THE REGULATION OF NITRATE REDUCTASE IN CORN ROOTS

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by

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SCOPE AND CONTENTS: The experiments described in this thesis were performed to study the:

1. Role of nitrate in the induction of nitrate reductase.
2. Effect of nitrate on the in vivo regulation of nitrate reductase.
3. Role of atrazine on the regulation of nitrate reductase.

The nitrate reductase assay system was improved up to 10 fold over that previously used (28). This improvement included the addition of oxalacetic acid and malic dehydrogenase to the system, thereby oxidizing all the NADH at the end of the reaction. NADH, which was a required co-factor in the assay system, interfered with the nitrite colour formation.

When the induction kinetics of the enzyme are followed in root tip sections (0 - 10 mm) of corn, a 25 minute lag period followed by a period of rapid nitrate reductase synthesis is seen. Neither the lag period nor the rate of increase in enzyme activity is affected by the concentration of the inducer used (1.0, 10.0 or 25.0 mM nitrate). However, with concentrations of nitrate from 0.1 mM to

to 10.0 mM, there is an increase in the final level of nitrate reductase. Nitrate levels between 10.0 mM to 100.0 mM did not alter this maximally induced level of enzyme.

In mature root sections (25 - 35 mm from the tip), a longer lag time and a slower rate of increase in enzyme activity is seen. The system is not saturated with nitrate concentrations up to 100.0 mM.

Cycloheximide, a protein synthesis inhibitor, was added to the roots after the enzyme had been maximally induced. This allowed one to study the _in vivo_ turnover of the enzyme. Nitrate did not alter the _in vivo_ turnover of nitrate reductase in corn roots.

Atrazine is a herbicide that has been reported to increase nitrate reductase levels in corn (34). When used in the experiments described here, it was found to be ineffective in altering the nitrate reductase activity of the roots and leaves of corn.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

A	atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine)
BSA	bovine serum albumin
CHX	cycloheximide ($C_{15}H_{23}NO_4$)
EDTA	ethylenediaminetetraacetic acid
FAD	flavinadenine dinucleotide
FMN	flavine mononucleotide
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
MDH	malic dehydrogenase
N	nitrate
NAD (H)	nicotinamide adenine dinucleotide (reduced form)
NADP (H)	nicotinamide adenine dinucleotide phosphate (reduced form)
NED	N-(1-naphthyl)-ethylenediamine dihydrochloride
Ni R	nitrite reductase (NAD(P)H: nitrite oxidoreductase, E.C. 1.6.6.4)
NR	nitrate reductase (NADH: nitrate oxidoreductase, E.C. 1.6.6.1)
OAA	oxalacetic acid
RNA (m)	ribose nucleic acid (messenger)

ERRATUM:

Replace the word turnover with the word degradation on the following pages:

p. iii, line 10 and 11

p. 4, line 24

p. 6, line 5

p. 101, line 6

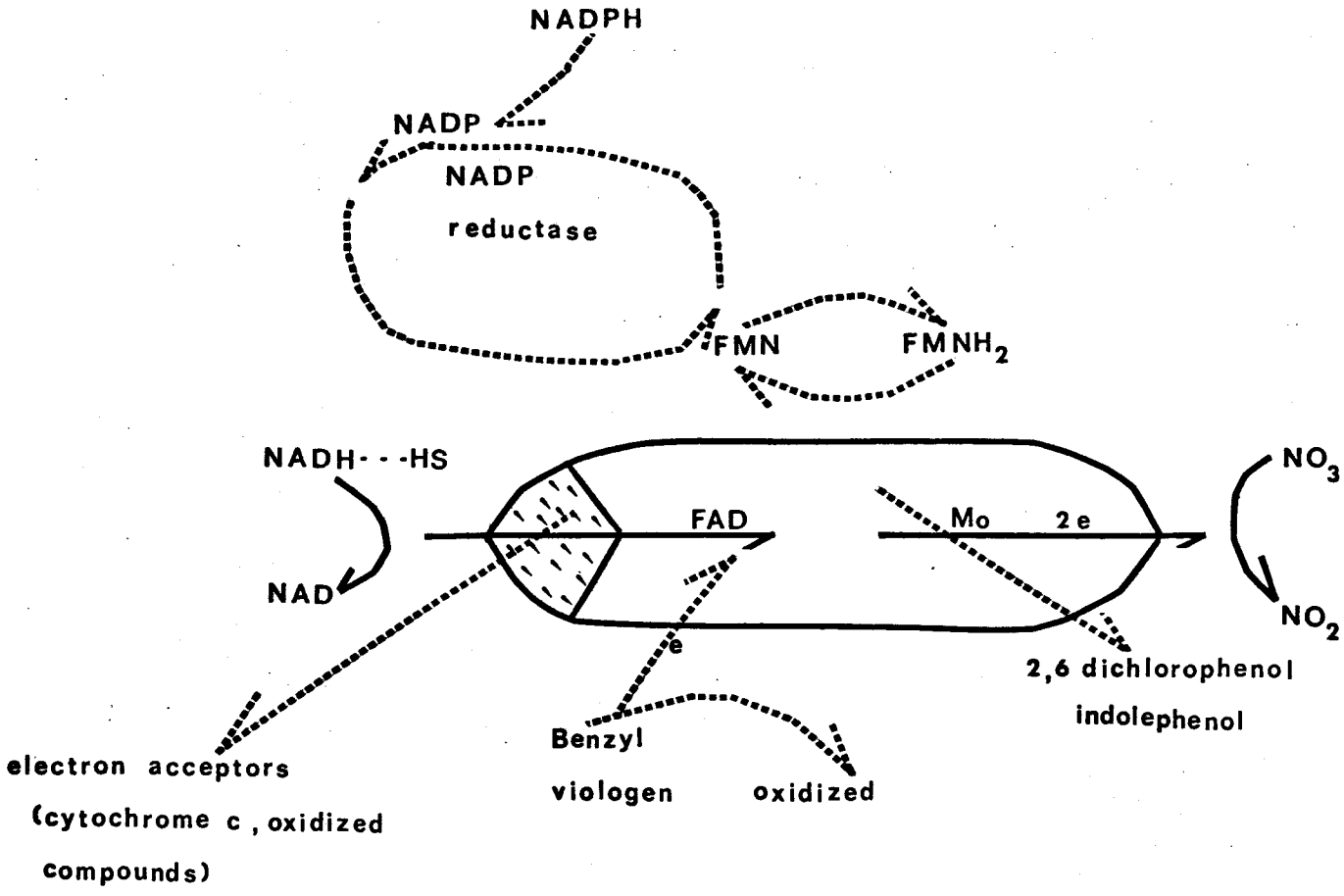
p. 105, line 17

INTRODUCTION

This study was undertaken in order to elucidate factors that regulate the enzyme nitrate reductase (NADH: nitrate oxidoreductase, E.C. 1.6.6.1) in Zea mays L. seedlings. This enzyme is the first enzyme in the nitrogen assimilatory pathway in plants. Nitrate is reduced initially to nitrite and then to ammonia. Eventually, it is incorporated into amino acids, which are required for protein synthesis. Several excellent reviews on nitrate reductase have been published (Hewitt and Nicholas, 1964, and Beevers and Hageman, 1969). Nitrate reductase is a molybdoflavoprotein. Figure 1 depicts the overall reaction of the enzyme. This scheme is a modified version of Beevers and Hageman's model (3). The overall reaction is designated as NADH-nitrate reductase. It is composed of at least two protein moieties, $FMNH_2$-nitrate reductase and NADH-diaphorase (48, 64). These proteins have different sedimentation coefficients and half lives. NADH is the preferred electron donor in higher plants (19). Reduced dyes such as benzyl viologen can act as an alternate electron source for the enzyme. The NADH-diaphorase was found to be heat labile. It contains thiol groups since the enzyme activity is protected by cysteine (35, 64) and is sensitive to p-chloro-mercuribenzoate, a thiol blocking agent (64). Cytochrome c or other oxidized compounds can act as artificical electron

Figure 1: A Model of the Nitrate Reductase Complex.

Figure 1 depicts a model of the enzyme nitrate reductase (NADH: nitrate oxidoreductase, E.C. 1.6.6.1). This scheme is a modified version of Beevers and Hageman's model (3). NADH is the preferred electron donor in higher plants. Reduced dyes such as benzyl viologen can act as an alternate source of electrons. The stippled area designates a heat labile portion of the enzyme, NADH-diaphorase. This portion contains sulfhydryl groups. Cytochrome c or other oxidized compounds can act as artificial electron acceptors for the NADH-diaphorase. The NADH-diaphorase is dependent on FAD (flavinadenine dinucleotide). NADPH can serve as an electron source for the overall reaction only by indirectly transfering its electrons to the enzyme via NADP reductase and FMN (flavin mononucleotide). The FMN is reduced by adding $Na_2S_2O_4$ to the assay. Molybdenum (Mo) is an intricate part of this portion of the nitrate reductase complex, designated $FMNH_2$-nitrate reductase. As artificial electron acceptor, oxidized 2,6-dichlorophenolindolephenol, can short circuit the $FMNH_2$-nitrate reductase before it reduces nitrate to nitrite. The overall reaction of the entire complex is the oxidation of NADH to NAD and the reduction of nitrate to nitrite.


NADPH
NADP
NADP
reductase
FMN
FMNH2
NADH- - -HS
NAD
FAD
Mo
2 e
NO3
NO2
e
2,6 dichlorophenol
indolephenol
Benzyl
viologen
oxidized
electron acceptors
(cytochrome c , oxidized
compounds)

acceptors for the NADH-diaphorase. The NADH-diaphorase was FAD dependent. NADPH could serve as an electron source only by transferring its electron indirectly via NADP reductase and FMN. The second part of the enzyme complex is $FMNH_2$-nitrate reductase. This portion is dependent on molybdenum, as seen by studies with its analog tungsten, which inhibits enzyme activity (60, 64). An artificial electron acceptor, oxidized 2,6-dichlorophenol-indolephenol, could short circuit the $FMNH_2$-nitrate reductase activity before it reduced nitrate to nitrite. It is also thought to be acting before the electrons reach the molybdenum complex (3). Reduced dyes such as benzyl viologen can act as alternate sources of the initial electron. The overall reaction of the entire complex is the oxidation of NADH to NAD with the subsequent reduction of nitrate to nitrite. The nitrite is then converted to ammonia via nitrite reductase. The ammonia goes initially to glutamic acid, and is eventually converted to the other amino acids (42, 51).

Nitrate reductase is induced by nitrate, its substrate. Increases in the enzyme activity are due to the de novo synthesis of the enzyme (3, 19, 65). The best method to date to prove conclusively that the enzyme was synthesized de novo is by the use of density labelling (65). This eliminates any possibility that increases in the enzyme activity were due to activation of nascent protein or to changes in the turnover of the enzyme.

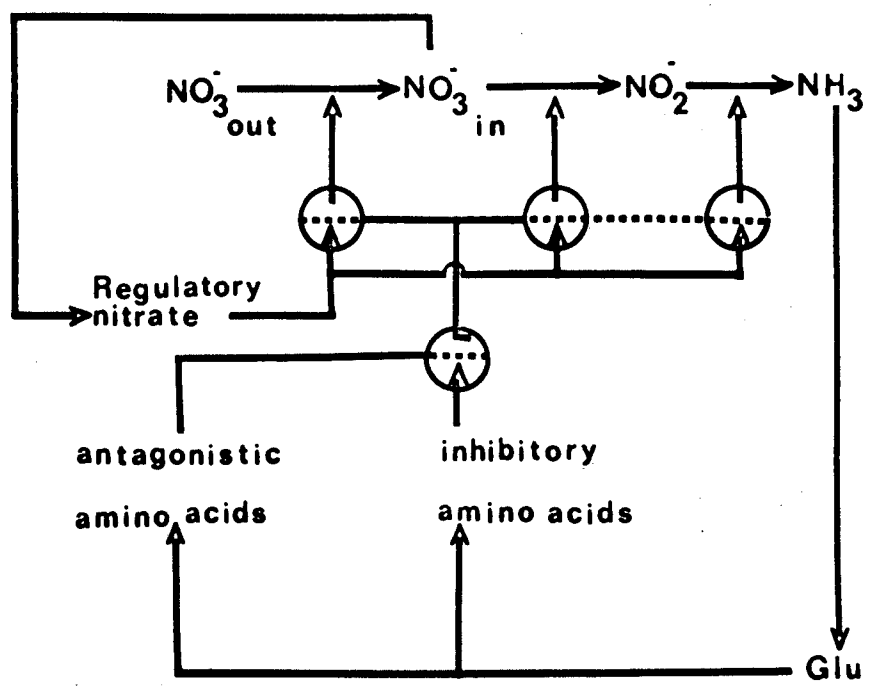
The technique involved growing the cultured tobacco cells on media containing ^{14}C-labelled arginine and ^{15}N-labelled nitrate. This radioactivity labels the pre-existing protein with ^{14}C, while the ^{15}N increases the buoyant density of the protein. The cells were then transferred to medium in which new proteins were synthesized from ^{14}N and were radioactively labelled with ^{3}H-arginine. Unlabelled amino acids were added to the induction media to dilute out pre-existing ^{15}N-amino acid pools. The buoyant density patterns showed that increases in nitrate reductase activity were caused by protein that had been synthesized de novo. Nitrate reductase has been found in such higher plants as the leaves of cauliflower, white mustard and sunflower (18), spinach leaves (31), the leaves of corn, marrow and spinach (39), radish cotyledons (20,21), cultured tobacco cells (17,65), rice (40), barley aleurone layers (6,7,8), suspension cultures of soybean and wheat (1,2), tomato roots and leaves (35), excised barley roots (44), and Chlorella (26,45,46). Nitrate reductase has also been found in the blue-green alga Anabaena cylindrica (29), in the fungi Neurospora crassa (48), and Aspergillus nidulans (33), and in the basidiomycete Ustilago maydis.(24).

The amount of nitrate reductase present in the system can be controlled at various molecular levels. For this substrate inducible enzyme, the nitrate could be a limiting factor. This would be due to controls on the nitrate uptake into the tissue, low nitrate concentrations, or limiting pools of nitrate that are available to

induce the enzyme (8). Filner, Wray and Varner (1969) suggested other possible control points for induced enzyme synthesis. These are at the levels of messenger RNA synthesis, m-RNA function, transfer-RNA function or polypeptide synthesis. In vivo inhibition of the enzyme and changes in the turnover rate of the enzyme could alter the levels of enzyme found. Zielke et al (65), using the buoyant density technique, found a constant turnover of nitrate reductase protein during the induction of the enzyme, after the enzyme was fully induced and during the decay of the enzyme. Filner (10) has outlined a model of the control relationships of the nitrate pathway in tobacco cells (Figure 2). He has represented the inhibitory amino acids as a grid potential which attenuates the inducing potential of the nitrate. The inhibitory amino acids that repress nitrate reductase formation when used individually were alanine, asparagine, aspartate, glutamate, glycine, histidine, leucine, methionine, proline, threonine and valine. Lysine and arginine were derepressors that had an antagonistic effect on the inhibitory amino acids, as indicated by their attenuating effect in the model. Cysteine and isoleucine acted as derepressors, but not in the presence of alanine or methionine. Figure 2 shows that controls can be exerted at the level of nitrate uptake, nitrate reduction to nitrite and nitrite reduction to ammonia. The ammonia goes on to form glutamate, which acts as a precursor for the other amino

Figure 2: Functional and Control Relationships of the Nitrate Pathway in Tobacco Cells.

Figure shows Filner's model of the functional and control relationships of the nitrate pathway in tobacco cells (10). The inhibitory amino acids were represented as a grid potential which attenuates the inducing potential of the nitrate. The inhibitory amino acids that repress nitrate reductase formation when used individually are alanine, asparagine, aspartate, glutamate, glycine, histidine, leucine, methionine, proline, threonine and valine. Controls can be exerted at the level of nitrate uptake, nitrate reduction to nitrite reduction to ammonia. The ammonia goes on to form glutamate, which acts as a precursor for the other amino acids. Lysine and arginine are derepressors that have an antagonistic effect on the inhibitory amino acids. This antagonistic effect is represented as a grid potential which attenuates the effect of the inhibitory amino acids. Cysteine and isoleucine also acted as derepressors, but not in the presence of alanine or methionine.


NO_3^- out
NO_3^- in
NO_2^-
NH_3
Regulatory nitrate
antagonistic amino acids
inhibitory amino acids
Glu

acids. The repressors specifically inhibited nitrate assimilation when nitrate was used as the nitrogen source (9). This causes a decrease in the reduced nitrogen entering the system, thereby starving the cells. Consequently, their overall growth was inhibited. If urea or γ-aminobutyric acid were used as alternate nitrogen sources, the repressors did not inhibit growth. The repressors were therefore specifically inhibiting the nitrate pathway. By using a threonine resistant cell line, XDR^{thr}, it was found that the resistence to the growth inhibiting amino acids was due to the inhibition of nitrate uptake by these amino acids. Lysine and arginine could also overcome this repression. Using corn seedlings, Schrader and Hageman (38) have shown that possible end products of nitrate reduction such as nitrite, ammonia, proline, glutamine, L-asparagine and L-serine did not inhibit nitrate reductase activity or synthesis.

Using corn root tips, Srivastava (50) has recently shown that arginine and lysine inhibited the induction of nitrate reductase by 39% and 49% respectively. Proline, leucine, alanine, glutamate, aspartate, casamino acids ammonium and urea did not affect the induction of nitrate reductase. The derepressors of the tobacco cells, arginine and lysine, repress the nitrate reductase formation in corn root tips. The problem of amino acid regulation in the intact corn seedling is made harder by the fact that there is a readily available supply of amino acids from the corn endosperm. Srivastava (50) followed up this possibility

by using an opaque-2 mutant of corn. This mutant is deficient in leucine, alanine and proline. In order to make up for this deficiency, one might expect a greater requirement for reduced nitrogen. This might be reflected in a higher nitrate reductase activity. He found an elevated level of nitrate reductase in the opaque-2 mutant when compared to the wild type activity. These studies show that the endosperm of corn might affect the nitrate reductase activity by altering the amino acid levels.

The role of ammonia in nitrate reductase regulation varies with the organism being studied. Ammonia is an end product of the nitrate reductase pathway. It was found to repress the in vivo enzyme activity in the basidiomycete Ustilago maydis (24) and in Neurospora crassa (52). In higher plant, ammonia is generally ineffective as seen by the lack of repression in corn (38) and radish cotyledons (21). Srivastava (50) has shown that ammonia has no effect on the induction of nitrate reductase in corn root tips. Chlorella (26,45) and excised barley root tips (44) are some of the few exceptions in higher plants were ammonia represses the nitrate reductase activity. Bayley, King and Gamborg (1,2) found that soybean cells in suspension cultures required nitrate and ammonia for good growth. The nitrate reductase activity increased after the ammonia was used up. The ammonia could be replaced by L-glutamine, L-alanine, or putrescine, but a source of reduced nitrogen was required for growth and subsequent development of

nitrate reductase activity. This reduced nitrogen requirement was not found in wheat suspension cultures (1,2). Thus, the components of the nitrogen assimilatory pathway can have a very diverse effect on nitrate reductase activity.

Various other factors influenced the nitrate reductase levels. Beevers et al (4,16) found that light caused an increase in nitrate reductase in radish cotyledons. This was due to the indirect effect of light enhancing nitrate uptake. Travis et al (54,55) showed that light was required to produce an increase in the nitrate reductase activity in barley leaves. They felt that the light was required by the leaves to maintain a high level of polyribosomes, thereby maintaining an active protein synthesizing apparatus. Upcroft and Done (59) felt that light influenced the nitrate reductase activity in wheat leaves by altering the transport of nitrate to the cytoplasm, thereby inducing the enzyme.

Ries et al (34,58) found that the addition of 9.0 μM simazine, an s-triazine herbicide, to corn roots caused a ten fold increase in the nitrate reductase levels in the leaves. This occurred at sub-optimal conditions such as low temperatures (22.5°C) coupled with low nitrate levels. Total nitrogen and dry weight increased by 20 to 25% under the same conditions. Results such as these suggest that the s-triazine herbicide was overcoming some regulatory mechanism, thereby altering the enzyme level. This could be due to a faster uptake of nitrate, an altered

distribution of nitrate within the cell, alterations at the transcriptional and translational levels of the enzyme or alterations in the turnover of the enzyme. Using bush bean, Singh and Salunkhe (43) have found a 2 fold increase of nitrate reductase when using simazine, and another related s-triazine, atrazine. Simazine and atrazine vary only in one substitution. In atrazine, an isopropylamino group on the 6 position replaces the ethylamino group of simazine (Figure 3). Corn is a resistant species to these herbicides. It converts the toxic s-triazine to its non-toxic 2-hydroxyanalog (23). More recently, atrazine-glutathione complexes were found to be the most common method of detoxification in corn (41). In the following studies, atrazine was preferentially used over simazine because of its higher degree of solubility (70.0 ppm versus 5.0 ppm).

