1987

Metabolite Control of Nitrate Reductase Synthesis in Cucurbita Maxima Var. Buttercup

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METABOLITE CONTROL OF NITRATE REDUCTASE SYNTHESIS IN *CUCURBITA MAXIMA* VAR. BUTTERCUP

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A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Science

October

1987

ACKNOWLEDGEMENTS

I want thank my thesis director Dr. John Smarrelli, Jr. and the members of my thesis committee, Dr. Domenic Castignetti and Dr. Warren Jones. For performing my Western blot analyses and acclimating me to laboratory work, I want to thank Michelle Watters. Also, I would like to thank Michelle and her husband, Craig Watters, for their emotional support and for making Loyola University and Chicago enjoyable.

I want to thank my parents, James and Barbara Langendorfer for their financial support throughout my college career. Last and most importantly, I want to thank Susan Martino. Without her assistance, prodding, and confidence in me, I would never have finished this thesis.

VITA

The author, Randall L. Langendorfer, is the son of James Langendorfer and Barbara Ann Langendorfer. He was born November 2, 1961, in Gary, Indiana. His elementary and secondary education were obtained from the Porter Township School System, Porter County, Indiana. He completed his secondary education in January, 1980, at the Portage High School, Portage, Indiana.

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REVIEW OF RELATED LITERATURE

Nitrate Assimilatory Pathway

Approximately 2% of a plant's dry weight consists of nitrogen (1). This organic nitrogen is generally derived from two main sources: atmospheric nitrogen (N_{2}) and nitrate (NO_{3}^{-}). The predominant form of nitrogen available to most higher plants is soil nitrate. The capability to assimilate nitrate is limited to a number of bacteria, fungi, algae, and higher plants (2). In higher plants, the incorporation of nitrate into organic nitrogen occurs primarily in the leaves (3).

The nitrate assimilatory pathway in plants is shown below:

nitrate reductase	nitrite reductase	glutamine synthetase	glutamate synthase

nitrate--->nitrite---->ammonium--->glutamine<---->glutamate

NADH + $2e^{-}$ NAD^{+} + $6e^{-}$ $3Fd_{red}$ Fd_{ox} glutamate ketotoglutarate (2)

As for most multi-step biochemical processes, the first step in the process is usually rate-limiting. This first step in the nitrogen assimilation pathway, the reduction of

nitrate to nitrite, is catalyzed by the enzyme nitrate reductase and is considered the rate-limiting step (4). Nitrite is then further reduced to ammonium which is converted into amino acids. A large amount of reducing power is required to complete the reduction of nitrate to ammonium. Therefore, this pathway is highly regulated to avoid excess energy expenditure when amino acids are present and as a result, much attention has been given to the enzyme nitrate reductase. Also, nitrite reductase and glutamine synthetase exhibit much greater activities than nitrate reductase (5).

Enzymology of Nitrate Reductase

Assimilatory nitrate reductases are soluble electron transferring proteins with molecular weights in the range of 200-300 Kd (4, 6, 7). Electron transfer is mediated by flavin adenine dinucleotide (FAD), cytochrome b_{557}, a molybdenum-pterin cofactor, and enzyme-bound heme iron (8). Electrons are transported between the physically separated pyridine nucleotide oxidation site and nitrate reduction site (6, 9).

Squash nitrate reductase is a homodimer with a subunit molecular weight of 115,000 daltons (10). Each subunit contains one equivalent of FAD, molybdenum-pterin cofactor, and cytochrome b_{557} (2). The physical arrangement of squash nitrate reductase is schematized in

Figure 1. The enzyme uses the pyridine nucleotide NADH as its reducing agent and it has an optimum pH for nitrate reductase of 7.5 (11). The amino acid composition of the enzyme in squash shows acidic amino acids predominating over basic amino acids (10). As a result, the squash nitrate reductase has an isoelectric point of 5.7 (10).

The physiological activity of the enzyme involves the reduction of nitrate to nitrite at the molybdenum cofactor site (12). The reducing power for this reaction is generated by a pyridine nucleotide which donates electrons to the enzyme through a specific enzyme sulfhydryl group (12). Most higher plants possess nitrate reductase enzymes that are able to utilize only NADH as the reducing agent (13). However, certain plants, including maize, barley, foxtail, Lemma minor, and soybeans, have been found that possess nitrate reductase molecules capable of utilizing both NADH and NADPH (13-16).

In addition to the physiological activity of nitrate reductases, nonphysiological activities have been found. Studies have shown that nitrate reductase is capable of reducing nitrate with electrons provided by viologen dyes, methylene blue or reduced flavins (2, 11). A second type of partial nonphysiological activivty is a dehydrogenase activity. This activity enables nitrate reductase to mediate the pyridine nucleotide-linked reduction of various electron acceptors such as dichlorophenolindolphenol,

ferricyanide, cytochrome c, ferrisidorophores, and other one and two electron acceptors (2).

