \times (K_A) \times [NR] \times [CN^-] + F \times [NR_{TOTAL}] - (F+FB) \times [NR]}{D \times K_A} \right] \times \frac{1}{[CN^-] \times (1+C)}$$

Combining terms

$$[NR] = \left[\frac{[NR] \times (D-F) \times (K_A) \times [CN^-] - (F+FB) + F \times [NR_{TOTAL}]}{D \times K_A} \right] \times \frac{1}{[CN^-] \times (1+C)}$$

Multiplying by $\frac{[CN^-] \times (1+C)}{[NR]}$ yields

$$[CN^-] \times (1+C) = \frac{[NR] \times ((D-F) \times (K_A) \times [CN^-] - (F+FB)) + F \times [NR_{TOTAL}]}{D \times K_A \times [NR]}$$

Separating terms

$$[CN^-] \times (1+C) = \frac{[NR] \times (D-F) \times (K_A) \times [CN^-]}{D \times K_A \times [NR]} - \frac{[NR] \times (F+FB)}{D \times K_A \times [NR]} + \frac{F \times [NR_{TOTAL}]}{D \times K_A \times [NR]}$$

Eliminating expressions of unity

$$[CN^-] \times (1+C) = \frac{(D-F) \times [CN^-]}{D} - \frac{(F+FB)}{D \times K_A} + \frac{F \times [NR_{TOTAL}]}{D \times K_A \times [NR]}$$

Multiplying by $D \times K_A$ and eliminating expressions of unity

$$[CN^-] \times D \times K_A \times (1+C) = K_A \times (D-F) \times [CN^-] - (F+FB) + \frac{F \times [NR_{TOTAL}]}{[NR]}$$

Rearranging

$$\frac{[NR_{TOTAL}]}{[NR]} = \frac{1}{F}\left[[CN^-] \times D \times K_A \times (1+C) - K_A \times (D-F) \times [CN^-] + (F+FB)\right]$$

$$\frac{[NR_{TOTAL}]}{[NR]} = \frac{[CN^-]\ (K_A)}{F}\left[(D \times (1+C) - (D-F)\right] + \frac{F+FB}{F}$$

Simplifying yields

$$\frac{[NR_{TOTAL}]}{[NR]} = [CN^-]\ \frac{(K_A)}{F}\ (D \times C+F)\ + \frac{F+FB}{F}$$

Therefore, if the effector is not cyanide, then the relationship $\frac{[NR_{TOTAL}]}{[NR]}$ vs. $[CN^-]$ will depend upon the relative contribution "C" of the activity of nitrate reductase with bound effector to the total activity in the extract. This relative contribution and therefore the slope of the above relationship will vary among extracts containing different concentrations of effector (that is, among cultures of different ages). This variation would be manifested as non-parallel slopes when $\frac{[NR_{TOTAL}]}{[NR]}$ is plotted vs. $[CN^-]$.

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