Of prime interest in this study was the effect of nitrate on the regulation of nitrate reductase in corn roots. This system involved the use of intact corn roots. Enzyme extractions were made from root tip sections (0 - 10 mm), a population of young, growing cells, and from mature root sections (25 - 35 mm back from the tip), a population of cells where growth was no longer a dominant features. Oaks, Wallace and Stevens (28) showed that the half-life of nitrate reductase from the mature root sections of corn was shorter than that of the root tip sections. Factors that control this turnover

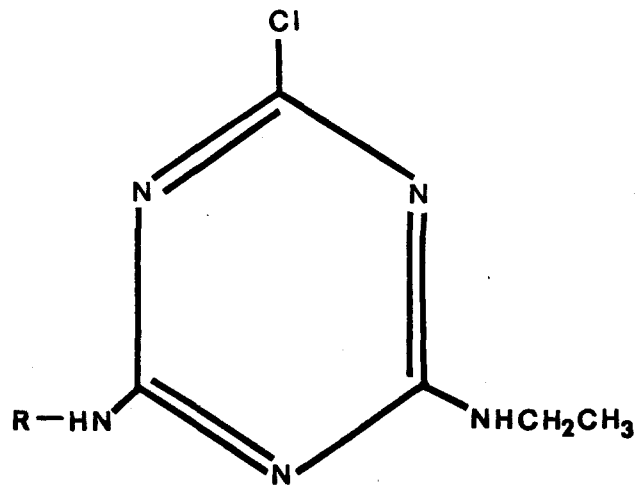


Figure 3: The Structure of the s-triazine herbicides.

R = -CH $(CH_2)_3$ for atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine)

R = $-C_2H_5$ for simazine (2-chloro-4,6-bis (ethylamino)-s-triazine)

were studied. Nitrate was required for the de novo synthesis of the enzyme. The effect of nitrate concentration on the induction kinetics of nitrate reductase was investigated. The role of nitrate in controlling the inducible levels of nitrate reductase and the in vivo stability was studied. The role of atrazine in regulating the nitrate reductase activity in corn was also studied.

CHAPTER I

MATERIALS AND METHODS

I) Seeds

Zea mays L. hybrids WF9 x 38-11, from the Agricultural Alumni Association (Lafayette, Indiana) and W64A x W182E, from the Warwick Seed Company (Blenheim, Ontario), were routinely used in the experiments. Hybrid W64A x W182E was chosen from several Warwick hybrid lines (Co 106 x Co 303, W59M x W117, W64A x W635, Warwick SL510, and A495 x Co 6124) on the basis of uniform growth patterns, straight roots and high nitrate reductase activity.

II) Growth Conditions

The corn seeds were surface sterilized for twenty seconds with Javex, a commercial bleach containing 6.00% sodium hypochlorite. They were then thoroughly rinsed with several changes of sterile distilled water. The seeds were planted embryo side down, with 18 seeds per petri plate. The plates were 9.0 cm in diameter and contained 50 to 60 ml of agar. The plates were placed in a tray covered by aluminum foil, and incubated at 26°C in the dark. Occasionally, the seeds were germinated on 0.9% agar, to which the desired concentration of nitrate had been added. For the induction experiments, seedlings were grown on sterile 0.9% agar (Difco Bacto-agar) at pH 5.8. The agar contained 1/10 strength Hoagland's salt solution (see appendix 1), a supplement of $Na_2MoO_4.2H_2O$ (0.207 μM), but no nitrate.

The roots of the seedlings remained straight and grew along the surface of the agar. A two to three fold excess of seeds were planted to insure that there would be an ample choice of seedlings. The seedlings were used when the roots were approximately 4.5 to 5.0 cm long. At this point, which required about 48 to 50 hours for hybrid WF9 x 38-11 and about 64 to 68 hours for hybrid W64A x W182E, the coleoptiles were just emerging.

When leaves were required, the seeds were grown in 18 x 11 cm trays containing a vermiculite-sand mixture. They were placed in a growth chamber at 26.5°C for a period of 7 to 10 days. The light regime was 18 hours light, 6 hours dark. They were watered daily, using the solutions indicated in the various figures and tables.

III) Nitrate Reductase Induction System

Originally, the induction experiments or pre-treatments were carried out according to the method of Oaks, Wallace and Stevens (28). The induction solution contained double distilled water, 1/10 strength Hoagland's salts with a molybdenum supplement (0.207 μM) and the treatment compound. Nitrate was omitted from the Hoagland's salt solution. The final pH was adjusted to 5.8. Originally, the seedlings were placed in 14 cm diameter petri plates and incubated for the specified times at 26°C in the dark. If a pre-treatment was involved, the roots were bathed in the pre-treatment solution, rinsed individually in double distilled water, and transferred to fresh medium for the

induction treatment. More recently, a modification of this method has been used. A 250 ml Pyrex beaker, containing a final volume of 230 ml of induction solution and topped with a plastic matting consisting of 0.75 x 0.75 cm openings, was used. The roots pass through the matting into the induction medium (Figure 4). The system was aerated. Up to fifty intact seedlings could be handled per treatment. Appropriate inducers or inhibitors could be added as required. This system made the transfer to various induction treatments more manageable and minimized handling of the roots.

IV) Nitrate Reductase Extraction

The enzyme nitrate reductase (NADH:nitrate oxidoreductase, E.C. 1.6.6.1) was extracted from either corn roots, or corn leaves. The root extractions were prepared from the 0 to 10 mm root tip region and from the mature root sections, which were taken from a region 25 to 35 mm back from the tip. Forty to fifty sections were routinely used per treatment. Immediately after harvesting, the samples were frozen in liquid nitrogen, weighed and stored overnight at -20°C. Leaves were designated as to their sequence of emergence (ie. primary leaf, second leaf) and to the number of days of germination. For the leaf extractions, ten representative leaves were chosen, frozen in liquid nitrogen and ground to a fine powder. From this sample, one gram of powder was actually extracted.

In the early experiments, the extracting buffer contained phosphate buffer (0.05 M, at pH 7.8), EDTA (5.0×10^{-4}M) and cysteine

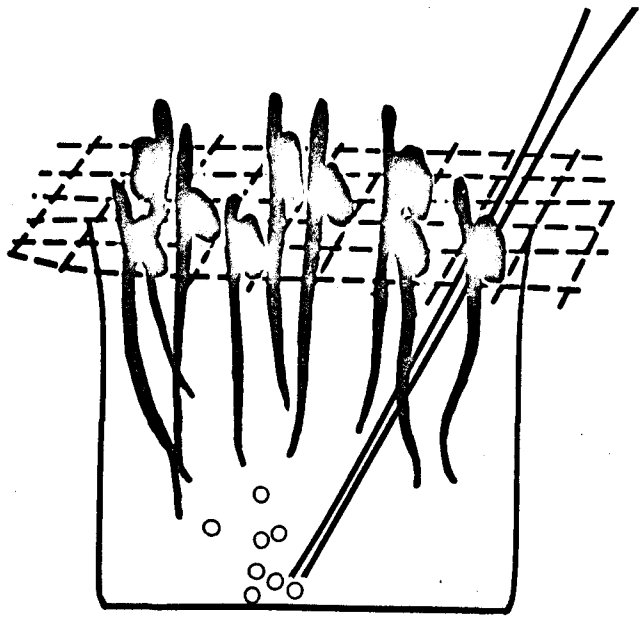


Figure 4: The Induction Apparatus.

Figure 4 shows the induction system. The induction apparatus consisted of a 250 ml Pyrex beaker which supported a plastic matting that had 0.75 x 0.75 cm openings. The roots passed through this matting into the induction solution. A final volume of 230 ml was used for each treatment solution. The system was aerated by passing air through a pasteur pipette that had been placed in the beaker. Up to fifty intact seedlings could be handled per treatment. The system was placed in an incubator, and kept in the dark. The temperature was maintained at 26°C.

(5.0×10^{-3}M). This will be designated Extraction Method A. Recently, the phosphate buffer was replaced by Hepes buffer (0.10M) at pH 7.4. This method will be called Extraction Method B. For each 1.0 gram fresh weight of plant material extracted, 4.0 ml of buffer was used. The samples were hand ground in a cold mortar and pestle. The extract was centrifuged at 30,900 x g for 30 minutes in a Sorval RC-2 centrifuge. The resultant supernatant was used for the assay.

The degree of variability in various nitrate reductase extractions was measured. In these experiments, the enzyme activities of tissue extracted and assayed just after freezing or two days later were measured (Table I). The seedlings of hybrid WF9 x 38-11 were grown for 3 days at 26°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution with a molybdenum supplement (0.207 μM) and a final nitrate concentration of 10.0 mM. The nitrate reductase values of tissue freshly assayed or assayed after being frozen for two days varied less than 4% from their mean values. This indicates that the extraction technique is reliable. In addition, the tissue can be frozen and stored for at least two days without loss in the extractable nitrate reductase.

V) Nitrate Reductase Assay System

Nitrate reductase activity was determined by measuring the production of nitrite. The assay was carried out according to the following protocol. Assay Method A included 110.0 μmoles phosphate

TABLE I. RELIABILITY OF EXTRACTION OF NITRATE REDUCTASE

Treatment	Fresh Weight (gm)	Absorbance (540 nm)
fresh	0.413	0.402
2 day frozen	0.420	0.428
2 day frozen	0.410	0.417

The seedlings of hybrid WF9 x 38-11 were grown for 3 days at 26°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution with a molybdenum (0.207 μM) and a final nitrate concentration of 10.0 mM. Forty root tips were used per extraction. Extraction Method A and Assay Method A, which did not include malic dehydrogenase, were used. Each assay contained 0.3 ml of extract.

buffer at pH 7.3 or pH 7.8, 1.12 µmoles NADH (freshly prepared), 20.0 µmoles KNO_3, and extract, all to a total volume of 1.5 ml. Assay Method B consisted of 16.0 µmoles KH_2PO_4, 0.564 µmoles NAD (fresh), 20.0 µmoles KNO_3, 130 µmoles Hepes buffer at pH 7.0 and extract, giving a total volume of 1.5 ml. The reaction was started by the addition of the extract and was routinely run at 28°C in a waterbath. The assay time was usually 30 minutes. The reaction was stopped by the addition of 0.1 ml lots of malic dehydrogenase and oxalacetate. This consisted of 10.0 µmoles of oxalacetic acid plus an aliquot of malic dehydrogenase that could completely oxidize all the exogenously added NADH in a 2 to 3 minute period.

To determine the amount of nitrite produced in the assay, the Griess-Ilosvay method (Hewitt and Nicholas, 1964) was used. This method involves the diazotisation of an aromatic amino compound (ie. sulfanilamide) by nitrite in an acid solution, and then coupling a suitable reagent N-(1-naphthyl)-ethylenediamine dihydrochloride (NED) to produce a colour (red). Thus, in each assay, 1.0 ml of 1.0% sulfanilamide in 1.0 N HCl was added, followed by the immediate addition of 1.0 ml of 0.2% NED. The reagents were thoroughly mixed. After 30 minutes, the absorbancy was read at 540 nm.

Results were routinely calculated as nmoles of nitrite formed per hour per milligram protein. Proteins were determined by the Lowry method (27) using bovine serum albumin as a standard. A unit of enzyme activity is defined as 1 nmole of nitrite formed per hour.

VI) Malic Dehydorgenase Extraction

Malic dehydrogenase was extracted from corn leaves of seedlings which had been grown for 7 to 10 days. After collection, the leaves were frozen in liquid nitrogen, and ground to a fine powder in a cold mortar and pestle. The samples were extracted with phosphate buffer (0.10 M, pH 7.0). A 1:2 fresh weight to volume of extraction medium was used. This extract was centrifuged for 15 minutes at 12,000 x g in a Sorval RC-2. The resultant supernatant was made up to a 33% ammonium sulphate solution, placed on ice for 20 minutes, and stirred using a magnetic stirrer. It was then centrifuged for another twenty minutes at 12,000 x g. The resultant solution was made up to a 66% ammonium sulphate solution, and placed on ice for twenty minutes. Upon re-centrifugation (12,000 x g for twenty minutes), the resultant precipitate was redissolved in a minimum amount of 0.1 M phosphate buffer (pH 7.0). This sample was run through a Sephadex G-25 (coarse) column, using 0.1 M phosphate buffer at pH 7.0 as the eluant. Alternately, it was dialyzed against the buffer. These last two procedures were carried out in a cold room at 2 to 5°C. The malic dehydrogenase activity was determined by measuring the oxidation of NADH. As the NADH becomes oxidized, there is a decrease in the absorbance peak at 340 nm. The criteria for subsequent dilutions of the malic dehydrogenase was a complete oxidation in 2 to 3 minutes of 1.12 µmoles of NADH. This was the amount of NADH that was routinely added to the nitrate

reductase assay. Suitable aliquots of the malic dehydrogenase extract were placed in small test tubes and stored at -20°C in the deep freeze.

VII) Materials

The reagents were obtained from the following sources: bovine serum albumin, cysteine, NADH, oxalacetic acid cycloheximide and sulfanilamide were obtained from Sigma Chemical Co., St. Louis, Missouri; Hepes buffer was from either Sigma or Calbiochem, San Diego, California, atrazine was a gift of CIBA-Geigy Agricultural Chemicals, Ardsley, New York; N-(1-naphthyl)-ethylene-diamine dihydrochloride and all inorganic chemicals were of reagent grade and were supplied by Fisher Scientific Co., Fairlawn, New Jersey.

CHAPTER II

RESULTS

I) The Nitrate Reductase Levels of Various Corn Hybrid Lines

Five hybrid lines of corn (Co 106 x Co 303, W64A x W182E, W59M x W117, W64A x A635, A495 x Co 6124) and the inbred line Warwick SL 510 were tested for their nitrate reductase levels. They were supplied by the Warwick Seed Company, Blenheim, Ontario. Hybrid WF9 x 38-11, from the Agricultural Alumni Association, Lafayette, Indiana, was also tested. The seedlings were grown in the dark for 60 hours at 26.0°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution with a molybdenum supplement of 0.207 μM and a final nitrate concentration of 10.0 mM. Both root tip sections (0 - 10 mm) and mature root sections (25 - 35 mm from the tip) were extracted and assayed. The following criteria were looked for: a high percentage of germination, regular growth, straight roots, and a high level of nitrate reductase activity. A comparison of the growth patterns in these various hybrids is shown in Table II. The seedlings were grown until the majority of the roots were 4.0 to 5.0 cm long. This was the root length used for the induction experiments. The root lengths were graded into four categories: between 4.0 to 5.0 cm., longer than 5.0 cm., shorter than 4.0 cm., and late germinated plus ungerminated. The latter category referred to seedlings that were shorter than 1.0 cm or had not germinated. Hybrid A495 x Co 6124 showed a very uneven germination pattern,

TABLE II. A COMPARISON OF GROWTH PATTERNS IN VARIOUS CORN HYBRIDS

Strain	Time Harvested (hours)	% Between 4.0 to 5.0 cm.	% Longer than 5.0 cm.	% Shorter than 4.0 cm.	% Late Germinated and Ungerminated
W64A x W182E	53.5	42.8%	4.8%	37.3%	15.1%
W59M x W117	57.0	46.0%	14.3%	26.2%	13.6%
Warwick SL 510	56.0	38.9%	3.2%	19.0%	38.9%
Co 106 x Co 303	57.0	45.2%	8.8%	19.8%	26.2%
A 495 x Co 6124	58.0	15.9%	38.1%	39.7%	6.4%
W64A x A635	52.0	46.0%	19.8%	19.8%	14.3%
WF9 x 38-11	51.0	34.2%	0.8%	29.4%	35.7%

100% represents 126 seeds.

The seedlings were grown at 26°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution with a molybdenum supplement (0.207 μM) and a final nitrate concentration of 10.0 mM. The seedlings were graded as to their length. The late germinated and ungerminated represents seedlings that were shorter than 1.0 cm.

with 38.1% of the roots being too long, 46.1% being too short and only 15.9% being the acceptable length. Inbred line Warwick SL 510 had a high percentage of late germination and ungerminated (38.9%). Its roots were very crooked. These two lines were the only ones considered to be unsuitable for use. The strains were then assayed for their nitrate reductase levels. The results in Table III show that hybrid lines W64A x W182E and W59M x W117 best met the criteria of a high nitrate reductase level when grown on a continuous nitrate medium. All the strains tested showed a higher level of nitrate reductase in the root tip sections than in the mature root sections when enzyme activities were calculated on a units per milligram protein basis.

These two lines, W64A x W182E and W59M x W117, were tested for their levels of inducible nitrate reductase. The seedlings were dark grown for 60 hours at 26.0°C on 0.9% agar. This agar contained 1/10 strength Hoagland's salt solution, plus a molybdenum supplement (0.207 μM) but lacked nitrate. The seedlings were induced in a solution containing 1/10 strength Hoagland's salt solution, a molybdenum supplement of 0.207 μM and a final nitrate concentration of 10.0 mM. The induction times were 0.0, 0.5, 3.0 and 5.5 hours. Inductions were carried out at 26.0°C in the dark. Table IV shows the results for the root tip and mature root sections. In these experiments, the level of nitrate reductase in root tip sections of hybrid W59M x W117 rose sharply from 0.5 to 3.0 hours and then at a slower rate for the next two

TABLE III. A COMPARISON OF NITRATE REDUCTASE ACTIVITIES IN VARIOUS CORN HYBRID LINES

Strain	Tip Sections (0-10mm)		Mature Sections (25-35 mm)	
	Soluble Protein (mg/ml)	NR Activity (units/mg protein)	Soluble Protein (mg/ml)	NR Activity (units/mg protein)
W64A x W182E	2.18	187.0	1.37	61.2
W59M x W117	3.08	171.0	1.07	21.6
Warwick SL 510	3.20	159.0	1.65	35.1
Co 106 x Co 303	2.51	152.0	1.11	45.8
A 495 x Co 6124	2.50	142.0	1.10	23.4
W64A x A635	3.31	113.0	1.37	31.3
WF9 x 38-11	3.00	87.8	1.16	36.2

The seedlings were grown for 2.5 days at 26°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution with a molybdenum supplement (0.207 μM) and a final nitrate concentration of 10.0 mM. Extraction was according to Method A. Fifty seedlings were used per sample. Assay Method A, which did not include malic dehydrogenase, was used.

TABLE IV. INDUCED NITRATE REDUCTASE ACTIVITY OF TWO HYBRID LINES

Strain	Induction Time	Tip Sections (0-10mm) Soluble Protein (mg/ml)	Tip Sections (0-10mm) NR Activity (units/mg protein)	Mature Sections 25-35 mm) Soluble Protein (mg/ml)	Mature Sections 25-35 mm) NR Activity (units/mg protein)
W59M x W117	0.0	2.79	12.2	1.15	5.9
	0.5	2.75	7.6	1.12	8.8
	3.0	2.75	105.2	1.14	52.3
	5.5	2.49	141.1	1.03	89.0
W64A x W182E	0.0	3.53	12.5	1.42	3.8
	0.67	3.00	24.0	1.23	5.8
	3.0	3.14	123.0	1.13	57.8
	5.5	2.75	114.0	1.16	101.0

.....continued

TABLE IV. (continued)

The seedlings were grown for 60 hours at 26°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution plus a molybdenum supplement (0.207 μM), but minus nitrate. Fifty seedlings per treatment were induced in large petri plates containing 1/10 strength Hoagland's salt solution, a molybdenum supplement and KNO_3 to a concentration of 10.0 mM. The final pH was 5.8. This was carried out at 26°C for the specified times. Extraction Method A was followed. The extracts were assayed according to Assay Method A, omitting the malic dehydrogenase system.

hours. In the tip sections of hybrid W64A x W182E, there was an initial low level of nitrate reductase activity for the first 2/3 of an hour, followed by a rapid increase in activity between 0.67 hours to 3.0 hours. There was relatively little change in enzyme activity between 3.0 to 5.5 hours. The mature root sections of both hybrids showed a continual increase in nitrate reductase activity throughout the 5.5 hour period. The pattern of induction using hybrid W64A x W182E was similar to that reported previously by Oaks, Wallace and Stevens (28) for the hybrid WF9 x 38-11. Because of this similarity, W64A x W182E was selected as the standard laboratory hybrid. The hybrid W59M x W117 may be important in studies concerning the regulation of the steady state levels of nitrate reductase since no plateau region was reached within the 5.5 hour period.