These activities can be selectively inhibited. Studies have shown that molybdenum-linked reactions are inhibited by metal binding agents, which have no effect on dehydrogenase activities (17). Several nitrate reductase specific antibodies have been shown to inhibit these partial activites (18, 19, 20). Also, dehydrogenase and pyridine nucleotide-linked reactions are inhibited by sulfhydryl binding agents such as p-hydroxymercuribenzoate (21). With the aid of these inhibitors, studies to determine the characteristics of nitrate reductase have been possible.

Transport of Nitrate and Location of Nitrate Reductase

Nitrate reductase can be found in almost every type of higher plant cell (2, 21). Nitrate reductase has been isolated from the root cells of various plants (14, 22). However, most nitrate reductase activity is found in the leaves (1, 23, 24). Nitrate absorbed by the roots of a plant must be transported to the leaves where it can be reduced. In most higher plants, it has been shown that nitrogen is mainly transported in the sap by nitrate and the amino acids glutamine and asparagine (25). Nitrate is actively transported into plant cells via membrane-bound nitrate transporters (53). Once nitrate has entered the

cell it is reduced by nitrate reductase. Nitrate reductase is located in the cytoplasm while the remainder of the nitrogen assimilation pathway is chloroplastic (26, 27).

Biochemistry of Nitrate Reductase Activity

Regulation of nitrate reductase has been studied extensively in fungi, most notably *Neurospora crassa* (28). Certain characteristsics of fungi facilitated these studies, particularly the availability of mutants in the nitrogen utilization system. Using the fungus *N. crassa*, Dunn-Coleman *et al.* (29) found that nitrate induced nitrate reductase activity and glutamine repressed nitrate reductase activity even when considerable nitrate was present. Several other reduced forms of nitrogen have been shown to repress nitrate reductase activity in *N. crassa*. For example, ammonium and various amino acids including glycine, proline, and glutamate repress nitrate reductase activity (29).

Regulation of nitrate reductase activities has also been studied in higher plants. Higher plant nitrate reductase mutants have provided a tool to study the regulation of nitrate reductase in much the same manner as that of fungal mutants. From these studies it is evident that separate genes exist for the nitrate reductase apoprotein and the molybdenum cofactor. Mutants of both types of deficiencies have been characterized in

Hordeum vulgare, N. tobacum, Datura innoxia, Glycine max, and Pisum sativum (16, 30-33).

Despite the availability of these mutants, the regulation of nitrate reductase activity in higher plants is much less clearly understood. Studies similar to those in N. crassa have been performed in higher plants, but a well defined pattern of nitrate reductase activity regulation has not emerged.

It has been established that added nitrate greatly increases nitrate reductase activity in both corn (34) and radish (35). Beevers et al. established that different amounts of nitrate in the media are required for optimal stimulation of nitrate reductase activity in various species of higher plants (36). However, there are also examples of nitrate not increasing nitrate reductase activity in higher plants (37, 38).

Little is known concerning the repression of nitrate reductase activity by reduced nitrogen compounds. In corn, Oaks et al. (39) have noted repressive effects on nitrate reductase activity upon the addition of various amino acids. It has also been shown that the amino acids glycine, glutamine, and asparagine strongly inhibit nitrate reductase activity in cotton roots but not in the leaves (40). Ammonium was shown to have only minor effects on nitrate reductase activity in corn roots (39). In radish cotyledons, Ingel et al. (35) found that added ammonium in

the presence or absence of nitrate increased nitrate reductase activity.

Mechanisms by which nitrate reductase activity is regulated other than by the availability of nitrate have also been studied. Most higher plants store large amounts of nitrate in vacuoles. As a result, studies have been performed to examine the role of nitrate flux through the cell as a means of controlling nitrate reductase activity (41). The uptake of nitrate has been viewed as a key regulatory step in the nitrogen assimilation pathway. Nitrate assimilation in plant cells is an active process (53). Nitrate transporters have been shown to be stimulated upon the addition of nitrate in barley (53). Studies of nitrate uptake systems in cultured tobacco cells (42) and barley seeds (43) have shown that these systems are also activated upon the addition of nitrate. Following an initial lag period, the rate of nitrate reduction was correlated with the rate of uptake.

Molecular Biochemistry of Nitrate Reductase Regulation

Much attention has focused on the molecular aspects of the regulation of nitrate assimilatory enzymes, most notably nitrate reductase. Two possible mechanisms for the regulation of nitrate reductase have been studied: activation/deactivation and synthesis/degradation. In *Chlorella*, changes in nitrate reductase activity are the

result of a lack of an enzymatically active nitrate reductase protein (44). In barley, however, Somers et al. concluded that nitrate reductase is regulated by de novo synthesis and degradation of protein, not activation/deactivation of a pre-existing protein (20). In this study, plants were grown under various nitrate and illumination conditions. Western blots performed on total proteins detected the presence of cross reacting material with anti-nitrate reductase antibodies only in the plants that received both light and nitrate. In vitro studies by Commere et al. (54) in maize and tobacco have also shown nitrate reductase to be regulated by de novo synthesis. They have shown that the nitrate reductase enzyme does not undergo a maturation process because the enzyme is not translocated out of the cytoplasm after synthesis.