II) Nitrate Reductase Assay Conditions

1) Factors Affecting Nitrite Colour Formation

In assaying nitrate reductase activity, the amount of nitrite present in the assay is determined colourimetrically. The presence of unoxidized NADH in the system reduces the formation of the red colour. Hewitt and Nicholas (19) attribute this loss of colour to the NADH reacting with the diazotised product of the nitrite and sulphanilimide complex. This produces a phenylhydrazine derivative which cannot couple with the naphthyl reagent, N-(naphthyl)-

ethylenediamine dihydrochloride. The effect of NADH, and its oxidized form, NAD, on nitrite colour formation was studied. The assays were carried out according to Assay Method A, with each assay containing a known amount of nitrite (30 nmoles), plus a known amount of NADH or NAD. Oxalacetate and malic dehydrogenase were omitted from the assay in order to keep the NADH in its reduced form. The results show that NAD does not have any effect on the colour formation, as seen by the constant absorbance values at 540 nm (Table V). NADH has a pronounced effect. Using the amount of NADH (1.128 μmoles) routinely added in Assay Method A, a 72% decrease in colour production would be seen if none of the exogenously added NADH was oxidized to NAD.

This system was explored further by investigating the effects of the components and the products of the nitrate reductase assay on the nitrite colour formation. To accomplish this, various known concentrations of nitrite were used, while several other assay parameters were altered in turn. The results are presented in Figure 5. It is clear that neither double distilled water, and 0.1 M phosphate buffer, nor NAD (1.13 μmoles) plus oxalacetate (10.0 μmoles) and malic dehydrogenase (.1 ml) interfered with the nitrite colour formation. When 1.13 μmoles of NADH were added to the reaction, there was a 65% decrease in nitrite colour formation. This was the amount of NADH routinely added to each assay. In order to decrease this colour loss, oxidation of the NADH to NAD,

TABLE V. THE EFFECT OF NADH AND NAD CONCENTRATIONS ON NITRITE COLOUR FORMATION.

[NADH] or [NAD] (μmoles)	Absorbance (540 nm) Using NADH	Using NAD
0.00	0.545	0.535
0.282	0.405	0.545
0.564	0.320	0.545
0.847	0.258	0.540
1.128	0.192	0.550
1.410	0.160	0.545
1.694	0.126	0.540
2.260	0.093	0.545

The assay was carried out in 0.1 M phosphate buffer at pH 7.8 and 28°C for 30 minutes. Each assay contained 30.0 nmoles of nitrite (KNO_2), plus a known amount of exogenously added NADH or NAD. Routinely, 1.128 μmoles of NADH were added to each assay.

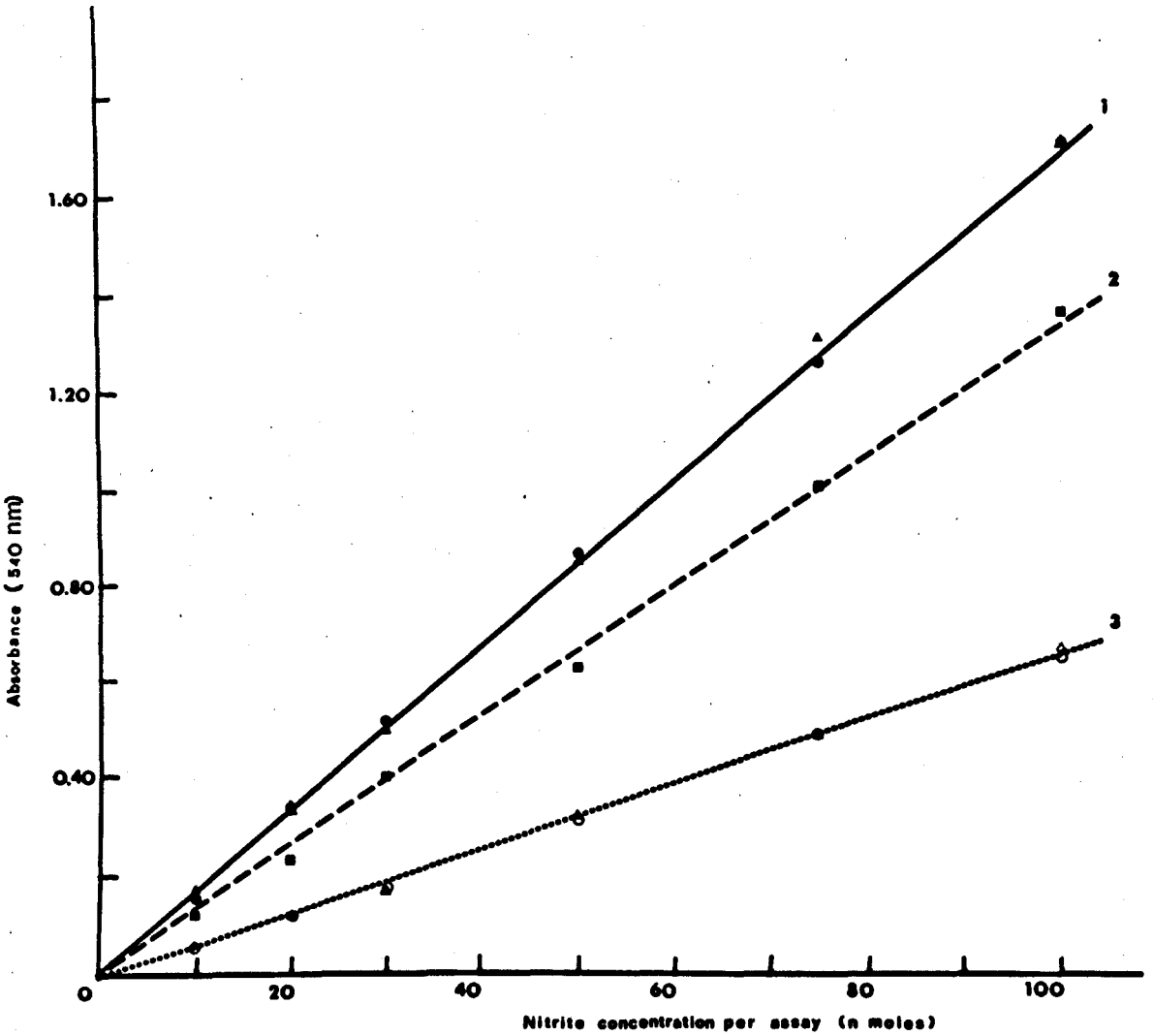
Figure 5: The Effect of the Nitrate Reductase Assay Parameters on Nitrite Colour Formation.

The effect of the nitrate reductase assay parameters on nitrite colour formation was studied. Using a known concentration of nitrite, several nitrate reductase assay parameters were altered in turn. The assay volume was made up to a constant volume of 1.5 ml by the addition of double distilled water. Then 1.0 ml of 1% sulfanilamide in 1.0N HCl, followed by 1.0 ml of 0.02% N-(1-napthyl)-ethylenediamine dihydrochloride, was added. After 30 minutes, the absorbance was read at 540 nm.

Figure 5 shows the effect of the components and the products of the nitrate reductase assay on the nitrite. colour formation.

The lines in Figure 5 represent:

1. Nitrite plus 110.0 µmoles of phosphate buffer and water (●) and nitrite plus 1.13 µmoles NAD, malic dehydrogenase and 10.0 µmoles oxalacetic acid (▲).
2. Nitrite plus 1.13 µmoles NADH, malic dehydrogenase and 10.0 µmoles oxalacetic acid (■).
3. Nitrite plus 1.13 µmoles NADH (O) and nitrite plus 1.13 µmoles NADH and 0.2 mmoles zinc acetate (Δ).


1.60
1.20
0.80
0.40
0
Absorbance (540 nm)
20
40
60
80
100
Nitrite concentration per assay (n moles)
1
2
3

which does not interfere with the colour, was tried. Enzymatic oxidation of the NADH with acetaldehyde (35 µmoles) and commercial preparations of alcohol dehydrogenase (5 µgm) is a commonly used method (1, 19). Another similar oxidation system, employing oxalacetic acid (10.0 µmoles) and malic dehydrogenase (0.1 ml), was used here. This enzyme was extracted from corn leaves. A malic dehydrogenase concentration that could oxidize 1.13 µmoles NADH in 2 to 3 minutes was used. NADH oxidation was seen by a decrease in absorbancy at 340 nm. Addition of the malic dehydrogenase and oxalacetic acid to the NADH increased the nitrite colour formation to 80% of the maximum values found with the NAD and water controls. Ample time had been allowed for the complete oxidation of the NADH. Precipitation of extraneous material by the addition of 0.2 mmoles of zinc acetate, as prescribed by Sanderson and Cocking (35) did not prevent the colour loss, even if extract was present. Subsequently, the malic dehydrogenase and oxalacetate were added to the routine assay. Figure 5 also shows that the nitrite colour formation is linear up to a nitrite concentration of 100.0 nmoles and an absorbance (540 nm) reading of 1.70.

2) Nitrate Reductase pH Profiles

i) Assay pH

The effect of pH on the nitrate reductase activity of leaf extracts is shown in Table VI. Originally, all assays were carried out as described in Assay Method A. This method used 0.1 M phosphate

TABLE VI. THE EFFECT OF ASSAY pH ON NITRATE REDUCTASE ACTIVITY OF LEAF EXTRACTS.

pH	Absorbance (540 nm) Phosphate Buffer (0.1 M)	Hepes Buffer (0.1 M)
6.8	0.353	0.435
7.0	0.350	0.614
7.2	0.340	0.523
7.4	0.518	0.543
7.6	0.515	0.536
7.8	0.293	0.422
8.0	0.336	0.291
8.2	0.162	0.195

Second leaves of hybrid W64A x W182E seedlings grown for 8 days at 26.5°C (18 hours light, 6 hours dark) were used. The seedlings had been watered with 1/12 strength Hoagland's salt solution. Extraction Methods A and B were used. Each assay contained 0.05 ml of extract. Assay Method A was used. Hepes buffer (0.1 M) was substituted for the phosphate buffer.

buffer at pH 7.8 and was essentially the method of Hageman and Flesher (1960). In order to test the pH dependence, both Hepes buffer (Good et al, 1966) and phosphate buffer were employed. Hepes buffer has a pK_a of 7.55 (at 20.0°C) and is therefore in a useful range for the nitrate reductase assay. Extracts were made from second leaves of hybrid W64A x W182E seedlings grown for 8 days at 26.5°C. A light regime of 8 hours light followed by 6 hours darkness was used. These seedlings had been watered with 1/2 strength Hoagland's salt solution. After extraction according to Methods A or B, they were assayed according to Assay Method A. Where appropriate, 0.1 M Hepes buffer was substituted for 0.1 M phosphate buffer in the assay. With 0.1 M phosphate buffer, the pH optimum of the leaf extracts was between pH 7.4 to 7.6 (Table VI). With Hepes buffer (0.1 M), a pH peak ranging from pH 7.0 to pH 7.6 was obtained. Similar results were seen with extracts from the primary leaves of seven and eight day old seedlings and from the second emerging leaves of eight, nine and ten day old seedlings.

To study the nitrate reductase activity in root sections, hybrid W64A x W182E seedlings were dark grown for 70 hours at 26.0°C on 0.9% agar. These seedlings were grown under continuous nitrate conditions in which the agar contained 1/10 strength Hoagland's salt solution, a molybdenum supplement of 0.207 μM and a final nitrate concentration of 5.0 mM. In root sections, the pH peak found using the 0.1 M phosphate buffer was between pH 7.0 and pH 7.2 for extracts of

root tip sections, and between pH 7.0 and pH 7.6 for extracts from the mature root sections (Table VIIa and VIIb). With Hepes buffer, the pH range was from pH 6.8 to pH 7.2 for the tip section extracts and from pH 6.8 to pH 7.6 for the mature section extracts. Extracts from leaf and root tip sections extracted and assayed with Hepes buffer exhibited a slightly higher activity (12 to 13%) than those prepared with phosphate buffer. With Hepes buffer, there was a two fold increase in the nitrate reductase activity found in the extracts from mature root sections. Because of the broadness of the assay peaks using Hepes buffer, and the inhibitory action of phosphate on nitrate reductase activity (as will be shown in Table XI), 0.1 M Hepes buffer at pH 7.0 was chosen as the routine assay buffer. This pH value of 7.0 was in the middle of the pH peak exhibited by the root tip extracts, and within the range of the broad peak exhibited by the mature root extracts.

ii) Extraction pH

Using the same buffer system as described in the new assay system, root sections were extracted using a wide pH range of Hepes buffer (0.1 M). The extraction buffer included EDTA (5.0×10^{-4} M) and cysteine (5.0×10^{-3} M). The results in Tables VIIIa and VIIIb indicate that the samples were most active when extracted between pH 7.4 to pH 7.8 for the root tip sections, and between pH 7.0 to pH 7.8 for the mature root sections. A two fold increase in enzyme

TABLE VIIa. THE EFFECT OF ASSAY pH ON NITRATE REDUCTASE ACTIVITY OF ROOT TIP SECTIONS.

Assay pH	Absorbance (540 nm) Extracted and Assayed with Phosphate Buffer (0.1 M)	Extracted and Assayed with Hepes Buffer (0.1 M)
6.8	0.106	0.168
6.9	-	0.206
7.0	0.115	0.213
7.1	-	0.182
7.2	0.169	0.188
7.4	0.132	0.136
7.6	0.115	0.147
7.8	0.051	0.100
8.0	0.027	0.056
8.2	0.033	0.044

Hybrid W64A x W182E seedlings were grown for 70 hours at 26°C on 0.9% agar. This agar contained a 1/10 strength Hoagland's salt solution, with a molybdenum supplement and a nitrate concentration of 5.0 mM. Tip sections (0-10 mm) were used. They were extracted with either phosphate or Hepes buffer. The assays were carried out using 0.05 ml of extract per assay. Assay Method A was used, with 0.1 M Hepes being substituted for the phosphate buffer.

TABLE VIIb. THE EFFECT OF ASSAY pH ON NITRATE REDUCTASE ACTIVITY OF MATURE ROOT SECTIONS.

Assay pH	Absorbance (540 nm) Extracted and Assayed with Phosphate Buffer (0.1 M)	Extracted and Assayed with Hepes Buffer (0.1 M)
6.8	0.104	0.274
7.0	0.112	0.251
7.2	0.140	0.280
7.4	0.120	0.251
7.6	0.120	0.237
7.8	0.080	0.205
8.0	0.046	0.163
8.2	0.040	0.133

Experimental conditions were described in Table VIIa. With extracts from mature root sections (25-35 mm), 0.2 ml of extract was used per assay.

TABLE VIIIa. THE EFFECT OF pH ON EXTRACTION OF ROOT TIP SECTIONS.

Extraction pH (0.1 M Hepes)	Fresh Weight (gm)	Soluble Protein (mg/ml)	NR Activity (units/mg protein)
6.0	0.39	1.54	112.0
6.2	0.41	1.41	167.5
6.4	0.36	1.60	214.0
6.6	0.39	1.77	265.5
6.8	0.34	1.92	431.0
7.0	0.38	1.74	442.0
7.2	0.39	1.82	418.0
7.4	0.35	1.87	602.0
7.6	0.33	1.97	545.0
7.8	0.38	1.75	567.0
7.8 (0.05 M phosphate)	0.36	1.36	306.0

Hybrid W64A x W182E was grown for 72 hours at 26°C on 0.9% agar. The agar included 1/10 strength Hoagland's salt solution plus a molybdenum supplement and a final nitrate concentration of 5.0 mM. Twenty-five tip sections were used per extract, and were extracted with Hepes buffer (0.1 M). The pH was adjusted as indicated. The assay was carried out using 130.0 µmoles Hepes buffer at pH 7.0, 16.0 µmoles KH_2PO_4, 0.564 µ moles NADH and 20.0 µmoles KNO_3.

TABLE VIIIb. THE EFFECT OF pH ON EXTRACTION OF MATURE ROOT SECTIONS.

Extraction pH (0.1 M Hepes)	Fresh Weight (gm)	Soluble Protein (mg ml)	NR Activity (units/mg protein)
6.0	0.49	0.783	9.5
6.2	0.51	0.665	23.9
6.4	0.42	0.945	25.8
6.6	0.46	0.810	32.6
6.8	0.45	0.945	33.0
7.0	0.50	1.020	57.7
7.2	0.51	0.828	50.6
7.4	0.53	0.940	54.8
7.6	0.50	1.020	60.4
7.8	0.47	1.036	49.1
7.8	0.54	0.921	93.7
(0.05 M phosphate)			

Experimental conditions were described in Table VIIIa.

activity was seen when Hepes buffer was used instead of phosphate buffer in the extraction. Thus, 0.1 M Hepes buffer at pH 7.4 was chosen as the standard extraction buffer. The 5.0×10^{-4} M EDTA and 5.0×10^{-3} M cysteine concentrations, as described by Oaks et al (28), were not altered in the extraction buffers.

3) The Effect of NADH, KNO_3 and KH_2PO_4 Concentrations on the Nitrate Reductase Assay System

Earlier results showed that NADH inhibited nitrite colour formation (Table V). The effect of NADH concentration on the enzyme activity was studied. The assay system was composed of 20.0 µmoles KNO_3, 130.0 µmoles Hepes buffer at pH 7.0 and various NADH concentrations, as indicated in Table IX. Oxalacetic acid (10.0 µmoles) and malic dehydrogenase, at a concentration that was capable of oxidizing all the exogenously added NADH within 2 to 3 minutes, were added after each assay was completed. The addition of exogenous NADH was required in the in vitro assay system since no enzyme activity was seen in its absence. The enzyme activities remained constant over a five fold range of NADH from 0.452 µmoles to 2.258 µmoles. Assay Method A employed 1.130 µmoles of NADH. This concentration was found to be unnecessarily high in order to maintain a maximum enzyme activity. The concentration was halved to 0.564 µmoles of NADH in Assay Method B.

The effect of nitrate concentration is shown in Table X. The Extracts were made using a 1:1 ratio of fresh weight to volume

TABLE IX. THE EFFECT OF NADH CONCENTRATION ON THE NITRATE REDUCTASE ASSAY.

[NADH] (µmoles)	Absorbance (540 nm)
0.00	0.000
0.226	0.293
0.452	0.328
0.677	0.347
0.940	0.335
1.130	0.329
1.355	0.338
1.580	0.319
1.806	0.326
2.032	0.330
2.258	0.313

Hybrid W64A x W182E was grown for 68 hours at 26°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution with a molybdenum supplement and a final nitrate concentration of 5.0 mM. Hepes buffer, pH 7.4, was used in the extraction. 0.1 ml of enzyme extract of the tip sections was used. The assay included KNO_3 (20.0 µmoles) and Hepes buffer (130.0 µmoles, pH 7.0).

TABLE X. THE EFFECT OF NITRATE CONCENTRATION ON THE NITRATE REDUCTASE ASSAY.

$[KNO_3]$ (nmoles)	Enzyme Activity (units/mg protein)
0.0	9.5
20.0	36.5
40.0	61.1
60.0	77.8
80.0	84.2
100.0	97.0
150.0	136.6
200.0	147.6
300.0	179.4
400.0	208.6
500.0	215.8

Second leaves of hybrid W64A x W182E seedlings grown for 8 days at 26.5°C (18 hours light, 6 hours dark) were used. The seedlings had been watered with a 1/2 strength Hoadland's salt solution. Extraction Method A was used. The sample was run through a Sephadex G-25 (coarse) column using Extraction Solution A as eluant, and maintaining the system at 2-5°C. Assay Method A was employed, using 0.2 ml of the resultant extract and the appropriate concentration of KNO_3.

of Extraction Medium A. Since the extracts were found to contain a large amount of endogenous nitrate, it was necessary to de-ionize the extracts by passing them through a Sephadex G-25 (coarse) column. The eluant and extraction buffer were identical. They contained phosphate buffer (0.1 M, pH 7.8), EDTA (5.0×10^{-4} M) and cysteine (5.0×10^{-3} M). The enzyme activity increased with increasing nitrate concentrations up to levels of 400 to 500 nmoles of nitrate. In Assay Methods A and B, 20.0 µmoles of KNO_3 were added to the assay. According to the above results this concentration saturated the system.

Nitrate was added to the assays in the form of its potassium salt, KNO_3. The effect of potassium on the assay was studied. Additions of levels of potassium of up to 60.0 µmoles KCl (ie. a three fold higher level than normally used) to the assay mixtures showed that potassium did not interfere with the enzyme activity.

Previously, it was noted that higher enzyme activities were found when the assays and extractions were done using Hepes buffer rather than phosphate buffer. Further studies on the effect of phosphate concentration on the enzyme assay system are shown in Table XI. The assay included 130.0 µmoles of Hepes buffer (pH 7.0), 20.0 µmoles KNO_3, 1.12 µmoles NADH, and extract, plus the designated phosphate concentration. Phosphate concentrations greater than 40.0 µmoles inhibited the enzyme activity. Phosphate levels between 4.0 to 40.0 µmoles saturated the enzyme system, giving a maximum

TABLE XI. THE EFFECT OF KH_2PO_4 CONCENTRATION ON THE NITRATE REDUCTASE ASSAY.