Using in vitro translation and immunoprecipitation procedures, similar results were noted for the regulation of nitrite reductase. Under the conditions of nitrate and light, Gupta and Beevers (45) found a protein corresponding to the molecular weight of nitrite reductase. In the absence of nitrate and light the protein was undetected. They concluded that increases in nitrite reductase activity were the result of increases in nitrite reductase protein synthesis.

MATERIALS AND METHODS

Plant Material

Squash seedlings, Cucurbita maxima, were grown in an environmental chamber which received 47 watts/m^2 of artificial illumination for 16 hours per day at 27 C. The seeds were germinated in vermiculite and grown in individual containers, 10 seeds per container, for 7 days. The seeds were watered on alternate days with tap water during this period. On day 7, seedlings were irrigated with a modified Hoagland's nutient medium (45). 5 mM KNO_3 was omitted from the medium and 5 mM $CaCl_2$ was substituted for 5 mM $Ca(NO_3)_2$. Varying concentrations and combinations of nitrate and reduced nitrogen sources were added to the Hoagland's solution to study their effects on nitrate reductase activity in squash cotyledons. The nitrate source was KNO_3 and the reduced nitrogen sources included NH_4Cl and the amino acids glutamine, glycine, arginine, asparagine, and phenylalanine.

In the experiments to test the effects of nitrate and glutamine on nitrate reductase activity, three nitrate concentrations were used: 50 mM, 10 mM, and 1 mM. Within each nitrate concentration, glutamine concentrations were increased to establish three nitrate:glutamine ratios. These ratios were 5:1, 1:1, and 1:5. In the experiments to

test the effects of different amino acids and ammonium on nitrate reductase activity, a constant 10 mM nitrate concentration was used. The concentrations of the other metabolites were 1 mM, 10 mM, and 50 mM. This established nitrate:metabolite ratios of 10:1, 1:1, and 1:5 respectively. Individual containers received nutrient solutions containing varying concentrations of nitrate and a reduced nitrogen source. Each container constituted a treatment group. Treatment group experiments were repeated three separate times.

The effects of various metabolites on nitrate reductase activity were measured using squash cotyledons as as a source of tissue. Nitrate reductase activity in squash cotyledons is zero in the absence of a nitrate source (unpublished results). Fully expanded cotyledons were randomly harvested from each treatment group at both 20 and 24 hours following irrigation on day 7. Peak nitrate reductase activity occurs between 20 and 24 hours following irrigation with a nitrate source (unpublished results). The 20 hour harvest took place 4-6 hours into the light cycle to eliminate the inhibitory effects of darkness on the nitrate reductase activity. Samples consisted of three randomly harvested cotyledons. These cotyledons were sealed in plastic bags and quick frozen in liquid nitrogen and stored at -70 C until assayed.

Samples were prepared for assaying by homogenizing

in a mortar and pestle in cold extraction buffer containing 0.1 M KH_2PO_4 and 1 mM EDTA at pH 7.5. This pH results in optimum activity of the NADH linked enzyme in squash (11). The homogenate was centrifuged at 13,000 rpm for 10 minutes to remove the cellular debris using a Sorval RC2-B centrifuge. The supernatant was designated as the crude enzyme extract.

Nitrate Reductase Activity Assays

NADH linked nitrate reductase activity in squash was assayed using a modification of the procedure of Hageman and Reed (24). The assay mixture contained 1.8 ml of 0.1 M KH_2PO_4 and 0.1 mM KNO_3 at pH 7.5 and 0.1 ml of crude enzyme extract. The reaction was initiated by the addition of 0.1 ml of a 2 mM NADH solution and terminated by the addition of 0.1 ml of a 1 M zinc acetate solution. Zinc acetate was used because it markedly enhanced the extent of nitrite color formation and apparent nitrate reductase activity (47). A control was also run to make sure that the zinc acetate was not being reduced and affecting the results.

Following centrifugation for 5 minutes to remove the precipitate formed upon addition of zinc acetate, the supernatant was used in the colorimetric determination of nitrite. One percent sulfanilamide in 20% HCl and 0.02% N-(naphtyl) ethylene diamine dihydrochloride (NED) were used as the colorimetric reagents. These reagents complexed

with nitrite to form an azo dye with a peak absorbance at 540 nm. All spectrophotometric readings were taken on a Bausch and Lomb Spectronic 21. The activity is expressed in nmoles nitrate reduced/minute/ml sample.

Protein Determination Assays

Following precipitation from the crude extract with 10% trichloroacetic acid (TCA), protein concentrations were determined by the method of Lowry et al. (48), using bovine serum albumin as the protein standard. Protein concentrations divided into the nitrate reductase activity assay results gave a final specific activity expressed in nmoles nitrate reduced/minute/mg protein.

Cellular Nitrate Determination Assays

Cotyledons from each treatment group were homogenized in 1 M NaOH using a mortar and pestle. The homogenate was boiled for ten minutes and used in the nitrate determination assay. A modified chromotropic acid procedure of West and Ramachandran (49) was used to quantitate endogenous nitrate concentrations. The assay mixture contained 1 drop of a 0.83 M sulfite/0.32 M urea solution and 0.8 ml antimony solution. The antimony solution contained 0.5 grams of the metal dissolved in 80% sulfuric acid. These two reagents masked the effects of nitrite and chloride respectfully. The colorimetric

reagents added were 0.8 ml of 0.1% chromotropic acid in H_2SO_4 and 2 ml of concentrated H_2SO_4. The chromotropic acid and H_2SO_4 complexed with nitrate to form a yellow color with a peak absorbance at 410 nm. A 1 M KNO_3 stock was used as the nitrate standard. The assay's results were expressed in umoles nitrate/gram fresh weight leaf tissue.

SDS-PAGE and Western Blots

Plant samples were prepared by combining equal parts crude enzyme extract and a 2X SDS treatment buffer consisting of 0.125 M tris-Cl (pH 6.8), 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol. The mixture was boiled for 3 minutes to denature the protein and to allow the SDS to combine with the protein. Seven and one half percent gels prepared as described by Laemmli (50) were run in duplicate at a constant current of 60 mA. Coomassie blue was used to stain the gels. Gels were placed in stain containing 0.125% Coomassie blue, 50% methanol, and 10% acetic acid for four hours. Gels were fixed for one hour in 50% methanol and 10% acetic acid. Gels were then transferred to a 5% methanol and 7% acetic acid solution for at least six hours at which time they were dried.

Western blots, as outlined by Maniatis (51), were done to visibly quantitate nitrate reductase protein present in the samples. Protein separated via SDS-PAGE were transferred to nitrocellulose using 70 V for 2.5-3

hours in a transblot apparatus. One tenth of a ml/100 ml buffer of an antiserum specific to the nitrate reductase protein was used to bind nitrate reductase. The second antibody, specific against the nitrate reductase antibody, was a goat anti-rabbit antibody. This antibody was linked to the indicator enzyme horse radish peroxidase (HRP). The substrates for HRP were added to produce the enzyme's product, a purple colored dye (51).

Data and Statistics

Each experiment was repeated three separate times; within each experiment, all nitrate reductase, protein concentration, and cellular nitrate concentration assays were performed in duplicate or triplicate for each treatment group. Means and standard deviations were calculated for all data. ANOVAs (Analysis of Variance) were performed on the results to determine if the differences were statistically significant (52).

Materials

Squash seeds (Cucurbita maxima var. buttercup) were obtained from W. Atlee Burpee Co. in Warminster, Pennsylvania. All chemicals used in assay mixtures and SDS-PAGE were reagent grade or better. Distilled deionized water was used for all procedures.

RESULTS

Nitrate and Glutamine Effects on Squash Nitrate Reductase

Preliminary studies were performed using nitrate, glutamine and a combination of these two metabolites as sole nitrogen sources. The effects of nitrate and glutamine concentrations on the amount of nitrate reductase activity present in whole plants are shown in Table 1. All the nitrate reductase activities listed are determined from the induction of nitrate reductase activity under the assay conditions noted in Materials and Methods. The activites are not the result of enzyme inhibition in whole plants.

The results in Table 1 show that in the presence of 50 mM nitrate, nitrate reductase activity increased by more than 40-fold over the control. In the presence of glutamine, nitrate reductase activity was similar to the control. When both 50 mM concentrations of nitrate and glutamine were present, intermediate nitrate reductase activity was observed. This activity was significantly lower ($p < 0.05$) than when only 50 mM nitrate was present. This showed that under nitrate conditions that are normally saturating (50 mM), glutamine is able to override these effects to a large degree. A 50 mM nitrate/50 mM glutamine combination yields only a 7-fold increase in nitrate reductase activity compared with the control. Under these

conditions, glutamine does not, however, completely repress nitrate reductase activity in the presence of nitrate. Therefore, it can be said that nitrate alone increases nitrate reductase activity, glutamine alone has no effect on activity and a combination of nitrate and glutamine exhibits an intermediate level of activity, all relative to the control.