[Phosphate] (μmoles)	Absorbance (540 nm)
0.0	0.361
4.0	0.427
8.0	0.435
10.0	0.444
16.0	0.447
20.0	0.438
30.0	0.419
40.0	0.409
60.0	0.367
80.0	0.323
100.0	0.284
120.0	0.255
140.0	0.232
160.0	0.205
180.0	0.193
200.0	0.171

.....continued

TABLE XI. (continued)

Hybrid W64A x W182E was grown for 68 hours at 26°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution with a molybdenum supplement, and a final nitrate concentration of 5.0 mM. Method B was used for the extraction. Assay Method B, with the designated phosphate concentration was used. Each assay included 1.128 μmoles NADH. Each assay contained 0.1 ml of tip extract. The nitrate reductase activity was measured by the absorbance at 540 nm.

activity . In Assay System A, 110.0 µmoles of phosphate buffer was used. This amount of phosphate was found to inhibit the in vitro assay by one third. Potassium did not alter the enzyme activity as previously shown. Because of these results, a level of 16.0 µmoles of phosphate was routinely added to each assay.

As a result of these tests, the assay method was altered to include 16.0 µmoles KH_2PO_4, 0.564 µmoles NADH (fresh), 20.0 µmoles KNO_3, 130.0 µmoles Hepes buffer at pH 7.0 and extract. The final volume was 1.5 ml. This altered method was called Assay Method B.

4) The Effect of Assay Times and Enzyme Concentration on the Nitrate Reductase Assay

The linearity of the in vitro nitrate reductase assay was tested using the two assay systems. Assay Method A consists of 110.0 µmoles phosphate buffer (pH 7.3), 1.12 µmoles NADH, 20.0 µmoles KNO_3 and extract, all in a total volume of 1.5 ml. Malic dehydrogenase and oxalacetic acid (10.0 µmoles) were included in the assay system. The extract tested was prepared from root tip sections (0 - 10 mm) of seedlings of hybrid W64A x W182E. These had been grown for 68 hours at 26.0°C on 0.9% agar. The agar contained a 1/10 strength Hoagland's salt solution with a molybdenum supplement (0.207 µM), but no nitrate had been added. These seedlings were then induced for 4 hours in

a solution containing 1/10 strength Hoagland's salt solution, the molybdenum supplement, and a final nitrate concentration of 1.5 mM. Each assay contained 0.1 ml of extract. The assay was run for various times at 28.0°C. The enzyme activity was measured by the absorbance at 540 nm. As seen in Figure 6, the assay was linear from 7.5 to 70.0 minutes and from an absorbance reading of 0.09 to 0.730. This indicates that the components of Assay Method A are not limiting for at least 70 minutes and absorbance readings up to 0.730.

Assay Method B contains 16.0 µmoles KH_2PO_4, 0.564 µmoles NADH, 20.0 µmoles KNO_3, 130.0 µmoles Hepes buffer (pH 7.0) and extract, all in a final volume of 1.5 ml. Malic dehydrogenase and oxalacete (10.0 µmoles) were added to each assay. The extract tested was prepared from root tip sections of seedlings of hybrid W64A x W182E. These had been grown for 68 hours at 26.0°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution, a molybdenum supplement (0.207 µM), and a final nitrate concentration of 5.0 mM. Each assay contained 0.1 ml extract. Enzyme activity was measured by the absorbance at 540 nm. The assay temperature was maintained at 28.0°C. Figure 7 shows that the assay was linear between 7.5 and 60.0 minutes, and between absorbance readings of 0.100 to 0.700. Beyond those ranges, the assay was no longer linear. For both Assay Methods A and B, the assays were considered to be linear if they were carried on for less than 70 minutes and were in an absorbance

Figure 6: The Effect of Time On Nitrate Reductase Activity When Using Assay Method A.

The assays were run for various times at 28°C using Assay Method A. These conditions included 110.0 µmoles of phosphate buffer at pH 7.3, 1.12 µmoles NADH, 20.0 µmoles KNO_3 and 0.1 ml of extract, all to a total volume of 1.5 ml. Oxalacetate (10.0 µmoles) and malic dehydrogenase (0.1 ml) were added at the end of each assay. Enzyme activity was measured by the absorbance at 540 nm. The extraction buffer contained 0.05 M phosphate buffer at pH 7.8, 5.0×10^{-3} M cysteine and 5.0×10^{-4} M EDTA. The extract was made from root tip sections of hybrid W64A x W182E seedlings that had been induced in the dark for 4 hours. The temperature had been maintained at 26°C. The induction solution had included 1/10 strength Hoagland's salt solution, a molybdenum supplement (0.207 µM) and a final nitrate concentration of 1.5 mM. Prior to induction, the seedlings were dark grown for 68 hours at 28°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution, plus a molybdenum supplement (0.207 µM), but lacked nitrates.

Figure 6 shows the linear relationship between the enzyme activities and the reaction times. Assay Method A was used.

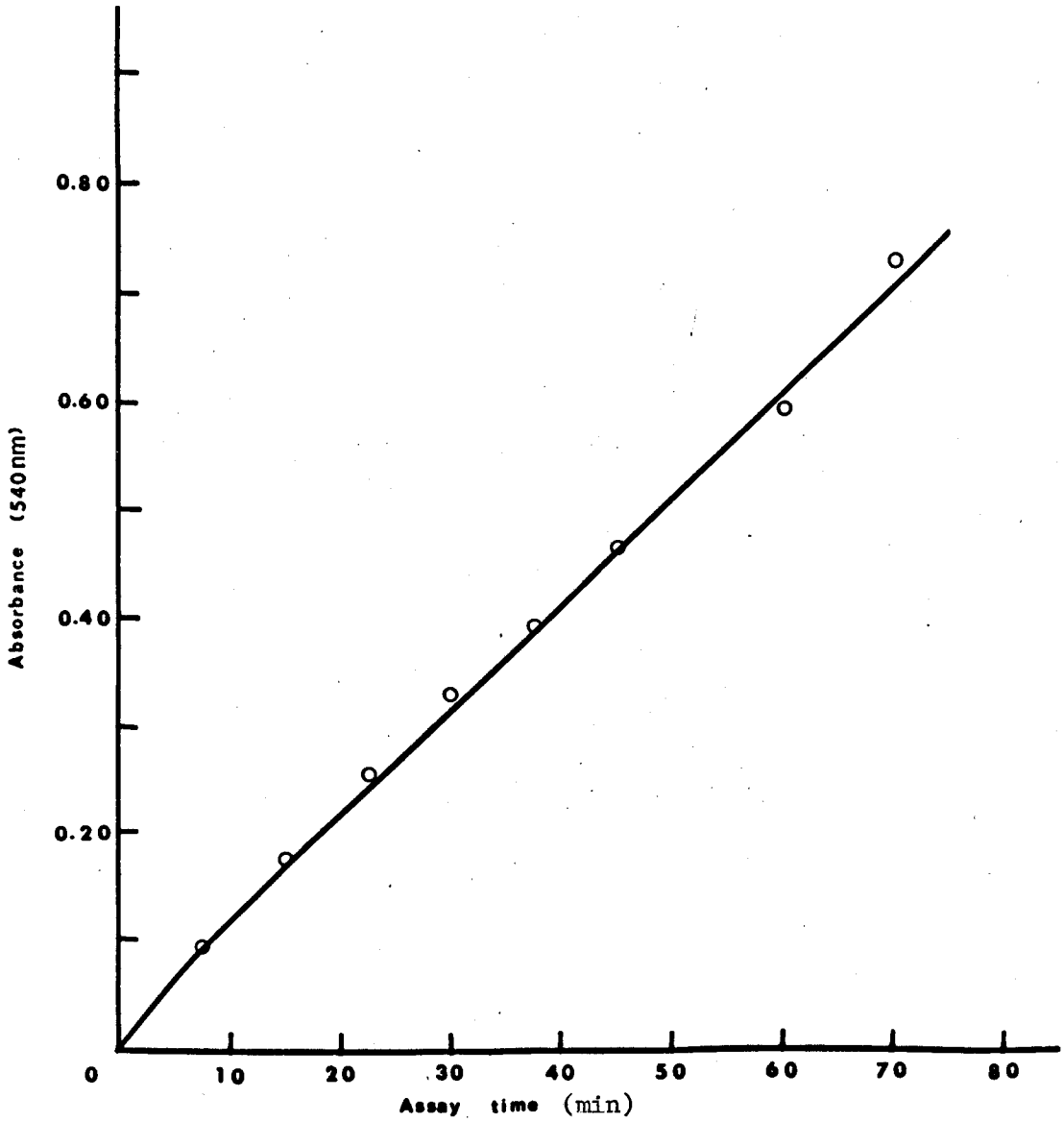
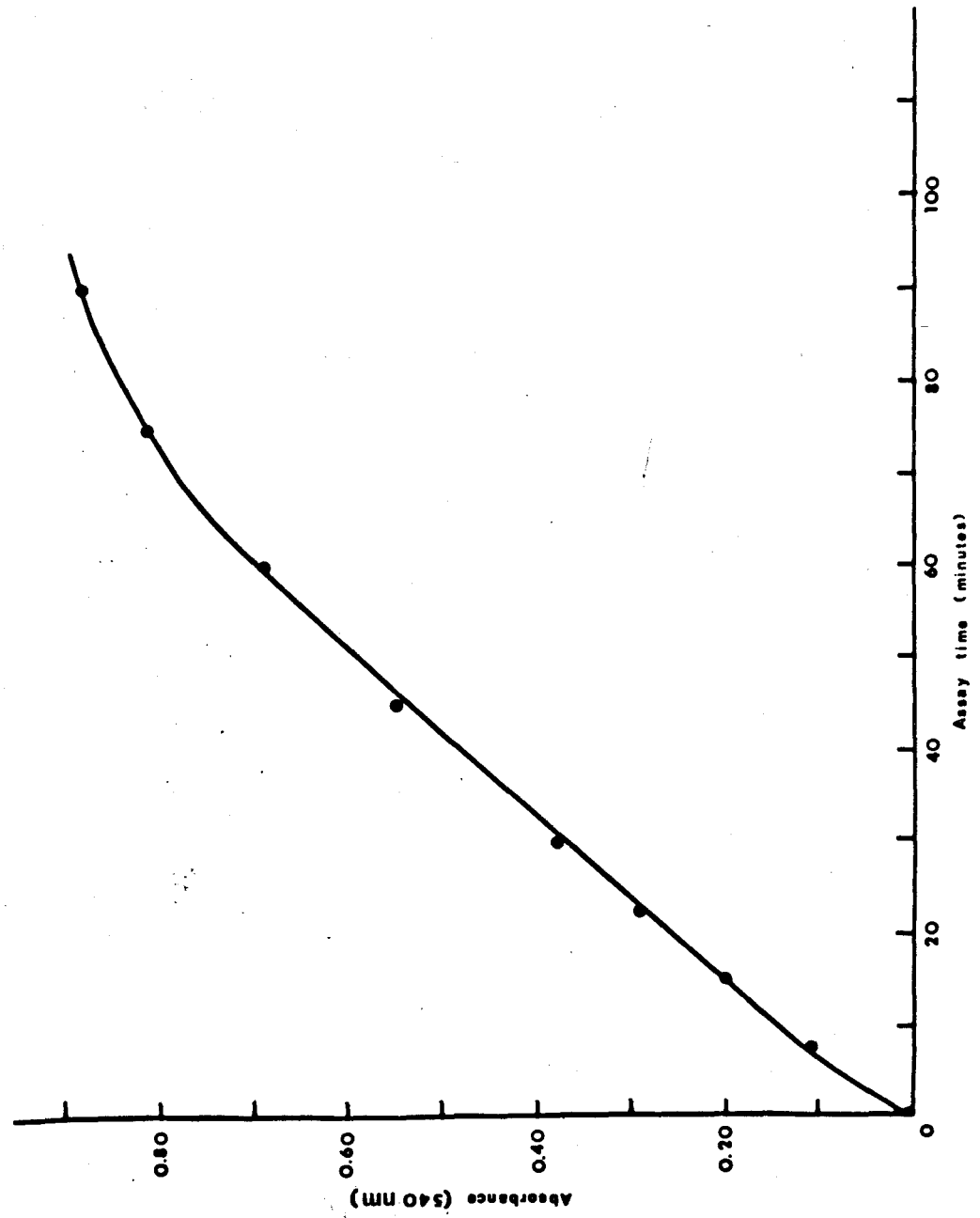

0.80
0.60
0.40
0.20
0
Absorbance (540nm)
10
20
30
40
50
60
70
80
Assay time (min)

Figure 7: The Effect of Time on Nitrate Reductase Activity When Using Assay Method B.

The assays were run for various times at 28°C using Assay Method B. This method included 16.0 µmoles KH_2PO_4, 0.564 µmoles NADH, 20.0 µmoles KNO_3, 130.0 µmoles Hepes buffer at pH 7.0 and 0.1 ml of extract, all to a total volume of 1.5 ml. Oxalacetate (10.0 µmoles) and malic dehydrogenase (0.1 ml) were added at the end of each assay. Enzyme activity was measured by the absorbance at 540 nm. The extraction buffer contained 0.1 M Hepes at pH 7.4, 5×10^{-3} M cysteine and 5.0×10^{-4} M EDTA. The extract was made from root tip sections of hybrid W64A x W182E seedlings dark grown for 68 hours at 28° on 0.9% agar. The agar contained 1/10 Hoagland's salt solution, a molybdenum supplement (0.207 µM) and a final nitrate concentration of 5.0 mM.

Figure 7 shows the linear relationship between the enzyme activities and the reaction times. Assay Method B was used.


Absorbance (540 nm)
0.80
0.60
0.40
0.20
0
20
40
60
80
100
Assay time (minutes)

range of 0.100 to 0.700.

The effect of varying the enzyme concentration on the linearity of the nitrate reductase assay was investigated. Seedlings of hybrid W64A x W182E were grown for 62 hours on 0.9% agar containing 1/10 strength Hoagland's salt solution. This included a molybdenum supplement (0.207 μM), but lacked nitrate. These seedlings were induced for periods of up to 8 hours in a solution containing 1/10 strength Hoagland's salt solution, the molybdenum supplement and a final nitrate concentration of 10.0 mM. Extracts were made from root tip samples. Methods A were used for the extraction and the assay. Malic dehydrogenase and oxalacetic acid (10.0μmoles) were added at the end of each assay to oxidize the NADH. Enzyme activity was measured by the absorbance at 540 nm. Figure 8 shows the linearity of the assay when different amounts of root tip extracts were assayed for 30 minutes at 28.0°C. These extracts were prepared after various induction times. For each extract, a linear relationship was seen between enzyme concentration and enzyme activity. This linearity indicates that the assay is reliable for enzyme additions of up to 0.3 ml. The assay system shows a linear relationship up to an absorbance reading of at least 0.780 units. Assays were routinely run in the 0.300 to 0.500 absorbance range, with the enzyme concentrations being adjusted to meet this criterion. This adjustment

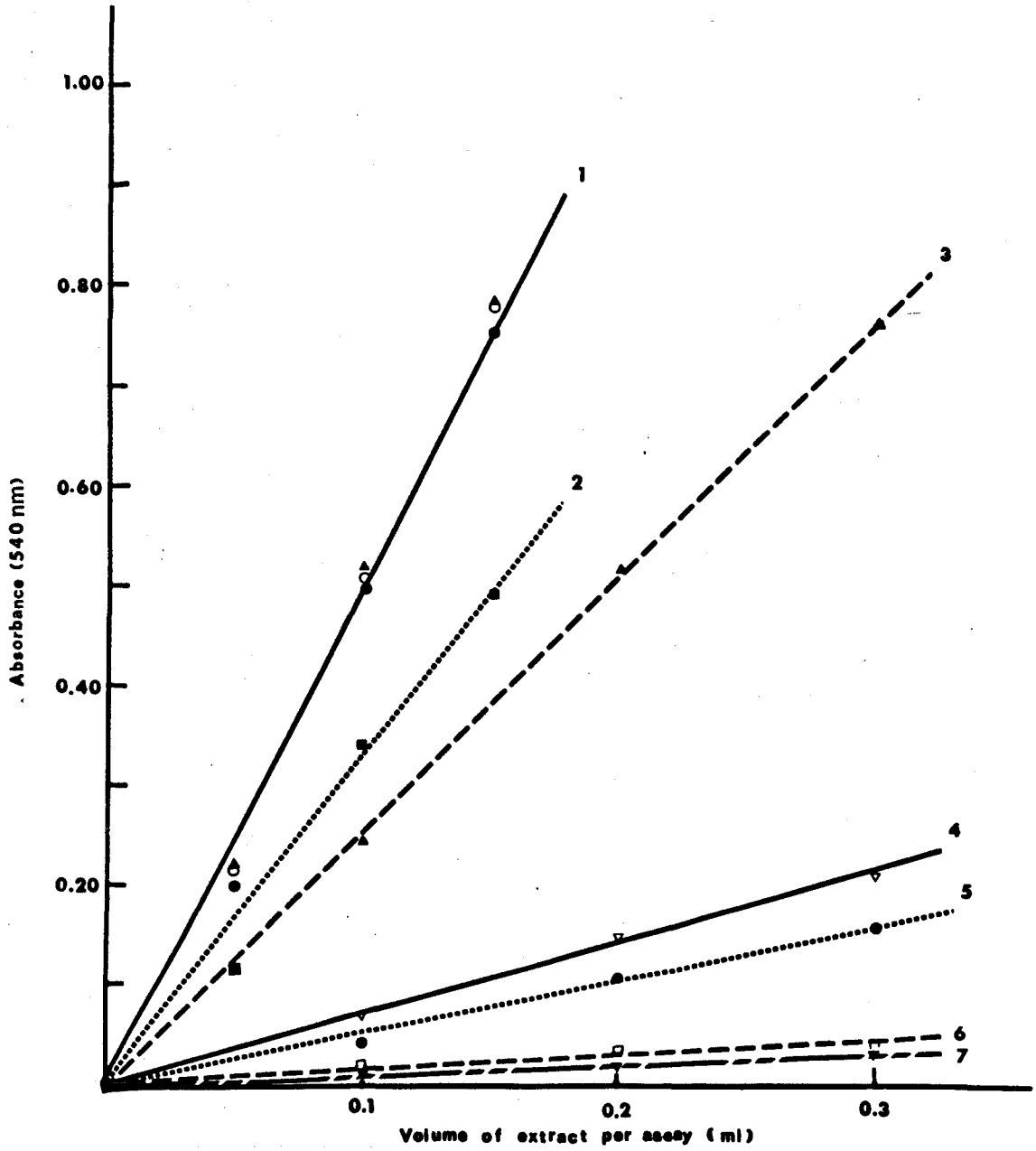
Figure 8: The Effect of Enzyme Concentration on Nitrate Reductase Activity.

Seedlings of hybrid W64A x W182E were dark grown for 68 hours at 26°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution plus a molybdenum supplement (0.207 μM), but lacked nitrate. Fifty seedlings per treatment were induced for various times in an induction solution. This contained 1/10 strength Hoagland's salt solution with a molybdenum supplement (0.207 μM), and a nitrate concentration of 10.0 mM. The incubation was carried out in the dark at 26°C. Fifty root tip sections per treatment were extracted according to Extraction Method A. Various amounts of extract were assayed for 30 minutes according to Assay Method A. Oxalacetate and malic dehydrogenase were added at the completion of each assay. Enzyme activity was measured by the absorbance at 540 nm.

Figure 8 shows the linear relationship between the enzyme activities and the amount of extract used.

The lines in Figure 8 represent the activities of the extracts of seedlings induced for:

1. 8 hours (▲), 4.7 hours (○) and 3 hours (●)
2. 2.1 hours (■)
3. 1.5 hours (▲)
4. 1.0 hours (▽)
5. 0.67 hours (●)
6. 0.42 hours (□)
7. 0.0 hours (▼)


1.00
0.80
0.60
0.40
0.20
Absorbance (540 nm)
1
2
3
4
5
6
7
0.1
0.2
0.3
Volume of extract per assay (ml)

did not alter the linearity of the system. By correcting the nitrate reductase activities on a per milligram protein basis, any measured difference in nitrate reductase activity would be due to the conditions imposed by the experiment, not on the total amounts of soluble protein (ie volume of the enzyme) added to the assay. Of interest is the fact that nothing was produced during the induction that interfered with the assay system since linearity was maintained with all induction times. This figure also indicates that the level of enzyme activity increased with an increase in induction time. Similar results were seen in the corresponding mature root section extracts of the 10.0 mM nitrate induction, and root tip and mature root section extracts of identical experiments induced with 1.0 mM and 25.0 mM nitrate.

The enzyme reactions were routinely carried out for only 30 minutes at 28.0°C. The extracts were maintained at a concentration that gave a final absorbance reading between 0.300 to 0.500. From the preceeding results, these conditions produced a linear relationship when either Assay Method A or B was used. This linearity was seen in both root tip and mature root extracts.

III) The Induction Kinetics of Nitrate Reductase

The effects of various nitrate concentrations on the induction kinetics of nitrate reductase in both root tip and

mature root sections are shown in Tables XIIa, XIIb and XIIc, and Figures 9a and 9b. Extracts were made from seedlings of hybrid W64A x W182E. These were grown for 68 hours at 26.0°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution, and a molybdenum supplement (0.207 μM), but lacked nitrate. The seedlings were induced for the appropriate times. The induction solutions included 1/10 strength Hoagland's salt solution, the molybdenum supplement and a final nitrate concentration of 0.0 mM, 1.0 mM, 10.0 mM or 25.0 mM. Nitrate reductase activity was expressed as units per milligram protein. These results show that for the root tip sections of hybrid W64A x W182E (Figure 9a), there is a time lag of 25 minutes before an increase in enzyme is seen, a period of rapid synthesis from 25 minutes to 4 hours, and a period after 4 hours when no further change is seen. Very long induction times of 24 hours show an overall decline in enzyme activity, when compared to the initial maximum peak. The seedlings induced in 0.0 mM nitrate maintained a constant low level of enzyme activity. In the tip sections, a twenty-five fold difference in nitrate concentration did not affect the times of the lag phase or period of rapid increase in enzyme activity. The only parameter changed was the final levels of activity obtained after 8 hours of induction. There were 389.0 units per mg protein for the 1.0 mM nitrate induction, 608.0 units per mg protein for the 10.0 mM nitrate induction, and 616.0 units

TABLE XIIa. INDUCTION KINETICS OF NITRATE REDUCTASE IN ROOT TIP SECTIONS (0-10 mm).

Induction Time (hours)	1.0 mM KNO_3 Induction		10.0 mM KNO_3 Induction		25.0 mM KNO_3 Induction	
	Soluble Protein (mg/ml)	NR Activity (units/mg protein)	Soluble Protein (mg/ml)	NR Activity (units/mg protein)	Soluble Protein (mg/ml)	NR Activity (units/mg protein)
0.0	1.69	22.6	2.50	52.0	2.45	50.7
0.33	1.95	46.6	2.50	50.8	1.93	47.7
0.67	2.12	50.4	2.72	62.5	2.02	74.7
1.0	2.07	69.8	2.49	97.1	2.47	70.3
1.5	2.00	117.8	2.52	132.8	2.40	146.6
2.0	2.05	202.0	2.53	226.5	2.06	285.0
3.0	2.29	368.0	2.61	397.0	2.61	373.0
4.0	2.27	318.0	2.66	528.0	2.76	559.0
8.0	2.61	389.0	2.51	608.0	2.58	616.0
24.0	2.60	220.0	2.45	408.0	2.35	360.0

.....continued

TABLE XIIa. (continued)

Hybrid W64A x W182E was grown for 68 hours at 26°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution, with a molybdenum supplement (0.207 μM). No nitrates were added. The seedlings were induced for the appropriate time. The induction solutions included 1/10 strength Hoagland's salt solution, a molybdenum supplement and a final nitrate concentration of 1.0 mM, 10.0 mM or 25.0 mM. Forty sections were used per treatment. Methods B were used for the extraction and assay. Oxalacetate and malic dehydrogenase were added at the completion of each assay. The nitrate reductase activity was calculated on a units per milligram protein basis.

TABLE XIIb. INDUCTION KINETICS OF NITRATE REDUCTASE IN MATURE ROOT SECTIONS (25-35mm).

Induction Time (hours)	1.0 mM KNO_3 Induction		10.0 mM KNO_3 Induction		25.0 mM KNO_3 Induction	
	Soluble Protein (mg/ml)	NR Activity (units/mg protein)	Soluble Protein (mg/ml)	NR Activity (units/mg protein)	Soluble Protein (mg/ml)	NR Activity (units/mg protein)
0.0	1.00	5.3	1.10	0.0	1.23	0.9
0.33	0.91	5.8	1.22	0.9	1.12	1.9
0.67	0.89	6.5	1.26	3.4	1.06	7.0
1.0	0.96	7.7	1.09	2.0	1.06	0.0
1.5	1.01	10.6	1.17	11.4	1.08	13.2
2.0	1.08	7.6	1.16	16.1	1.09	24.9
3.0	1.07	19.8	1.25	27.1	1.01	49.5
4.0	0.96	32.6	1.43	35.4	1.17	45.7
8.0	1.11	95.3	1.34	187.0	1.09	122.3
24.0	0.91	98.8	1.10	389.0	0.92	226.5

Experimental conditions were described in Table XIIa.

TABLE XIIc. NITRATE REDUCTASE INDUCTION KINETICS WHEN INDUCED UNDER ZERO NITRATE CONDITIONS

Time (hours)	Tip Sections (0 - 10 mm) Soluble Protein (mg/ml)	Tip Sections (0 - 10 mm) NR Activity (units/mg protein)	Mature Sections (25 - 35 mm) Soluble Protein (mg/ml)	Mature Sections (25 - 35 mm) NR Activity (units/mg protein)
0	2.50	52.0	1.10	0.0
4	2.36	63.3	0.987	6.4
6	2.25	60.7	0.858	7.4

The experimental conditions were described in Table XIIa. In this case, a 0.0 mM KNO_3 induction solution was used.

Figure 9a: The Induction Kinetics of Nitrate Reductase in the Root Tip Sections (0 - 10 mm) of Corn.

The experimental conditions are described in Table XIIa. Forty root tip samples were used per extract.

Figure 9a shows that the nitrate reductase activity of root tip samples increases as the time of induction is prolonged.

The induction kinetics are shown for 0.0 mM, 1.0 mM, 10.0 mM and 25.0 mM nitrate.

The lines in Figure 9a represent the activities of the extracts of seedlings induced in:

1. 25.0 mM nitrate (○)
2. 10.0 mM nitrate (□)
3. 1.0 mM nitrate (△)
4. 0.0 mM nitrate (●)

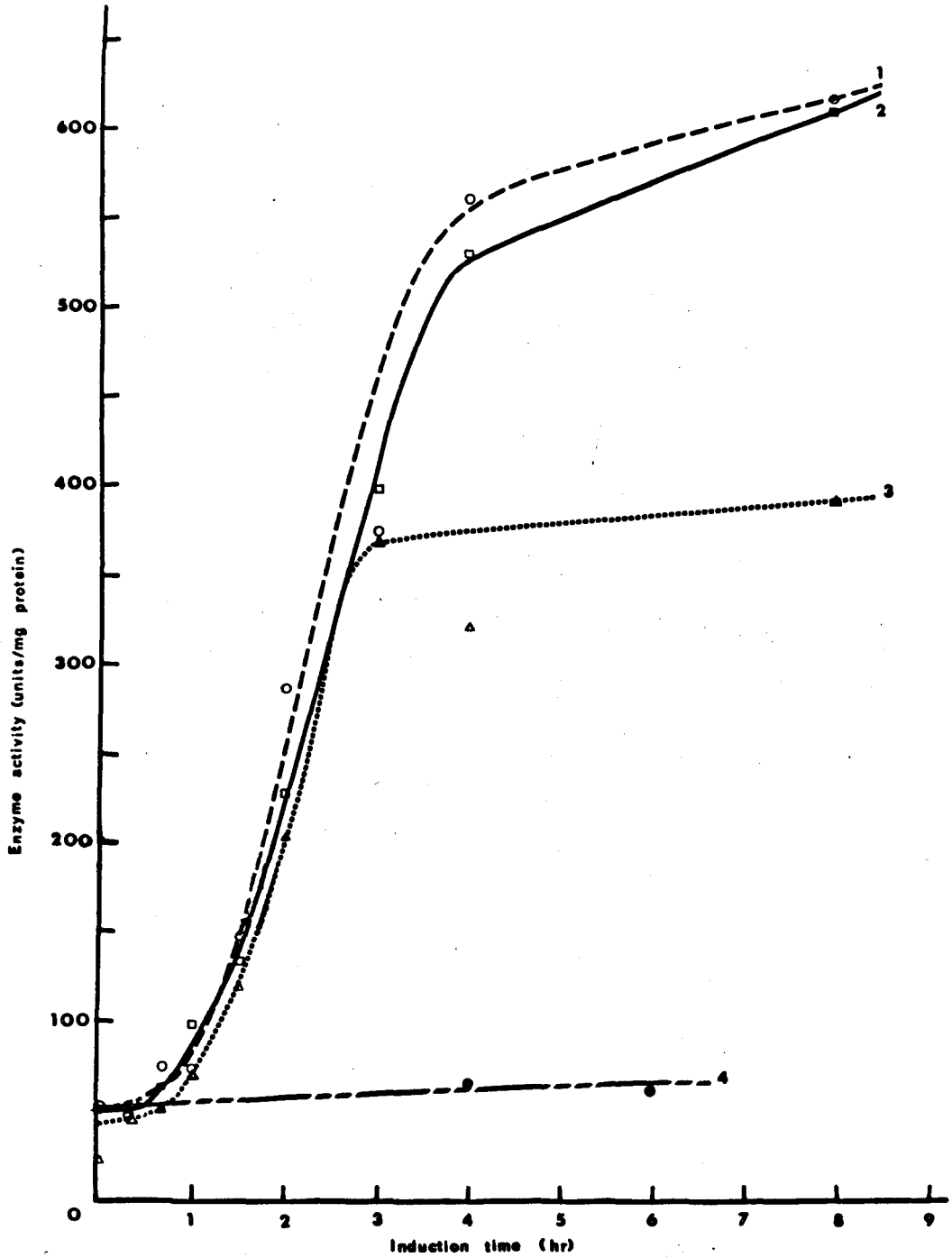

Enzyme activity (units/mg protein)
600
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
Induction time (hr)
1
2
3
4

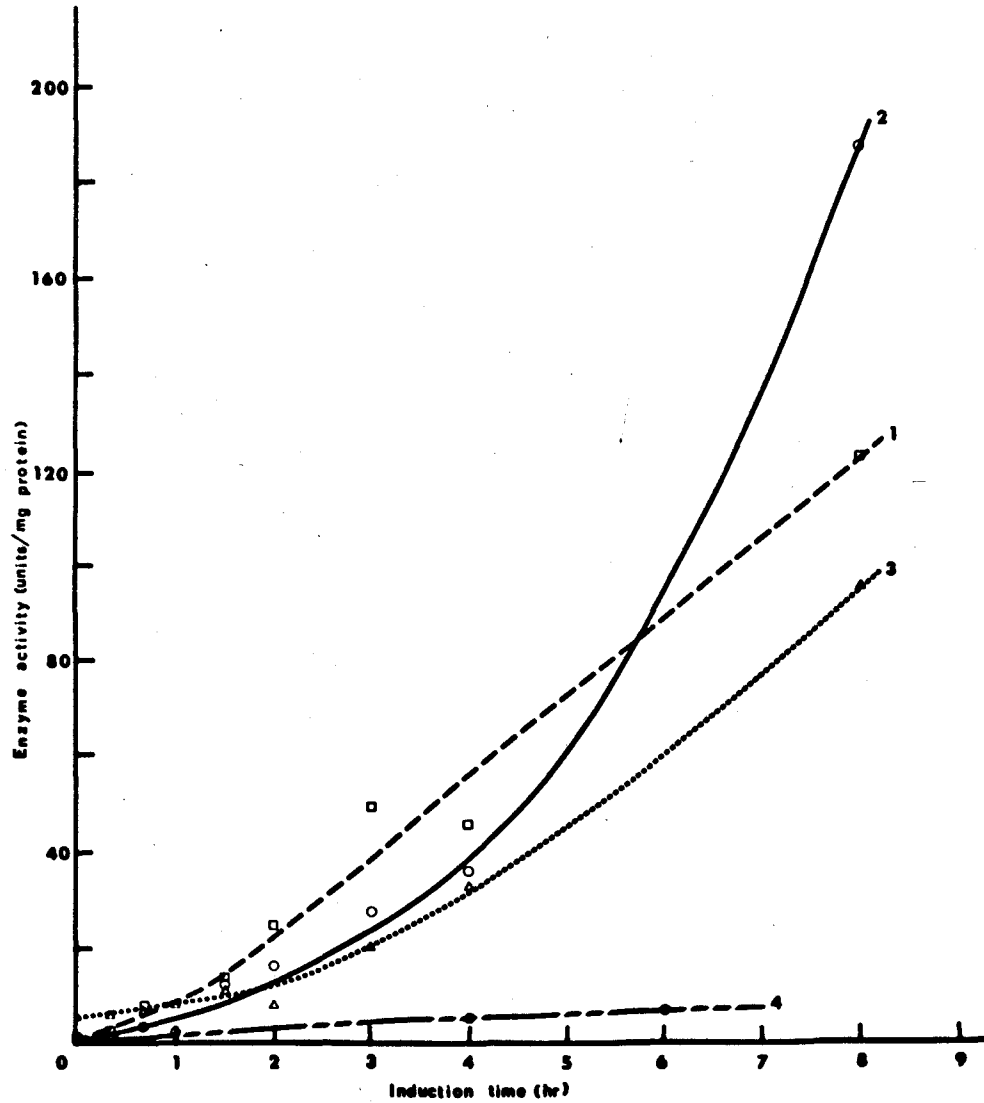
Figure 9b: The Induction Kinetics of Nitrate Reductase in the Mature Root Sections (25 - 35 mm) of Corn.

The treatment conditions, are described in Table XIIa. In this case, forty mature root sections per treatment were extracted and assayed.

Figure 9b shows that there is an increase in nitrate activity with time in the mature root sections. The induction kinetics seen with 0.0 mM, 1.0 mM, 10.0 mM and 25.0 mM nitrate are shown.

The lines in Figure 9b represent the activities of the extracts of seedlings induced in:

1. 25.0 mM nitrate (□)
2. 10.0 mM nitrate (O)
3. 1.0 mM nitrate (△)
4. 0.0 mM nitrate (●)


200
160
120
80
40
0
1
2
3
4
5
6
7
8
9
Enzyme activity (units/mg protein)
Induction time (hr)
1
2
3
4

per mg protein for the 25.0 mM nitrate induction. The 1.0 mM, 10.0 mM and 25.0 mM nitrate induction kinetics paralleled each other closely for the initial 3 hours. At this point, no further increase in enzyme activity was seen in the 1.0 mM nitrate sample. The enzyme levels found in the 10.0 mM and 25.0 mM nitrate samples continued to increase up to the fourth hour of induction. At this point, the enzyme activities were maintained at a constant level for an additional four hours. The 1.0 mM nitrate solution induced an enzyme level 37% below that maximally induced by the two higher nitrate concentrations. At all three concentrations of nitrate, the twenty-four hour induction values declined by substantially the same degree (33 to 44% below their relative 8 hour values).

As indicated in Figure 9b, the mature root sections showed no substantial change in nitrate reductase activity during the initial 1.5 hours of induction. After an induction period of 4 hours, the mature root sections had reached enzyme levels of 32.6 units per milligram protein with the 1.0 mM nitrate induction, 35.4 units per milligram protein with the 10.0 mM nitrate induction and 45.7 units per milligram protein with the 25.0 mM nitrate inductions. This was the point at which a plateau level had been reached with the root tip sections. In the mature sections there was a steady rise in enzyme activity for at least eight hours. There were no plateau regions seen. Again, the presence of nitrate was required to induce the enzyme. At

twenty-four hours, the enzyme levels have almost doubled over the 8 hour levels for both the 10.0 mM and the 25.0 mM KNO_3 induction samples. The value for the 1.0 mM KNO_3 sample remained unchanged at that time. In the mature root sections, the 10.0 mM KNO_3 induction was capable of maintaining a higher level of nitrate reductase activity at 8 and 24 hours than the 25.0 mM nitrate sample. At the induction times of 8 and 24 hours, perhaps the higher nitrate concentrations are toxic in mature root sections, but not in the actively growing root tip sections. No overt decrease in overall growth, as measured by root length, was seen.

A further analysis on how nitrate concentrations affect the plateau level of nitrate reductase was undertaken. Seedlings of W64A x W182E were grown as described in the previous section. Extracts were made from roots that had been induced for 4 hours in 1/10 strength Hoagland's salt solution, a molybdenum supplement (0.207 μM) and various nitrate concentrations. The results are shown in Tables XIIIa, XIIIb and Figure 10. In the root tip sections, the extremely low concentration of 0.1 mM nitrate induced an enzyme level four fold that of the 0.0 mM nitrate sample, but only half the value of the maximum level found with the higher nitrate concentrations. During the four hour period, the levels of induced enzyme increased as the nitrate concentration increased. The enzyme level reached a maximum when 10.0 mM KNO_3 was used in the induction solution. This level of enzyme inducibility remained constant over a further 10 fold increase in nitrate concentrations

TABLE XIIIa. THE EFFECT OF KNO_3 CONCENTRATION ON A 4 HOUR INDUCTION OF ROOT TIP SECTIONS (0 - 10 mm)

$[KNO_3]$ (mM)	Fresh Weight (mg)	Soluble Protein (mg/ml)	NR Activity (units/mg protein)
0.0	0.42	2.36	63.3
0.10	0.41	2.24	282.0
1.0	0.38	2.18	367.0
5.0	0.41	2.28	401.0
10.0	0.36	2.24	462.0
25.0	0.40	2.31	474.0
50.0	0.43	2.18	412.0
75.0	0.42	2.17	522.0
100.0	0.48	2.06	462.0

Seedlings of W64A x W182E were grown for 66 hours at 26°C on 0.9% agar. This agar contained 1/10 strength Hoagland's salt solution plus a molybdenum supplement, but minus nitrate. The seedlings were induced for 4 hours. The induction medium contained 1/10 strength Hoagland's salt solution, a molybdenum supplement, plus the appropriate nitrate concentration. Extraction and Assay Method B were used. Oxalacetate and malic dehydrogenase were added at the end of each assay. The nitrate reductase activity was calculated on a units per milligram protein basis.

TABLE XIIIb. THE EFFECT OF KNO_3 CONCENTRATION ON A 4 HOUR INDUCTION OF MATURE ROOT SECTIONS (25-35 mm)

$[KNO_3]$ (mM)	Fresh Weight (mg)	Soluble Protein (mg ml)	NR Activity (units/mg protein)
0.0	0.75	0.99	6.4
0.10	0.75	0.98	12.9
1.0	0.72	1.00	20.2
5.0	0.73	0.98	43.7
10.0	0.71	1.01	50.8
25.0	0.76	0.94	63.3
50.0	0.76	0.97	85.9
75.0	0.76	0.86	72.2
100.0	0.78	0.97	94.1

The experimental conditions were described in Table XIIIa. Mature root sections (25 - 35 mm) were used in this case.