To expand on these preliminary results, studies were undertaken using an expanded range of nitrate and glutamine concentrations. Three constant nitrate series were established; 50 mM, 10 mM, and 1 mM. Table 2 and Figure 2 show the effects on nitrate reductase activity when glutamine concentrations were increased relative to these constant nitrate concentrations. Again, it should be noted that the activities seen are the result of the induction of nitrate reductase activity under the assay conditions and not the result of enzyme inhibition in intact plants.

The results show that nitrate alone within each fixed series exhibited maximal activity. When glutamine was present and concentrations were increased, the nitrate reductase activities observed were significantly decreased ($p < 0.05$). There is an inverse relationship between glutamine concentration and nitrate reductase activity when nitrate concentrations remain constant.

For example, this relationship can be seen in the 10 mM nitrate series. Nitrate alone gave a maximal nitrate

reductase activity of 3.5 nmoles nitrate reduced/minute/mg protein. As the glutamine concentration was increased from 2 mM to 50 mM, the nitrate reductase activity was decreased from 1.18 to 0.22 nmoles nitrate reduced/minute/mg protein respectfully. Again it should be noted that the presence of glutamine during the day prior to harvest did not completely repress nitrate reductase activity in the presence of nitrate. The inability of glutamine to abolish nitrate reductase activity could be best seen when 250 mM glutamine was added. At 50 mM nitrate/250 mM glutamine the activity was 0.21 nmoles nitrate reduced/minute/mg protein. This activity was a 2-fold increase compared to the control.

A relationship has been shown to exist between glutamine and nitrate reductase activity. This relationship was substantiated by two factor analyses of variance (ANOVA). The ANOVAs were performed on the data obtained from the 50 mM and 10 mM nitrate series'. This data is presented in Table 2.

The ANOVAs were used to determine the effects of each metabolite (nitrate and glutamine) on nitrate reductase activity. The results showed that the nitrate concentration was not the controlling factor affecting nitrate reductase activity. Both 50 mM and 10 mM nitrate concentrations were saturating and had the same effect on nitrate reductase activity.

The ANOVAs showed that glutamine concentrations significantly affected nitrate reductase activity ($p > 0.5$). An increase in glutamine concentration reduced nitrate reductase activity. This confirmed the conclusions made earlier concerning Table 2 and Figure 2. It was also determined that there was not an interaction between nitrate and glutamine concentrations on nitrate reductase activity. This can be clearly shown using the concept of ratios. Within the fixed nitrate series', three ratios of nitrate:glutamine were established, 5:1, 1:1, and 1:5. If a significant interaction between nitrate and glutamine existed, the effect of a ratio on nitrate reductase activity should have remained constant from series to series.

For example, the effect on nitrate reductase activity of the 1:1 ratio in the 50 mM nitrate series should have been the same as the effect of the 1:1 ratio in the 10 mM nitrate series. The ANOVAs showed that these ratios had no effect on nitrate reductase activity ($p < 0.05$). From these analyses, glutamine concentration was the controlling factor affecting nitrate reductase activity. It took a large quantity of glutamine to override the stimulatory effects of nitrate. However, even large nonphysiological concentrations of glutamine (50 mM and 250 mM) could not completely abolish nitrate reductase activity.

Figure 3 shows the plot of the cellular nitrate concentration for the 50 mM and 10 mM nitrate series'. These two groups exhibited similar results. The treatment groups lacking glutamine had maximal cellular nitrate concentrations. The figure clearly shows that as the concentration of glutamine was increased, the concentration of nitrate in the cells was reduced. Plants receiving no nitrate or glutamine had a nitrate concentration of 6.5 umoles nitrate/gram fresh weight leaf tissue. This is the lowest concentration observed. When nitrate was added, cellular nitrate concentrations increased proportionally. The 50 mM series had a much higher cellular nitrate concentration than the 10 mM and 1 mM series'. When glutamine was added and its concentration increased, cellular nitrate concentrations within each series decreased. There is an inverse relationship between glutamine concentration and cellular nitrate concentration within each fixed nitrate series. However, glutamine, even at high nonphysiological concentrations, did not reduce cellular nitrate concentrations to those of the control.

To determine if the decrease in cellular nitrate concentrations resulted in changes in cellular protein content, SDS-PAGE was performed on the treatment groups. Figure 4 is a 7.5% SDS-PAGE gel. Samples run were total squash cotyledon proteins from the 50 mM nitrate series. Protein banding patterns were similar in all treatment

groups. The major band, consisting of 20-40% of celllular protein, ribulose biphosphate carboxylase, is in the 50 kilodalton (Kd) range.

The band position of the nitrate reductase protein subunit is 115 Kd. This position was localized using molecular weight standards and a partially purified nitrate reductase protein sample. The Coomassie blue staining procedure used was not capable of detecting quantitative differences in nitrate reductase protein. Western blotting was used to increase sensitivity.