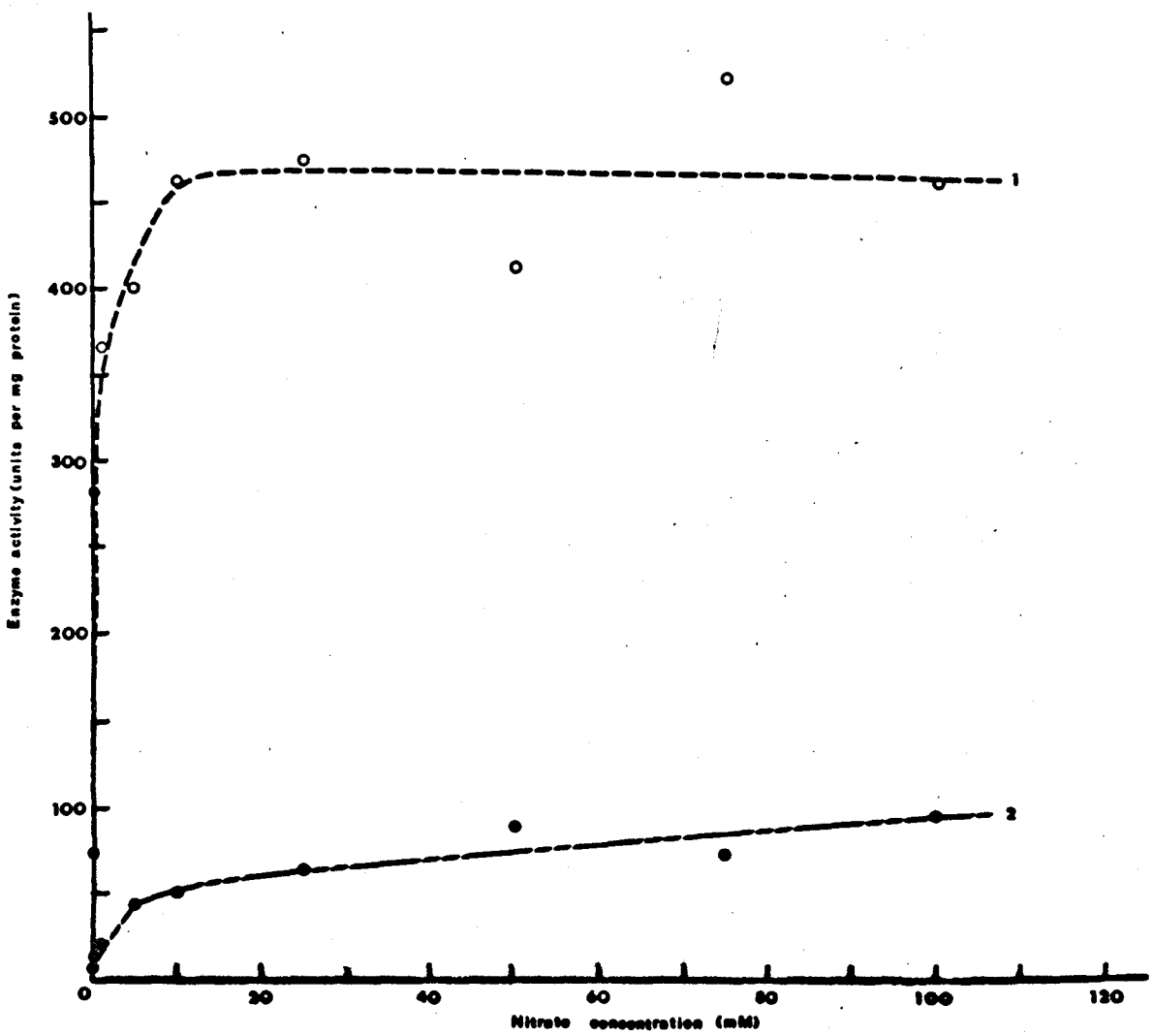
Figure 10: The Effect of Nitrate Concentrations on the Nitrate Reductase Activity Found in Root Tip and Mature Root Sections after a 4 Hour Induction.

The treatment conditions are described in Table XIII. Either forty root tip or forty mature root samples were used per extract.

Figure 10 shows the nitrate reductase levels induced after four hours in various nitrate concentrations. Both root tip (0 - 10 mm) and mature root samples (25 - 35 mm) were tested.

The lines in Figure 10 represent the activities of the extracts from:

1. root tip sections (0 - 10 mm) (O)
2. mature root sections (25 - 35 mm) (●)


Enzyme activity (units per mg protein)
500
400
300
200
100
0
20
40
60
80
100
120
Nitrate concentration (mM)
1
2

of up to at least 100.0 mM. The nitrate reductase levels of the root tip sections, after a 4 hour induction, agree within 7% of the mean value of the levels found with the induction kinetics (Table XIIa).

In contrast to the root tip sections, the mature root sections that had been induced for 4 hours showed a very slow increase in nitrate reductase activity between the nitrate concentrations of 5.0 mM to 100.0 mM. This is seen in Table XIIIb and Figure 10. When the nitrate concentration was less than 5.0 mM, the enzyme levels induced after 4 hours were very small. Higher levels of nitrate gave progressively higher enzyme levels. No level of nitrate used saturated the inducing system. From the induction kinetics experiments (Table XIIb and Figure 9b), at 4 hours the nitrate reductase activities of the mature root sections had not reached maximum levels, as had the enzyme levels in the root tip sections. Thus, it appears that under conditions that saturated the nitrate reductase induction in the root tip sections, the mature root sections remained unsaturated.

One could interpret these results as corresponding to a period of synthesis of messenger RNA which is required specifically for the enzyme production, and an initial period of protein synthesis. These are followed closely by a very rapid period of synthesis. Then, there is a period where synthesis is equal to degradation (ie the plateau region).

This final period was absent in the mature root sections of hybrids W64A x W182E, WF9 x 38-11 and W59M x W117. In the root tip sections, a nitrate concentration of 10.0 mM is sufficient to saturate the nitrate reductase induction system. Nitrate concentrations of up to 100.0 mM do not saturate the induction system in the mature root. These concepts will be discussed later in relation to the in vivo regulation of nitrate reductase.

IV) The Effect of Nitrate on the in vivo Turnover of Nitrate Reductase

As shown in the previous section, the presence of nitrate caused the induction of the enzyme. In order to see whether nitrate had any effect on the in vivo stability of nitrate reductase, the enzyme was induced until it had reached a maximum level. At this point, new protein synthesis was stopped by the addition of cycloheximide, a protein synthesis inhibitor. At various times following that treatment, the levels of nitrate reductase were determined. No maximum levels of nitrate reductase were induced in the mature root sections when using long induction times of 8 hours or very high nitrate concentrations of 100.0 mM. Since maximum enzyme levels were reached in the root tip sections, they were subsequently used to study the in vivo turnover of nitrate reductase. The seedlings of hybrid WF9 x 38-11

were grown for 50 hours at 26.0°C on 0.9% agar. Similarly, seedlings of hybrid W64A x W182E were grown for 68 hours. The agar contained 1/10 strength Hoagland's salt solution, a molybdenum supplement (0.207 μM), but lacked nitrate. The pre-treatment and treatment solutions contained 1/10 strength Hoagland's salt solution, the molybdenum supplement and the stated nitrate concentration. In addition, the treatment solution included cycloheximide. Oaks et al (28) have shown that cycloheximide, at a concentration of 17.8 μM or greater, caused an immediate inhibition of nitrate reductase synthesis in corn roots. In the following experiments, a cycloheximide concentration of 35.6 μM was used. Enzyme activity is expressed as a relative percent of the 3 hour pre-treatment sample. To investigate the effect of nitrate on the in vivo stability of nitrate reductase, a 3 hour pre-treatment in 10.0 mM nitrate, followed by treatment with cycloheximide (35.6 μM) ± KNO_3 (10.0 mM), was used. The enzyme activity was measured for times up to 4 hours following the treatment. The cycloheximide minus KNO_3 treatment closely paralleled the cycloheximide plus KNO_3 treatment, as Figure 11 indicates. In the root tip sections of hybrid WF9 x 38-11, there was a 35 to 42% decrease in enzyme activity during the initial 50 minutes of treatment. This was followed by a much slower decrease, only 10%, for the remainder of the 4 hour treatment. This indicates that in hybrid WF9 x 38-11, the presence or absence of nitrate does not alter the degradation

Figure 11: The Effect of Nitrate on the *in vivo* Turnover of Nitrate Reductase After a 3 Hour Pre-treatment in 10.0 mM nitrate.

The seedlings of hybrid WF9 x 38-11 were dark grown for 50 hours at 26°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution, a molybdenum supplement (0.207 μM), but lacked nitrate. The pre-treatment and treatment solutions contained 1/10 strength Hoagland's salt solution and a molybdenum supplement (0.207 μM). The pre-treatment solution also contained 10.0 mM nitrate. The pre-treatment time was 3 hours. Included in all the treatment solutions was 35.6 μM cycloheximide. The presence of or absence from the treatment solution of 10.0 mM nitrate is indicated below. The treatment was followed for up to 4 hours. Fifty root tips per treatment were extracted according to Extraction Method A. These extracts were assayed using Assay Method A. Malic dehydrogenase and oxalacetic acid were omitted from the assays. The nitrate reductase activities are shown as relative percents of the activity of the 3 hour pre-treatment sample. One hundred percent represents 139 units per milligram protein.

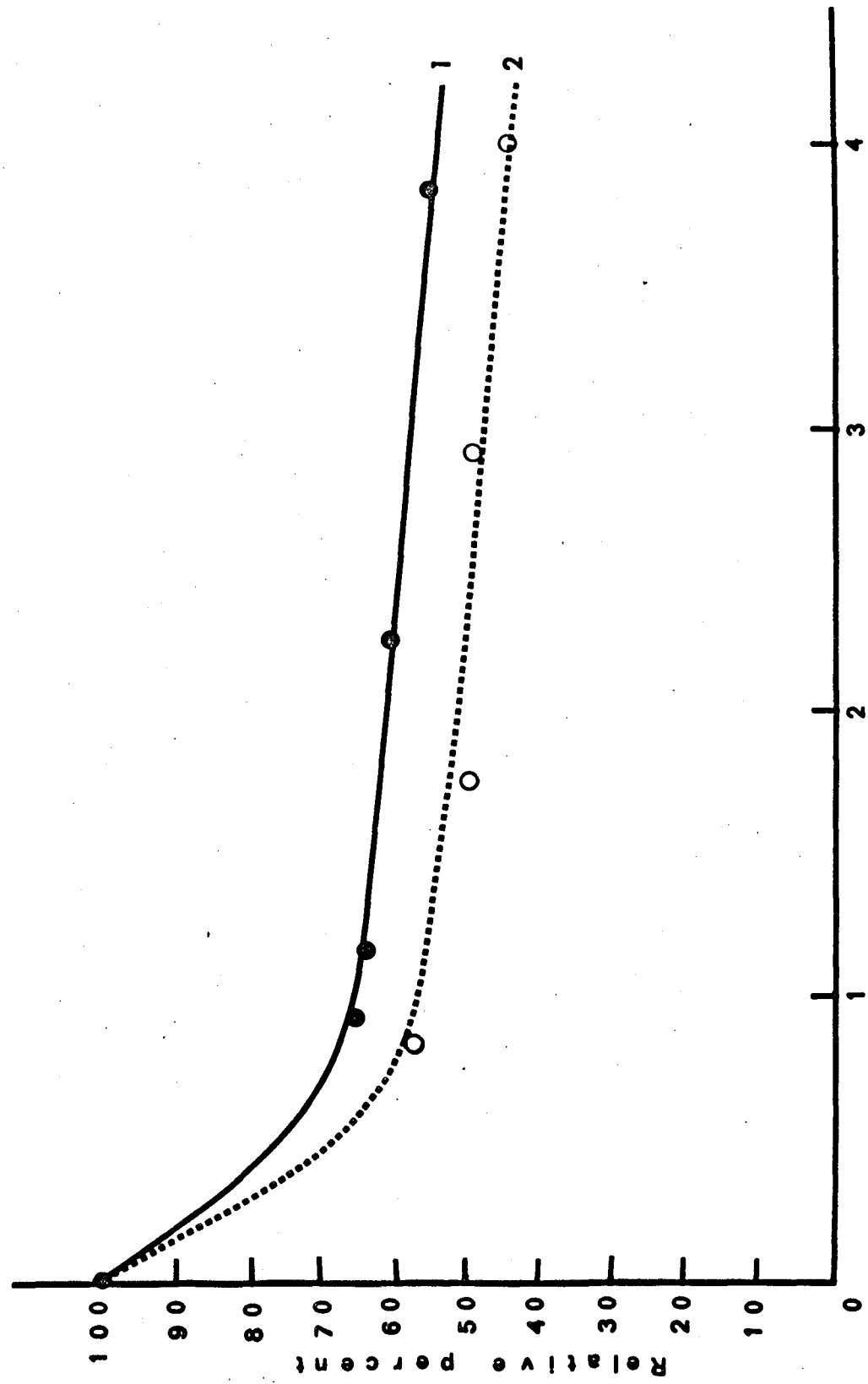
Figure 11 shows the effect that 10.0 mM nitrate has on the *in vivo* turnover of nitrate reductase after a 3 hour pre-treatment in 10.0 mM nitrate. New protein synthesis had been stopped at this point by the addition of 35.6 μM cycloheximide.

.....continued

Figure 11: (continued)

The lines in Figure 11 represent:

1. 35.6 μM cycloheximide plus 10.0 mM nitrate (●)
2. 35.6 μM cycloheximide minus nitrate (O)


1
2
100
90
80
70
60
50
40
30
20
10
0
Relative percent
0
1
2
3
4
Treatment time (hr)

pattern of nitrate reductase, after protein synthesis has been arrested.

In order to see if the presence of nitrate influenced the nitrate reductase stability throughout the entire plateau region, the system was studied further by inducing for a longer time. Seedlings of hybrid WF9 x 38-11 were pre-treated for 6 hours in 10.0 mM KNO_3, and treated in cycloheximide (35.6 μM) ± KNO_3 (10.0 mM) for periods up to an additional 6 hours. As demonstrated in Figure 12, there was an initial rapid decrease in enzyme activity. Again, there was no gross difference in enzyme stability for the root tip samples with either the plus or minus nitrate samples. Thus, the presence of nitrate does not affect the stability of the induced enzyme from various parts of the plateau region. Figure 13 shows the effect of nitrate after a 3 hour pre-treatment in a very high concentration of nitrate (25.0 mM) followed by a treatment with ± cycloheximide (35.6 μM) ± KNO_3 (25.0 mM). This high nitrate concentration was chosen in order to totally saturate the system with nitrate. This would eliminate any possibility that 10.0 mM nitrate was not a high enough concentration to affect the turnover of the enzyme. Root tip sections from hybrid WF9 x 38-11 were used. The presence or absence of nitrate did not alter the decay in the cycloheximide treated samples. In this figure, the effectiveness of cycloheximide

Figure 12: The Effect of Nitrate on the *in vivo* Turnover of Nitrate Reductase after a 6 Hour Pre-treatment in 10.0 mM Nitrate.

The experimental conditions are described in Figure 11. In this case, a pre-treatment time of 6 hours in 10.0 mM nitrate was used. The treatment conditions were followed for up to another 6 hours. The nitrate reductase activities are shown as relative percents of the activity of the 6 hour pre-treatment sample. One hundred percent represents 158 units per milligram protein.

Figure 12 shows the effect that 10.0 mM nitrate has on the *in vivo* turnover of nitrate reductase after a 6 hour pre-treatment in 10.0 mM nitrate. After the pre-treatment period, new protein synthesis had been arrested by adding 35.6 μM cycloheximide.

The lines in Figure 12 represent:

1. 35.6 μM cycloheximide plus 10.0 mM nitrate (●)
2. 35.6 μM cycloheximide minus nitrate (○)

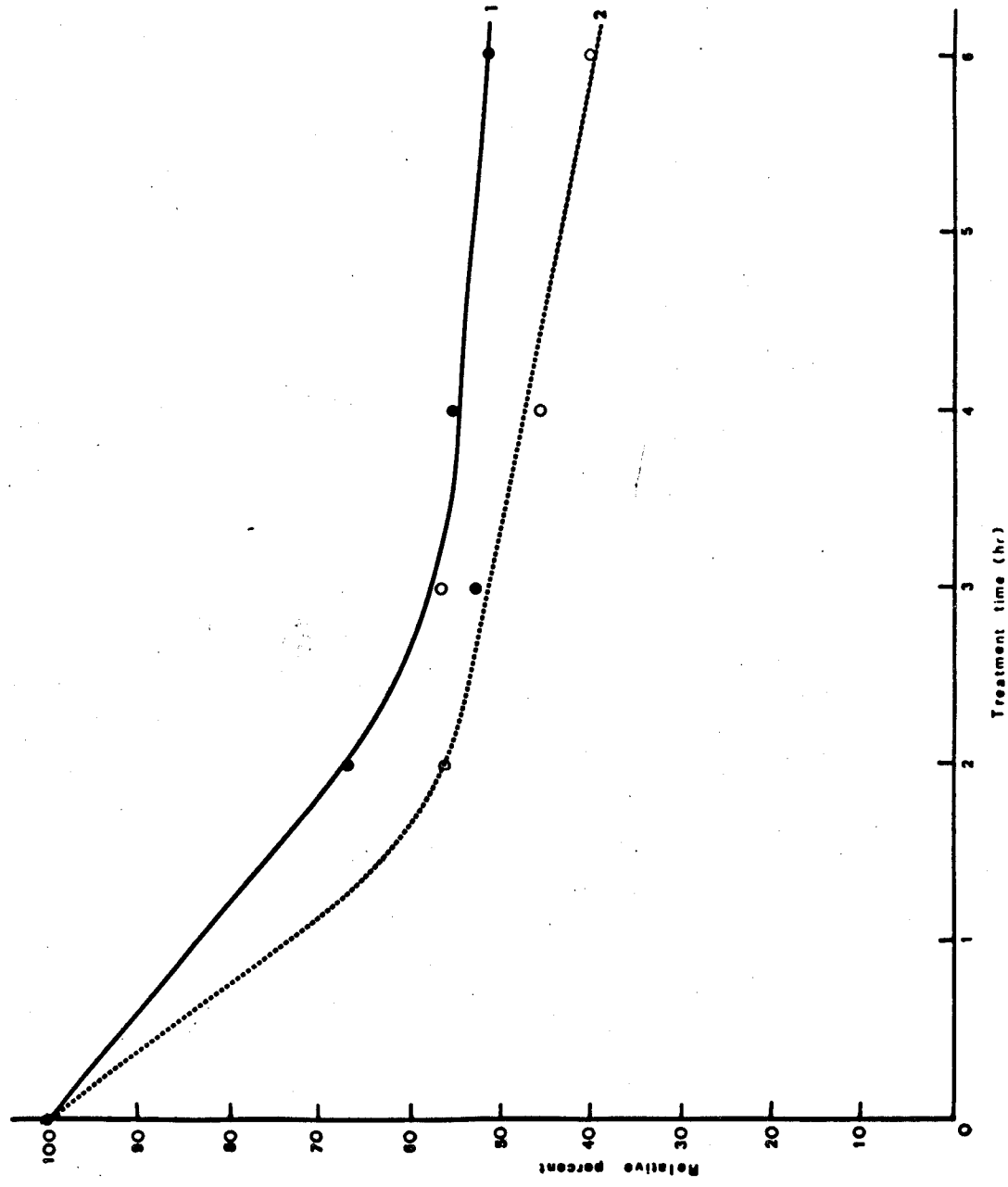

1
2
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
Relative percent
Treatment time (hr)

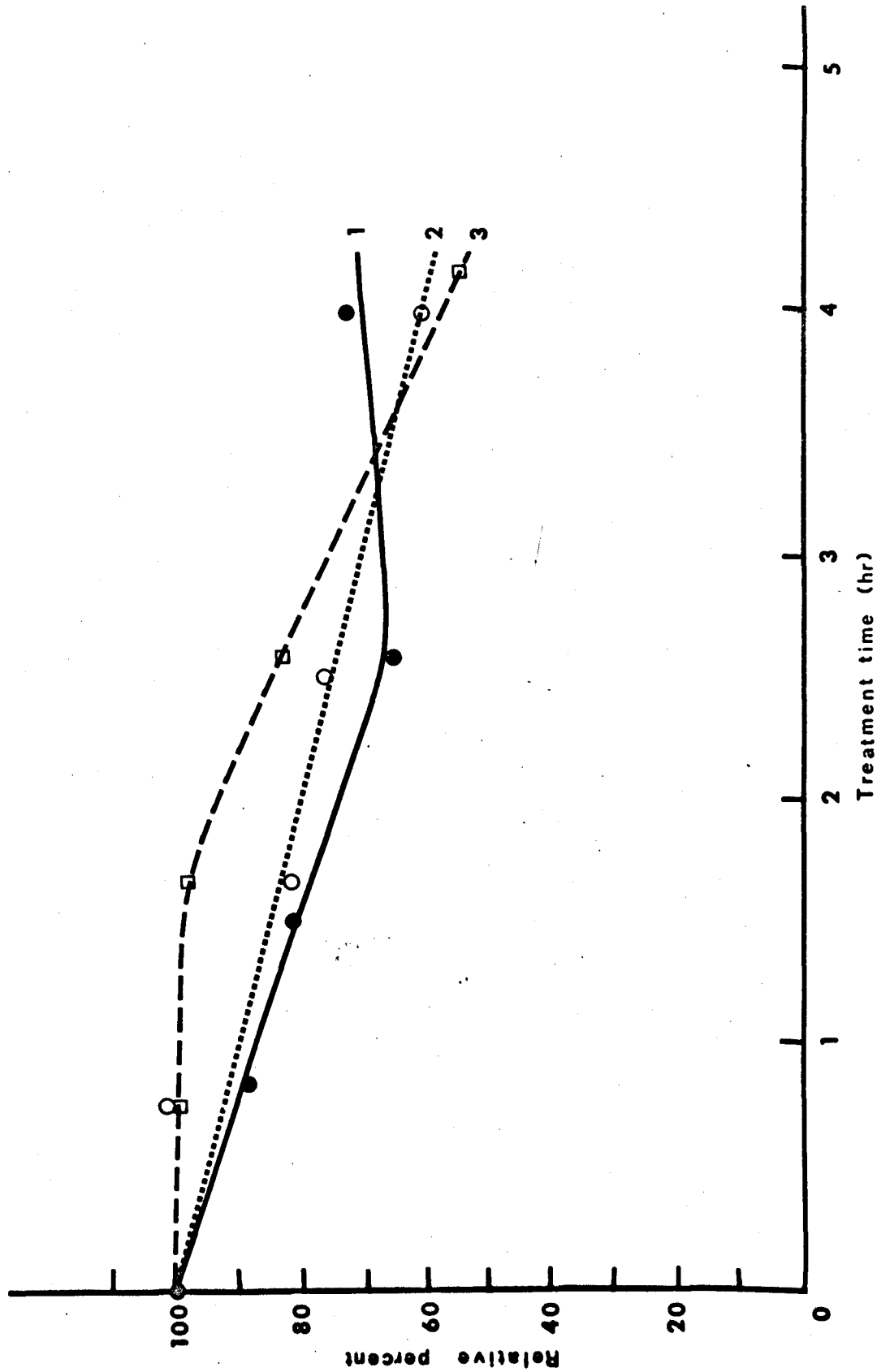
Figure 13: The Effect of Nitrate on the *in vivo* Turnover of Nitrate Reductase after a 3 Hour Pre-treatment in 25.0 mM Nitrate.