Proteins transferred to nitrocellulose were immunologically assayed to determine quantitative amounts of nitrate reductase protein present. Figure 5 shows the results of a Western blot made from a dulpicate of the gel shown in Figure 4. The degree of nitrate reductase antibody binding was proportional to the amount of nitrate reductase protein present. The binding capacity also relates to nitrate reductase activities (Table 2). The sample that had the highest amount of nitrate reductase activity, 50 mM nitrate/0 mM glutamine, showed the most pronounced antibody binding. Therefore, there is a correlation between amount of nitrate reductase protein and actual nitrate reductase activity.

Several other important observations can be made from the blot. When nitrate was absent from the treatment group, nitrate reductase protein was not present. There

was no antibody binding seen. In the presence of nitrate, nitrate reductase protein was observed. Antibody binding is seen in all samples that contained nitrate. Nitrate must therefore be present to stimulate the production of nitrate reductase protein.

In the presence of glutamine as the sole source of nitrogen, protein was again not observed. Clear differences can be observed when different concentrations of glutamine were used in the presence of nitrate. The enzyme concentrations were visably decreased as the concentration of glutamine was increased from 10 mM to 250 mM. At 0 mM nitrate/250 mM glutamine, no protein can be seen. Glutamine reduced the amount of nitrate reductase specific protein being made even in the presence of nitrate.

Other Amino Acid Effects on Nitrate Reductase Activity

The amino acids glycine, arginine, asparagine, phenylalanine and glutamine (control) were employed in experiments identical to those where only glutamine was used. These amino acids, like glutamine, are reduced forms of nitrogen and end products of the nitrogen assimilatory pathway. Table 3 shows the effects of the amino acids on nitrate reductase activity at two concentrations, 50 mM and 10 mM. The nitrate concentration was fixed at 10 mM. The percent activity is relative to the control which lacked

any amino acid. This control had 100% activity. The results show that all the amino acids examined decrease nitrate reductase activity even in the presence of nitrate. This reduction in activity increased as the amino acid concentration was increased. For example, when 10 mM glycine was present, the activity was only 66% that of the control. When the glycine concentration was increased to 50 mM, the nitrate reductase activity was reduced to 38%. None of the amino acids tested , however, had as dramatic an effect on nitrate reductase activity as that of glutamine.

Table 4 summarizes the cellular nitrate concentrations determined in the presence of amino acids. The nitrate concentration is expressed relative to the treatment group lacking amino acid, that is, 10 mM nitrate/0 mM amino acid. When nitrate and amino acids were added in combination, the cellular nitrate concentrations were decreased as the concentration of amino acid increased. This occurred for all amino acids tested. There was an inverse relationship between amino acid and cellular nitrate concentrations. In the presence of glutamine the cellular nitrate concentration was the lowest.

Ammonium Effects on Nitrate Reductase Activity

Figure 6 and Table 5 show the effects of ammonium, a

nitrogen assimilatory pathway intermediate, on nitrate reductase activity in the presence of nitrate. The nitrate concentration was constant at 10 mM. Figure 6 plots the activities obtained at both the 20 and 24 hour harvests. Ammonium had little effect on nitrate reductase activity. An increase in the ammonium concentration caused neither a significant decrease nor increase in nitrate reductase activity. The activities were in the same range as those found for the controls in the previous experiments, the 10 mM nitrate/0 mM glutamine treatment group. This showed that ammonium did not increase the nitrate reductase activity to the same degree even though its concentration increased. Table 5 shows the percent activities of the data presented in Figure 6. The control for each time period again served as the reference, 100% activity. From the data it appeared that ammonium had a slight enhancing effect on nitrate reductase activity. This effect is, however, not statistically significant.

Table 6 shows the cellular nitrate concentrations found at various ammonium concentrations. The levels are expressed in percent nitrate relative to the control. There was a slight decrease in nitrate concentrations compared with the control. However, as ammonium concentration increased there was no change in the cellular nitrate concentrations.

DISCUSSION

Tables 1 and 2 indicated squash nitrate reductase activities are greatly increased in the presence of exogenous nitrate. In the absence of added nitrate, nitrate reductase activity is found to be zero. Also, glutamine, in the presence of nitrate, significantly inhibits squash nitrate reductase activity (Table 2, Figure 2). An increase in glutamine concentration causes a decrease in nitrate reductase activity. These effects are determined from the induction of nitrate reductase activity under the assay conditions outlined in Materials and Methods. These assay conditions eliminate the effects of any enzyme inhibition that may be resulting from the metabolites in the intact plant.

This enhancement of nitrate reductase activity by nitrate and the repression of nitrate reductase activity by glutamine are consistent with other nitrate studies in both higher plants and fungi. In *N. crassa*, Dunn-Coleman *et al.* found added nitrate to increase nitrate reductase activity and glutamine to repress this activity. Similar results were found in corn and radish by Hageman *et al.* (34) and Ingel *et al.* (35) respectfully.

ANOVAs show that glutamine concentration is the overriding factor controlling nitrate reductase activity.

Squash plants given equal 50 mM concentrations of nitrate and glutamine show 25% the nitrate reductase activity of plants given only 50 mM nitrate. Saturating nonphysiological amounts of glutamine are not able to completely eliminate nitrate reductase activity. It has recently been shown in our laboratory that the glutamine concentrations inside the cell correlate with the concentrations given to the plant (Michelle Watters, personal communication).

Nitrate reductase activities are dependent on the quantity of nitrate reductase protein. This correlation is shown in Table 2 and Figure 4. By comparing the nitrate reductase protein levels and activities observed in each treatment group it is evident that the greater the amount of nitrate reductase protein present, the greater the nitrate reductase activity. Therefore, glutamine must decrease nitrate reductase activity by reducing the quantity of active protein.

The control of nitrate reductase synthesis involves both nitrate and glutamine. Nitrate is required to stimulate nitrate reductase synthesis. This is evident from the SDS-PAGE and the Western blot results (Figures 4 and 5). When nitrate is absent nitrate reductase is not produced. Upon the addition of nitrate (50 mM), the <u>de novo</u> synthesis of nitrate reductase occurs. Nitrate does not appear to activate a nitrate reductase precursor.

If a precursor existed, it would probably also be detected by the nitrate reductase specific antibody and appear on the blot even when no nitrate is present. Somers et al. (20) found similar results in barley seeds. In their experiment plants were grown under various nitrate and light conditions. Western blots using nitrate reductase specific antibodies showed that only those plants that received nitrate and light had synthesized nitrate reductase enzyme. They concluded that nitrate reductase is regulated by de novo production and degradation of protein, not activation/inactivation of a pre-existing protein (20).

Glutamine, in the presence of nitrate, reduces the quantity of nitrate reductase protein synthesized. As the glutamine concentration is increased, the amount of nitrate reductase protein produced is reduced. Glutamine is not deactivating previously synthesized enzyme molecules. A glutamine inactivated nitrate reductase molecule would probably also be detected on a Western blot using nitrate reductase specific antibodies. Under constant nitrate conditions, the same quantity of nitrate reductase would be seen despite the concentration of glutamine present. Increasing glutamine concentrations would inactivate more nitrate reductase molecules but they would probably still react with nitrate reductase specific antibodies and appear on the blot. Figure 5 shows the quantity of nitrate reductase protein is decreased as the concentration of

glutamine is increased. Glutamine does not inactivate a precursor but reduces the actual quantity of nitrate reductase protein synthesized. Glutamine inhibits the stimulatory effects of nitrate on nitrate reductase production. These findings are consistant with those found by Somers et al. (20).

These experiments suggest that glutamine's repressive effects on the nitrogen assimilation pathway involve some aspect of glutamine metabolism or glutamine directly. When glutamine is present in sufficient quantities, the cell does not need to utilize the nitrogen assimilation pathway. The cell avoids expending large amounts of energy necessary to reduce nitrate to glutamine when it is not needed. Glutamine or some aspect of glutamine metabolism may therefore act as a direct repressor of this pathway, more specifically, repress the synthesis of the nitrate reductase gene.

Dunn-Colemann et al. (29) have proposed the mechanism of direct repression in N. crassa. They have stated that nitrogen metabolite repression is ultimately governed by glutamine or glutamine sythetase. They have discovered the existance of two forms of glutamine synthetase, an octomer and a tetramer. Using N. crass mutants, they have postulated that glutamine synthetase can bind the nit-2 gene, the gene believed to code for nitrate reductase, and thereby cause the effects of nitrogen

metabolite repression. They found that when glutamine is in sufficient quantities, the octomeric form is predominant. This form if able to bind the nitrate reductase gene and repressed nitrate reductase synthesis would stop the acquisition of unnecessary nitrogen assimilation. However, when nitrogen is limiting, the tetrameric form is predominant. This form, which if unable to bind the nitrate reductase gene, could not halt the utilization of the nitrogen assimilation pathway. Nitrogen utilization and nitrate reductase activity would then be observed.

Glutamine and glutamine synthesis may be able to take on a similar role in higher plant nitrate reductase regulation. There may be two forms of glutamine synthetases in the cell with the predominant form depending upon the amount of nitrogen present. When nitrogen is abundant, the predominant form would bind the nitrate reductase gene and repress the nitrogen utilization pathway. However, when nitrogen is limiting the predominant form would be unable to bind the gene and the pathway would be derepressed. This would be consistant with the results obtained in this study.