The experimental conditions are described in Figure 11. In this case, a pre-treatment time of 3 hours in 25.0 mM nitrate was used. The treatment conditions included the addition or deletion of 35.6 μM cycloheximide and the addition or deletion of 25.0 mM KNO_3, as indicated below. The nitrate reductase activities are shown as relative percent of the activity of the 3 hour pre-treatment sample. One hundred percent represents 92.6 units per milligram protein.

Figure 13 shows the effect that 25.0 mM nitrate has on the *in vivo* turnover of nitrate reductase after a 3 hour pre-treatment in 25.0 mM nitrate. After the pre-treatment period, new protein synthesis had been arrested by adding 35.6 μM cycloheximide.

The lines in Figure 13 represent:

1. 35.6 μM cycloheximide plus 25.0 mM nitrate (●)
2. 35.6 μM cycloheximide minus nitrate (O)
3. minus cycloheximide, minus nitrate (□)


Relative percent
100
80
60
40
20
0
1
2
3
4
5
Treatment time (hr)

in stopping protein synthesis is clearly shown. The plateau region is that period when synthesis is equal to degradation, as indicated by a lack of increase or decrease in the amount of the enzyme. There is a turnover of the enzyme on the plateau region, as seen by the decay when new protein synthesis was arrested with cycloheximide. Thus, maintenance of the plateau region requires a continual synthesis of the enzyme. Previously, it had been shown that nitrate was required to induce the enzyme. By just removing the nitrate, one might expect the induction machinery to cease. It was found that the minus cycloheximide minus KNO_3 treatment maintained this plateau for an additional 1.25 hours. After this time, the rate of degradation exceeded the rate of synthesis. This produced an overall decline in enzyme activity. This indicates that enough nitrate was present in the seedlings of the minus nitrate sample to maintain equal rates of synthesis and degradation for at least 1.25 hours. A possible explanation for the rapid decrease in the nitrate reductase activity in the cycloheximide treated samples would be that cycloheximide itself causes the rapid decline in activity. An alternate explanation is that protein is being rapidly turned over, and the immediate decline in activity with the cycloheximide just indicates that all new protein synthesis has ceased.

By pre-treating with a low level of KNO_3 (1.0 mM), it was thought that the effect of various nitrate concentrations on the

enzyme stability could be seen, since the system would not be saturated with nitrate. Saturation in this case was taken to be the lowest level of KNO_3 that could maximally induce the enzyme system. From Table XIIIa and Figure 10 this level was found to be 10.0 mM KNO_3. After a 4 hour pre-treatment in 1.0 mM nitrate, the system was treated with cycloheximide (35.6 μM) and various nitrate concentrations. As Figure 14 indicates, no significant difference in stability was seen after 2 hours in the presence of various KNO_3 concentrations (0.0, 1.0, 10.0, and 25.0 mM) in the presence of cycloheximide. The enzyme level remained constant for two whether the inducer was present or absent, as seen with the minus cycloheximide ± 1.0 mM KNO_3. Again, enough nitrate must have been present in the tissue of the minus nitrate treatment to maintain the synthesis of the enzyme, since no net turnover was observed. These results suggest that nitrate affects the system by inducing enzyme formation, but is not required to maintain the in vivo stability of the enzyme.

V) The Effect of Atrazine on Nitrate Reductase Levels

A slightly modified version of Ries' earlier work (58) was repeated, using atrazine instead of simazine. Primary leaves of hybrid W64A x W182E seedlings grown for 9 days at 22.5°C (16 hours light) and 17.0 °C (8 hours dark) were used. Tweedy and Ries (58) referred to these as sub-optimal conditions.

Figure 14: The Effect of Nitrate on the *in vivo* Turnover of Nitrate Reductase after a 4 Hour Pre-treatment in 1.0 mM Nitrate.

The seedlings of hybrid W64A x W182E were dark grown for 68 hours at 26°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution, a molybdenum supplement (0.207 μM), but lacked nitrate. The pre-treatment and treatment solutions contained 1/10 strength Hoagland's salt solution and a molybdenum supplement (0.207 μM). The pre-treatment solution also contained 1.0 mM nitrate. The pre-treatment time was 4 hours. The treatment conditions included the addition or deletion of cycloheximide (35.6 μM) and the addition of various nitrate concentrations (0.0, 1.0, 10.0 or 25.0 mM), as indicated below. Forty root tips per treatment were extracted according to Extraction Method B. These extracts were assayed using Assay Method B. Malic dehydrogenase and oxalacetic acid were added at the end of each assay. The nitrate reductase activities are shown as relative percents of the activity of the 4 hours pre-treatment sample. One hundred percent represents 352 units per milligram protein.

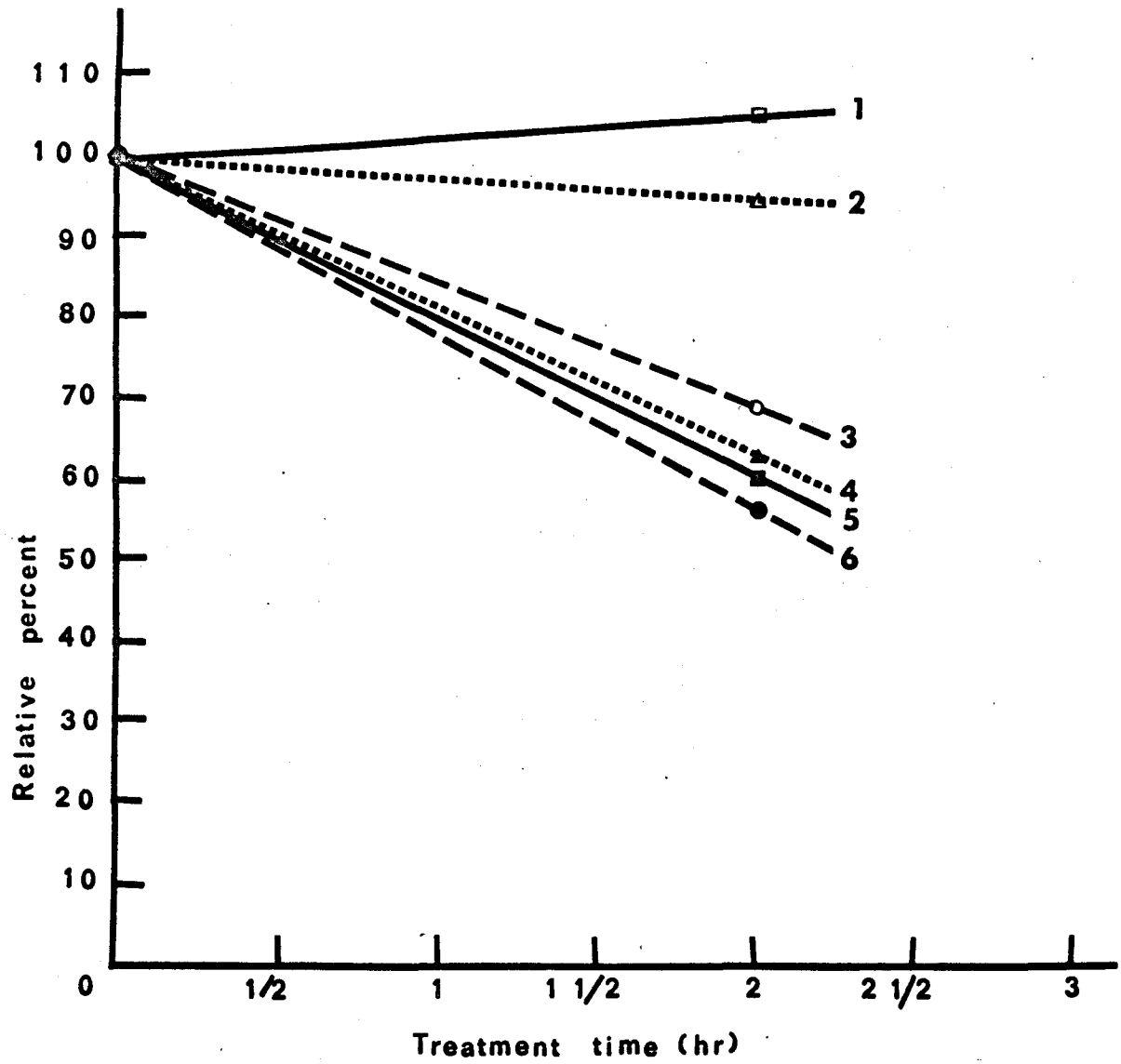
Figure 14 shows the effect that various nitrate concentrations have on the turnover of nitrate reductase after a 4 hour pre-treatment in 1.0 mM nitrate. In some cases, new protein synthesis was stopped by the addition of 35.6 μM cycloheximide.

.....continued

Figure 14: (continued)

The lines in Figure 14 represent:

1. no cycloheximide plus 0.0 mM nitrate (□)
2. no cycloheximide plus 1.0 mM nitrate (△)
3. 35.6 μM cycloheximide plus 0.0 mM nitrate (○)
4. 35.6 μM cycloheximide plus 1.0 mM nitrate (▲)
5. 35.6 μM cycloheximide plus 10.0 mM nitrate (■)
6. 35.6 μM cycloheximide plus 25.0 mM nitrate (●)


110
100
90
80
70
60
50
40
30
20
10
0
Relative percent
1/2
1
1 1/2
2
2 1/2
3
Treatment time (hr)
1
2
3
4
5
6

The seedlings had been watered daily with 200 ml of treatment solution, which included 1/2 strength Hoagland's salt solution with no nitrate, plus the treatments. The treatments were as follows: minus nitrate ± atrazine (10.0 μM), plus low nitrate (3.0 mM) ± atrazine (10.0 μM), and plus high nitrate (12.0 mM) ± atrazine (10.0 μM). Initially, the seedlings were watered with distilled water to insure even germination conditions. After the coleoptiles had emerged on day 5, the seedlings were watered with the treatment solutions. Ries had used 17 day old corn seedlings that had their endosperms removed at day 10. This system used 9 day old leaves from intact seedlings. Table XIV shows the effect of nitrate and atrazine on the level of nitrate reductase in seedlings grown under sub-optimal conditions. Atrazine produced only minor effects. The 39% increase in nitrate reductase activity seen in the low nitrate plus atrazine sample was not of the same magnitude as the 4 fold one reported by Tweedy and Ries (58). Atrazine did not alter the nitrate levels in the high nitrate and the minus nitrate samples. This lack of increase in enzyme activity was not due to the fact that the enzyme was already maximally induced. The low nitrate induced a level of nitrate reductase twice that of the zero nitrate control, but only one-half that of the value of the high nitrate level. Thus, a maximum level had not been reached in the low nitrate sample. Similar results were found with primary leaves of 10,

TABLE XIV. THE EFFECT OF ATRAZINE TREATMENT AT LOW TEMPERATURES ON NITRATE REDUCTASE ACTIVITY IN PRIMARY LEAVES

Treatment	Soluble Protein (mg/ml)	NR Activity (units/mg protein)	% of -A control
- N - A	2.90	186.2	99.6%
- N + A	2.28	185.2	
+ low N - A	2.75	404.0	139.0%
+ low N + A	2.80	563.0	
+ high N - A	3.06	868.0	114.0%
+ high N + A	2.71	992.0	

Primary leaves of hybrid W64A x W182E seedlings grown for 9 days at 22.5°C (16 hour light) and 17.0°C (8 hours dark) were used. The seedlings had been watered daily with 200 ml of treatment solution, which included 1/2 strength Hoagland's salt solution minus nitrates, plus the indicated treatments. 'A' designated an atrazine concentration of 10.0 μM. Low N indicated KNO_3 concentration of 3.0 mM, whereas high N indicated a KNO_3 concentration of 12.0 mM. Extraction and Assay Methods B were used. Oxalacetate and malic dehydrogenase were added at the end of each assay. The nitrate reductase activity was calculated on a units per milligram protein basis.

11, 12 and 13 day old seedlings grown under sub-optimal conditions. The experiment was repeated using more optimal conditions. These conditions were the normal growth conditions of 26.5°C and a light regime of 16 hours of light, followed by 8 hours of darkness. Again, atrazine did not affect the levels of nitrate reductase. The first, second and third leaves of 8, 9, 10, and 11 day old seedlings were tested, and gave similar results. Thus, atrazine did not alter the nitrate reductase levels in corn leaves to the significant level found by Tweedy and Ries using simazine, a similar s-triazine herbicide.

Further studies were made to see how atrazine affects the nitrate reductase levels in corn roots. Seedlings of hybrid W64A x W182E were grown for 72 hours at 26.0°C on 0.9% agar. This agar contained 1/10 strength Hoagland's salt solution, a molybdenum supplement and the treatment solutions. The treatments used were: 5.0 mM KNO_3 plus atrazine (0.0, 1.0, or 10.0 ppm), and 25.0 mM KNO_3 plus atrazine (0.0, 1.0, or 10.0 ppm). The results are shown in Table XV. In the root tip sections, the changes due to the presence of atrazine were very small. Atrazine concentrations of 1.0 and 10.0 ppm increase the 5.0 mM nitrate treatment activity by only 29 to 35%. No effect was seen when atrazine was added with the 10.0 mM nitrate samples. With the highest nitrate concentration used (25.0 mM), only a slight increase of 22% was seen in the nitrate reductase activity due to the presence of atrazine (10.0 ppm). Atrazine did not change the

TABLE XV. THE EFFECT OF ATRAZINE ON NITRATE REDUCTASE LEVELS IN ROOTS GROWN ON CONTINUOUS ATRAZINE AND NITRATE

Treatment on agar	Tip Sections (0 - 10 mm)		Mature Sections (25 - 35 mm)	
	Soluble Protein (mg/ml)	NR Activity (units/mg protein)	Soluble Protein (mg/ml)	NR Activity (units/mg protein)
5.0 mM KNO_3 + 0.0 ppm atrazine	1.80	356.0	0.81	65.1
5.0 mM KNO_3 + 1.0 ppm atrazine	1.77	462.0	0.80	68.7
5.0 mM KNO_3 + 10.0 ppm atrazine	1.94	483.0	0.71	59.2
10.0 mM KNO_3 + 0.0 ppm atrazine	1.67	429.0	0.76	130.0
10.0 mM KNO_3 + 1.0 ppm atrazine	1.70	440.0	0.78	144.5
10.0 mM KNO_3 + 10.0 ppm atrazine	1.71	452.0	0.77	139.0
25.0 mM KNO_3 + 0.0 ppm atrazine	1.98	405.0	0.91	101.2
25.0 mM KNO_3 + 1.0 ppm atrazine	1.93	372.0	1.33	98.7
25.0 mM KNO_3 + 10.0 ppm atrazine	1.44	498.0	0.87	124.2

.....continued

TABLE XV. (continued)

The seedlings of W64A x W182E were grown for 72 hours at 26°C on 0.9% agar. This agar contained 1/10 strength Hoagland's salt solution, a molybdenum supplement and the designated nitrate and atrazine concentrations. The KNO_3 concentrations used were 5.0 mM, 10.0 mM and 25.0 mM. The atrazine concentrations were 0.0 ppm, 1.0 ppm, and 10.0 ppm. Fifty root tip sections per sample were used. Extraction and Assay Methods B were used. Oxalacetate and malic dehydrogenase were added at the end of each assay. The nitrate reductase activity was calculated on a units per milligram protein basis.

nitrate reductase levels in the mature root sections when added together with 5.0 mM, 10.0 mM or 25.0 mM nitrate. Similar results were obtained with hybrid WF9 x 38-11, under identical conditions.

The effect of atrazine on the inducible levels of nitrate reductase in corn roots was studied. The seedlings of hybrid W64A x W182E were grown for 72 hours at 26.0°C on 0.9% agar. The agar contained 1/10 strength Hoagland's salt solution, a molybdenum supplement (0.207 μM) and an atrazine concentration of 0.0, 1.0, or 10.0 ppm. No nitrate was present. The seedlings were induced for four hours in a solution containing 1/10 strength Hoagland's salt solution, the molybdenum supplement, and final nitrate concentrations of 0.0 mM, 1.0 mM, 10.0 mM or 25.0 mM. Atrazine was included at a concentration of 0.0, 1.0 or 10.0 ppm. Table XVI shows that the inducible levels of nitrate reductase were not altered by the various atrazine treatments, for all the nitrate concentrations used. This was found in both the root tip and the mature root sections. This lack of effect was not due to the enzyme being maximally induced at the lower nitrate concentrations, even in the presence of atrazine. The levels for the 1.0, and 10.0 mM KNO_3 samples plus atrazine were below those of the 25.0 mM KNO_3 inductions. If atrazine had affected the induction, one might have expected the enzyme levels induced by the lower nitrate concentrations to approach those of

TABLE XVI. THE EFFECT OF ATRAZINE ON NITRATE REDUCTASE ACTIVITY IN ROOTS INDUCED FOR FOUR HOURS IN NITRATE

Treatment (on agar)	Treatment (induction)	Tip Sections (0 - 10 mm) Soluble Protein (mg/ml)	Tip Sections (0 - 10 mm) NR Activity (units/mg protein)	Mature Sections (25 - 35 mm) Soluble Protein (mg/ml)	Mature Sections (25 - 35 mm) NR Activity (units/mg protein)
0.0 ppm atrazine	0.0 mM KNO_3	2.98	50.2	1.33	7.6
1.0 ppm atrazine	0.0 mM KNO_3	2.62	59.4	1.18	8.1
10.0 ppm atrazine	0.0 mM KNO_3	2.85	53.4	1.12	8.1
0.0 ppm atrazine	1.0 mM KNO_3	2.81	274.0	1.23	27.6
1.0 ppm atrazine	1.0 mM KNO_3	2.94	283.0	1.22	30.0
10.0 ppm atrazine	1.0 mM KNO_3	2.62	337.0	1.14	29.3
0.0 ppm atrazine	10.0 mM KNO_3	2.94	346.5	1.03	62.7
1.0 ppm atrazine	10.0 mM KNO_3	2.82	310.0	1.26	61.8
10.0 ppm atrazine	10.0 mM KNO_3	2.65	331.0	1.36	49.2

.....continued

TABLE XVI. (continued)

Treatment (on agar)	Treatment (induction)	Tip Sections (0 - 10 mm) Soluble Protein (mg/ml)	Tip Sections (0 - 10 mm) NR Activity (units/mg protein)	Mature Sections (25 - 35 mm) Soluble Protein (mg/ml)	Mature Sections (25 - 35 mm) NR Activity (units/mg protein)
0.0 ppm atrazine	25.0 mM KNO_3	2.94	384.0	1.18	86.7
1.0 ppm atrazine	25.0 mM KNO_3	2.36	378.0	1.21	66.3
10.0 ppm atrazine	25.0 mM KNO_3	2.74	309.0	1.32	39.4

The seedlings of W64A x W182E were grown for 72 hours at 26°C on 0.9% agar. This agar contained 1/10 strength Hoagland's salt solution, a molybdenum supplement, and an atrazine concentration of 0.0 ppm, 1.0 ppm, or 10.0 ppm. Nitrate was not added. The seedlings were induced for 4 hours in a solution containing 1/10 strength Hoagland's salts, a molybdenum supplement and a final nitrate concentration of 0.0 mM, 1.0 mM, 10.0 mM, or 25.0 mM. Atrazine was also included at concentrations of 0.0 ppm, 1.0 ppm, or 10.0 ppm. Forty root tip sections per treatment were used. Extraction and Assay Methods B were used. Oxalacetic acid and malic dehydrogenase were added at the end of each assay. The nitrate reductase activity was calculated on a units per milligram protein basis.

the higher nitrate concentrations. This was not seen. Atrazine, up to a concentration of 0.232 mmoles, did not affect the *in vitro* enzyme assay. Thus, atrazine does not affect the nitrate reductase levels in both the roots and the leaves of corn. This was seen under inducing conditions, and conditions of continuous nitrate and atrazine.

CHAPTER III

DISCUSSION

I) Enzyme Regulation

A number of studies have been carried out to study the regulation of transcription and translation of inducible enzymes. Transcription refers to the formation of a ribonucleic acid message (m-RNA) that is specific for the induced enzyme. Translation refers to the reading of this message and the formation of a specific polypeptide, which is the enzyme. Using Neurospora, Turner et al (56,57) were able to distinguish between the effect of the inducer molecule on the transcription and the translation of the inducible enzyme, kynureninase. They used Actinomycin D to arrest messenger-RNA synthesis (ie. transcription) and cycloheximide to stop new protein synthesis (ie. translation). In the presence of cycloheximide and any one of the inducers kynurenine, N-formylkynurenine or tryptophan, they found an accumulation of the capacity to synthesize the enzyme, kynureninase. This represented a build-up of messenger. Upon removal of the protein synthesis inhibitor and the inducer, and the addition of Actinomycin D, the enzyme was translated. This subsequent expression of the kynureninase did not require RNA synthesis since the presence of Actinomycin D did not inhibit the enzyme synthesis at this point. The inducer molecule was not required at the translational level since its addition or deletion at this point did not alter the rate or the amount of enzyme synthesized. A maximum rate of induced

synthesis occurred after 10 minutes.

Several regulatory sites were seen with the induction of the enzyme δ-aminolevulinic acid synthetase, the rate limiting enzyme of heme biosynthesis. Sassa and Granick (36) showed that the natural steroid etiocholanolone specifically induced δ-aminolevulinic acid synthetase at the level of transcription, but it had no effect at the translational level. Other inducing chemicals such as allylisopropylacetamide and γ-hexachlorocyclohexane, acted only at the level of translation by increasing the amounts of enzyme present. Hemin, a stable form of the end product heme, inhibited the translation of δ-aminolevulinic acid synthetase by competing with the translational inducers. It did not alter the turnover of the enzyme. Actinomycin D was used to stop m-RNA formation, and cycloheximide was used to stop protein synthesis, thereby separating the action of these chemicals at the transcriptional and translational levels of control. These results show that enzyme induction can be regulated by several inducing agents each acting at various levels of protein synthesis (ie. transcription or translation).

Positive effectors, that are capable of inducing enzyme synthesis for long periods of time, have been found. Kaufman et al (22), while studying the turnover of invertase in Avena stem segments, found that they required the continuous presence of sucrose and gibberellic acid, a plant hormone, for the induction and maintenance

of high levels of invertase activity. Sucrose and gibberellic acid together also caused more than an additive growth response, as seen by a 4 fold increase in shoot length over the controls. By adding cycloheximide at various times during the induction of invertase, and studying the decay kinetics, it was found that sucrose and gibberellic acid did not alter the turnover of the enzyme. Kaufman suggested that sucrose and gibberellic acid were acting as positive effectors.

A similar enhancement effect was seen by Schimke, Sweeney and Berlin (12,37). They administered ^{14}C-leucine to rats and then measured the amount of radioactivity in the protein that was precipitated with a specific antibody to their enzyme, trypotphan pyrrolase. They found that the inducer hydrocortisone increased the rate of de novo synthesis of tryptophan pyrrolase. The enzyme's substrate, tryptophan, did not alter the rate of synthesis of the enzyme, but it did decrease the rapid rate of degradation. The addition of tryptophan, when added together with hydrocortisone, increased the total amount of enzyme, but did not cause an increase in the radioactive incorporation into the enzyme. This indicated that the overall increase in enzyme activity seen with tryptophan was due to an increase in enzyme stability along with an accumulation of enzyme that was being continuously synthesized. This, is a case where the inducer, cortisone, alters the rate of de novo enzyme synthesis, and the substrate, tryptophan, alters the rate of degradation of the enzyme.

Factors that affect the integrity of the enzyme itself may regulate the enzyme by altering its turnover rates. Using E. coli, studies have been carried out on the turnover of nonsense fragments of incomplete chains of β-galactosidase (13,25,62). Amber and ochre mutants of the z gene of E. coli produced nonsense fragments of inactive, incomplete chains of the enzyme β-galactosidase. These enzymatically inactive chains were detected by their ability to cross react with antiserum prepared with the native β-galactosidase. Partial diploids were made from various strains that lacked complementary parts of the structural gene. The resultant partial diploids produced two inactive polypeptides which then combined noncovalently in vivo, forming active β-galactosidase. The degradation rates were determined by growing the E. coli in the inducer, isopropyl β-D-thiogalactoside, then washing the cells, and following the degradation in the absence of the inducer. The incomplete chains were degraded very rapidly in vivo, whereas the active wild type β-galactosidase and the active complemented chains of the nonsense polypeptides were found to be stable. Thus, factors that alter the integrity of the enzyme can also change the turnover of the enzyme. These alterations could possibly be in the tertiary or quaternary structure of the enzyme.

Subramanian and Sorger have recently studied the regulation of nitrate reductase in Neurospora crassa (52,53). They separated

the transcriptional and translational roles of nitrate by using Actinomycin D and cycloheximide respectively. They found that a low concentration (0.2 M) of the inducer, nitrate, was needed for the formation of the messenger. A high nitrate concentration (20.0 mM) was required to stabilize the newly translated nitrate reductase. Ammonia, a potential end product of the reaction, inactivated the enzyme in vivo. By using the nitrate non-utilizing mutants nit-1 (NADPH-cytochrome c reductase) and nit-3 (reduced benzyl viologen-nitrate reductase), they were able to show that the integrity of the active nitrate reductase complex was required for the in vivo inactivation to occur. The partial activities of the nit mutants were not inactivated by either the removal of nitrate from the medium or by the addition of ammonium to it. Lewis and Fincham (24) also found ammonium inactivated nitrate reductase in vivo in Ustilago maydis. Srivastava (50), using corn roots, found no alteration in the inducible levels of nitrate reductase when ammonium was included in the induction medium. Thus, ammonium, an end product of nitrogen assimilation, does not affect the regulation of the enzyme in vivo. Vennesland's group have recently found that nitrate reductase activity can be activated from an induced proenzyme. This was done by incubating the preparation in nitrate, phosphate, magnesium and ADP. They had used extracts of Chlorella vulgaris, Berlin strain (47,61). All these preceding systems showed how the inducing agent can influence

the turnover of the newly formed protein. Studies were undertaken in order to elucidate the effect that the inducing agent, nitrate, has on regulating the plateau levels in corn.

II) The Effect of Nitrate on the Induction of Nitrate Reductase

The effect of various nitrate concentrations on the induction kinetics of nitrate reductase was studied. In the corn root tip extracts, there is an initial lag time of 25 minutes. This represented the period of nitrate uptake and m-RNA formation. This is followed by a rapid synthesis of nitrate reductase from 25 minutes to 4 hours. No change is seen in the enzyme levels after four to eight hours induction in various concentrations of nitrate. This nitrate reductase lag time was identical to that seen by Subramanian and Sorger (52) in Neurospora crassa, by Lewis and Fincham (24) in Ustilago maydis and by Oaks, Wallace and Stevens (28) in Zea mays L. The four hours required to reach a maximum level of enzyme activity was one to two hours longer than that reported for Ustilago (24) and Neurospora (52). In the kynureninase (56,57) and the tryptophan pyrrolase (27) system, the enzyme levels continually increased until the supply of inducer was exhausted. With δ-aminolevulinic acid synthetase (36), invertase (22), and nitrate reductase (24,28,52), the induced enzyme activities eventually leveled out. A twenty-five fold change in nitrate concentration from 1.0 mM to 25.0 mM did not alter these induction characteristics in corn root tips as

shown in Figure 7a, and Table XIIa. This infers that the amount of m-RNA formed is of the same order of magnitude for all the three nitrate concentrations used (1.0, 10.0 and 25.0 mM). Similarly, the rates synthesis must be equivalent since the rates of increase of the enzyme paralled each other for the three nitrate concentrations used. The 1.0 mM nitrate could only maintain this increase in nitrate reductase activity for three hours, at which time a plateau region was reached. The 10.0 and 25.0 mM nitrate inductions plateaued after 4 hours, maintaining a level of activity 57% greater than that of the 1.0 mM nitrate induction. The enzyme level reached a maximum when nitrate concentrations between 10.0 to 100.0 mM were used. Nitrate levels below this did not maximally induce the enzyme, but maintained plateau regions of lower nitrate reductase activities (Table XIIIa and Figure 10). The plateau represents a region where degradation is equal to synthesis, as indicated by the constant level of enzyme. Zielke and Filner (65) have previously shown that there was a constant turnover of the nitrate reductase during the initial increase, the steady state and the decay period. In this system, nitrate reductase activity declined upon addition of cycloheximide, a protein synthesis inhibitor (Figures 9, 10, 11, and 12). This indicates that the enzyme was being turned over.

In mature root sections (25 - 35 mm from the tip), the induction kinetics followed a different pattern. No change was seen in the enzyme levels for the first 1.5 hours. This was

followed by a steady increase in enzyme activity. A plateau region was never reached in the mature root sections, even when long induction times of 8 hours were used (Figures 7b and Table XIIb) or when high nitrate concentrations of up to 100.0 mM were used (Figure 8 and Table XIIIb). The reason that the nitrate reductase was not maximally induced could be due to a poorer protein synthesizing apparatus in the older root sections. Travis and Key (55), using dark grown corn seedlings, found a decrease in the polyribosome content in 10 day old leaves when compared to younger 3 day old shoots. They attributed this loss in the polyribosomal level as the reason there was a significant increase of two to four hours in the lag phase of nitrate reductase induction. For a system that can not synthesize protein as efficiently in time, one would still expect it to become saturated. This would lead to a steady level of nitrate reductase, but the level of the enzyme would be lower than that of the actively synthesizing root tips. The fact that the enzyme level in the mature root sections never plateaued, and was at a very low level when compared to the activity of the root tip sections could be caused by two possibilities. Firstly, the mature root enzyme might be controlled differently from that of the root tip sections. Pan and Marsh (30) have recently found a protein-like molecule from 10 day old corn roots that

specifically inhibits nitrate reductase activity. Perhaps this is causing the low enzyme activities in mature root sections. A second possibility could be that nitrate is not absorbed efficiently by the mature root sections. Nitrate uptake studies along the root of peas (15) showed that the greatest uptake occurred at the tip (0 - 1.0 cm). The minimum amount of uptake was seen in the more mature regions (1.0 to 4.5 cm). Thus, in corn root tip sections, the final levels of enzyme activity, and not the induction kinetics, were dependent on the concentration of nitrate used. In mature root sections, no plateau regions were reached, even with very high nitrate concentrations. Consequently, most of the work on the effect of nitrate on the regulation of nitrate reductase was done using corn root tips, since they could be maximally induced.

III) The Effect of Nitrate on the Degradation of Nitrate Reductase

Experiments were carried out to test the effect of nitrate on the degradation of nitrate reductase. Nitrate concentrations between 10.0 to 100.0 mM maximally induced the nitrate reductase activity (Table XIIIa and Figure 8). Lower concentrations of nitrate (5.0, 1.0 and 0.10 mM) induced lower enzyme levels. The initial rates of synthesis were not affected by nitrate concentrations, as seen by the induction kinetics experiments. The various plateau levels of nitrate

reductase seen after 4 hours could have been caused by a change in the stability of the enzyme due to the concentration of nitrate used. The effect of nitrate on the *in vivo* stability of nitrate reductase was studied.

In the corn root system, nitrate, the inducer, did not affect the *in vivo* stability of nitrate reductase. Once new protein synthesis had been stopped by using cycloheximide (35.6 μM), the maximally induced enzyme decayed to the same extent whether or not nitrate was present (Figures 9, 10, 11, and 12). This was seen for enzyme preparations induced for different times on the plateau region (3 and 6 hours), and for seedlings induced in low (1.0 mM) or saturating amounts (10.0 and 25.0 mM) of nitrate. In one study, the system had been pre-treated with unsaturating amounts of nitrate (1.0 mM). Upon stopping new enzyme synthesis with cycloheximide, various concentrations of nitrate were added to the system. The degradation of the enzyme was then followed. As seen in Figure 12, even saturating amounts of nitrate (10.0 and 25.0 mM) did not alter the degradation. Thus, nitrate does not appear to affect the *in vivo* stability of nitrate reductase in corn roots. Oaks, Wallace and Stevens (28) have recently shown that the mature root extracts have a higher *in vitro* turnover rate of nitrate than do the root tip extracts. The half-lives were 2 and 3 hours respectively. Oaks (unpublished) mixed

extracts of the root tips and mature roots and found no increase in the loss of root tip enzyme. This suggests that the difference in turnover between the root tip and the mature root sections was not due to the specific nitrate reductase inhibitor found by Pan and Marsh (30). This *in vitro* loss might be due to an inactivation of the enzyme. Vennesland (47,61) has been able to activate nitrate reductase activity in extracts of *Chlorella vulgaris*, Berlin strain by incubating the extract in nitrate, phosphate, magnesium and ADP. Srivastava (50) showed that in corn root system, contrary to that seen in *Neurospora* (52) and barley root tips (44), ammonium, a possible end product, did not inhibit the enzyme formation. Another possible reason for this difference in *in vitro* degradation rate could be due to conformational changes in the enzyme from the mature root sections. Increased decay rates were seen for incomplete β-galactosidase polypeptides in *E. coli* (13,25,62).

IV) The Effect of Atrazine on Nitrate Reductase Levels

S-triazine herbicides were found to produce high levels of nitrate reductase in corn grown under suboptimal conditions such as low nitrate and low temperatures (58). Thus, the effect of atrazine, an s-triazine, was studied in the corn root system in order to help ascertain how nitrate regulated the nitrate reductase levels. It was originally thought that if the low non-saturating nitrate concentrations (ie. those less than 10.0 mM) were limiting the induction, then atrazine might be able to overcome these

limitations. This might have increased the nitrate reductase levels up to those levels found under saturating nitrate conditions. The atrazine could have done this by causing a faster uptake of nitrate, altering the distribution of nitrate within the cell, influencing the transcriptional or translational mechanisms or altering the turnover of the enzyme. In our system, atrazine was found to be ineffective in altering the nitrate reductase levels. (Tables XIV, XV and XVI).

V) How Nitrate Can Affect the Nitrate Reductase Levels

The question of how nitrate regulates the different plateau regions still exists. Nitrate concentrations below 10.0 mM induced lower levels of nitrate reductase than those induced by higher nitrate concentrations. No difference was seen on the initial rates of induction or the lag times. Even after twenty-four hours of induction, the enzyme levels still decrease by the same relative amounts. On the plateau regions, one sees no overall change in enzyme activity. Nitrate was shown to have no influence on the *in vivo* degradation of nitrate reductase. On the plateau region, the rates of synthesis are equal to the rates of degradation for all the nitrate concentrations used since no change in enzyme levels were seen. Since the degradation rates were unaltered by nitrate concentration, this infers that the rates of enzyme synthesis must be the same, regardless of the nitrate concentration used. If the

synthesis rates are identical, as seen by the same induction kinetics under saturating and non-saturating nitrate conditions, and the steady state regions, then, nitrate concentrations do not affect the translational level directly. Therefore, the primary effect of nitrate on the inducible level of nitrate reductase has to be expected at the transcriptional level. The uptake of nitrate is not a limiting feature since there was no alteration in the lag time for the low, non-saturating nitrate concentrations (1.0 mM), when compared to the lag time of the 10.0 mM and 25.0 mM inductions. The nitrate concentration could affect the total amount of nitrate reductase messenger available, the turnover rate of the m-RNA, or the efficiency of the m-RNA translation. For the first alternative of the nitrate controlling the total amount of m-RNA to hold true, one would expect to see an overall decrease in enzyme production for the 1.0 mM nitrate samples. This would be due to smaller amounts of messenger being produced. This would manifest itself in an increased lag time, and a decrease rate of initial synthesis. These features were not seen. If the translational mechanism was limiting, then perhaps the higher nitrate concentrations could induce more m-RNA. This would allow the higher nitrate treatments to maintain the initial rate of synthesis for a longer time since the messenger would not be limiting. The initial rate of induction would be the same for both high and low inductions since the limiting step would

be at the translational level. In order to see a higher enzyme level at the higher nitrate concentrations, for the lower nitrate concentrations, the amount of m-RNA at the lower nitrate concentrations would have to become limiting. This would be due to the turnover of the m-RNA. This would cause new enzyme synthesis to slow down. When the synthesis rate equaled the degradation rate, the enzyme level would plateau. The only difference between the various nitrate inductions would be their final plateau levels. This argument does not hold true in the corn root tip system. The nitrate concentrations did not affect the degradation of the enzyme when cycloheximide was added to stop protein synthesis. Thus all the synthesis rates are the same since the degradation rates were identical. Identical synthesis rates would preclude alterations in the total amount of the specific m-RNA present. If the translational mechanism was not limiting, and if more m-RNA was produced by the higher nitrate concentrations, then onw would expect a higher rate to enzyme synthesis. This would eliminate the plateau region since the degradation rates remained the same at all nitrate concentrations used. This effect was not seen. This leaves the possibility of nitrate concentration affecting the stability of the m-RNA. Nitrate concentrations between 10.0 to 100.0 mM were capable of inducing a large amount of nitrate reductase. If the lower nitrate concentrations were capable of inducing these same high levels of nitrate reductase, an increase in the turnover of the m-RNA at low nitrate concentrations

could indeed account for the lower enzymes levels. The third alternative, a differential efficiency of the messenger, is hard to prove in an *in vivo* system. As previously shown in Table III, hybrid W59M x W117 did not exhibit a plateau region throughout the 5.5 hour induction. Warner *et al* (63) have recently described a corn inbred line, Oh 43, that has an unstable nitrate reductase. Perhaps these varieties will help to shed some light on the regulation of the steady state levels of nitrate reductase.

SUMMARY

1) The nitrate reductase assay was improved up to 10 fold over that previously used by Oaks, Wallace and Stevens (1972). These improvements included a redefinition of the assay conditions. The new assay included 16.0 µmoles KH_2PO_4, 0.564 µmoles NADH, 20.0 µmoles KNO_3 and 130.0 µmoles Hepes buffer (pH 7.0), all in a total volume of 1.5 ml. The reaction was stopped by the addition of oxalacetic acid (10.0 µmoles) and malic dehydrogenase in order to oxidize all the NADH.

2) For the induction kinetics of the corn root tip sections (0 - 10 mm), the concentrations of nitrate used (1.0, 10.0, or 25.0 mM) did not affect the 25 minute lag period or the period of rapid synthesis of nitrate reductase. With nitrate concentrations between 0.1 mM and 10.0 mM, the only parameter changed was the final level of enzyme activity. Nitrate concentrations of 10.0 mM or greater did not induce nitrate reductase levels above the maximum.

3) In mature root sections (25 - 35 mm from the tip), there was a longer lag period of 1.5 hours, followed by a slow increase in nitrate reductase activity for the remainder of the 8 hour induction period. The enzyme levels were not saturated by nitrate concentrations up to 100.0 mM.

4) With the addition of cycloheximide, a protein synthesis inhibitor, there was an immediate decrease in nitrate reductase activity. The

rate of loss of enzyme was not influenced by nitrate.

5) Atrazine, a herbicide, has been found to increase nitrate reductase levels in corn grown under the suboptimal conditions of a low nitrate source and a low temperature (58). In the system described here, it was found to be ineffective in altering the enzyme levels in leaves and roots of corn.

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APPENDIX 1

Modified Hoagland Solution that is Lacking Nitrate, as Adapated from the Earhart Laboratory.

Nutrients		Nutrient Concentration	
		In stock solution (M)	In 1/10 solution (mM)
Solution A			
Calcium chloride	$CaCl_2.H_2O$	1.246 M	0.498 mM
Sequestrene	NaFe, 13%	0.063 M	0.0254 mM
Solution B			
Potassium phosphate	KH_2PO_4	0.252 M	0.1008 mM
Potassium chloride	KCl	1.25 M	0.50 mM
Magnesium sulphate	$MgSO_4.7H_2O$	0.512 M	0.204 mM
Zinc sulphate	$ZnSO_4.7H_2O$	0.195 mM	0.0778 µM
Manganous sulphate	$MnSO_4.H_2O$	2.31 mM	0.925 µM
Copper sulphate	$CuSO_4.5H_2O$	0.0942 mM	0.0377 µM
Boric acid	H_3BO_3	0.0117 M	4.68 µM
Molybdic acid	$MoO_3.2H_2O$	0.236 µM	0.0945 mM

For 1/10 strength Hoagland's solution, mix 0.4 ml of solution A with 0.4 ml solution B in a total volume of 1000 ml double distilled water. A molybdenum supplement of 0.207 µM $Na_2MoO_4.2H_2O$ was also added.