From these experiments, it also appears that glutamine inhibits the stimulatory effects of nitrate by blocking the transport of nitrate into the plant cell. Glutamine inhibits the net influx of nitrate. Nitrate

assimilation in plant cells is an active process (53). Nitrate transporters are believed to be stimulated upon the addition of nitrate. These receptors, located in the cell membrane and dependent on metabolic energy, bind nitrate molecules, and actively transport nitrate into the cell (53). Glutamine molecules may also bind to the nitrate stimulated receptors and reduce their efficiency. This in turn reduces the net influx of nitrate across the membrane. This reduced net nitrate influx would reduce the nitrate stimulated production of nitrate reductase.

Figure 3 supports this idea. As the concentration of glutamine increases, the cellular nitrate concentration decreases. The presence of glutamine inhibits nitrate from entering the cell. The possibility that the cell may have a greater net nitrate exflux than influx in the presence of glutamine exists, however.

ANOVAs show that it is the absolute concentration of glutamine that is required to reduce nitrate reductase activity. Many glutamine molecules would be needed to bind all the available nitrate receptors and cause a decrease in nitrate reductase activity. If glutamine and nitrate competitively bind the receptors, as the concentration of glutamine increases, the number of receptors unable to transport nitrate would increase. The nitrate induced nitrate reductase synthesis would decrease and this would result in a decrease in nitrate reductase activity.

Competitive binding would enable nitrate to enter the cell and stimulate nitrate synthesis as long as nitrate is present. The results show that nitrate reductase activity is never completely eliminated with the glutamine concentrations studied. Nitrate reductase activities do not approach the levels seen in the absence of nitrate. Cellular nitrate levels (Figure 3) also show that nitrate concentrations increase even under extremely high glutamine concentrations. This data support competitive binding of both nitrate and glutamine to the nitrate receptors.

Other amino acids besides glutamine were chosen in order to test their effects on nitrate reductase activity and synthesis. Like glutamine, these amino acids are end products of the nitrogen assimilation pathway. Glycine, arginine, asparagine, and phenylalanine were chosen because they represent the different properties of amino acids: size, charge, aromaticity, extra amino groups. The results obtained from these amino acids should be representative of all amino acids.

The data show that the amino acids act similarly to glutamine. They decrease nitrate reductase activity in the presence of nitrate and inhibit the net influx of nitrate into the plant cell (Tables 3 and 4). Amino acids have also been shown to repress nitrate reductase activity in other higher plants. Oaks et al. have found that various amino acids repress nitrate reductase activity in

corn (39). Similarly, the amino acids glycine, glutamine, and asparagine, have been shown to strongly repress nitrate reductase activity in cotton roots (40). Nitrate reductase activity, however, was not inhibited in the cotton leaves.

Since various amino acids have repressed nitrate reductase activity in higher plants, it seems likely that these amino acids would all function, like glutamine, by restricting the net influx of nitrate. All amino acid may be able to bind the nitrate transport receptors to restrict the net nitrate influx. Receptor binding efficiency would vary between amino acids, none having as great an affinity for the receptors as glutamine. This is seen in Table 4. Glutamine inhibits net nitrate influx more severely than any other amino acids tested.

An indirect method of restricting net nitrate influx by amino acids may also occur. Glutamine may be the only amino acid capable of binding the receptors. Other amino acids may be quickly converted to glutamine, which then binds the nitrate receptors. It would take each amino acid a different length of time for this conversion. This time variation could also account for the discrepancies in the net nitrate influx inhibition. Whatever the mechanism, all amino acids inhibit net nitrate influx. The decrease in cellular nitrate causes a decreases in the stimulation in nitrate reductase synthesis and a decrease in nitrate reductase activity.

Ammonium, an intermediate in the nitrogen assimilatory pathway, is toxic to plant cells in high concentrations. To protect the plant, it is quickly incorporated into glutamine. Unlike amino acids, ammonium has no effect on nitrate reductse activity and nitrate uptake in squash cotyledons. This is consistant with the results found by Oaks et al. in corn roots (39). They demonstrated that ammonium had only minor effects on nitrate reductase activity. However, in radish cotyledons, Ingel et al. (35) determined that ammonium in the presence or absence of nitrate increased nitrate reductase activity.

Because ammonium is quickly incorporated into glutamine, it should be able to decrease nitrate reductase activity and net nitrate influx. This may not be seen because in the presence of a sufficient pool of amino acids, the ammonium may be sequestered in the cell following its uptake. This ammonium may never be converted to amino acids, especially glutamine, and may not have any effect on nitrate reductase activity or nitrate reductase protein activity.

Figure 1. SQUASH NITRATE REDUCTASE SCHEMATIC. Nitrate Reductase is depicted as an electron shuttle system with separate sites for NADH and nitrate reduction. Electrons are donated by the pyridine nucleotide NADH to an enzyme bound sulfhydryl group (SH). The electrons are then transferred to the nitrate reduction site via flavin adenine dinucleotide (FAD), cytochrome b (CYT-B_{557}), and the molybdenum cofactor (MO).

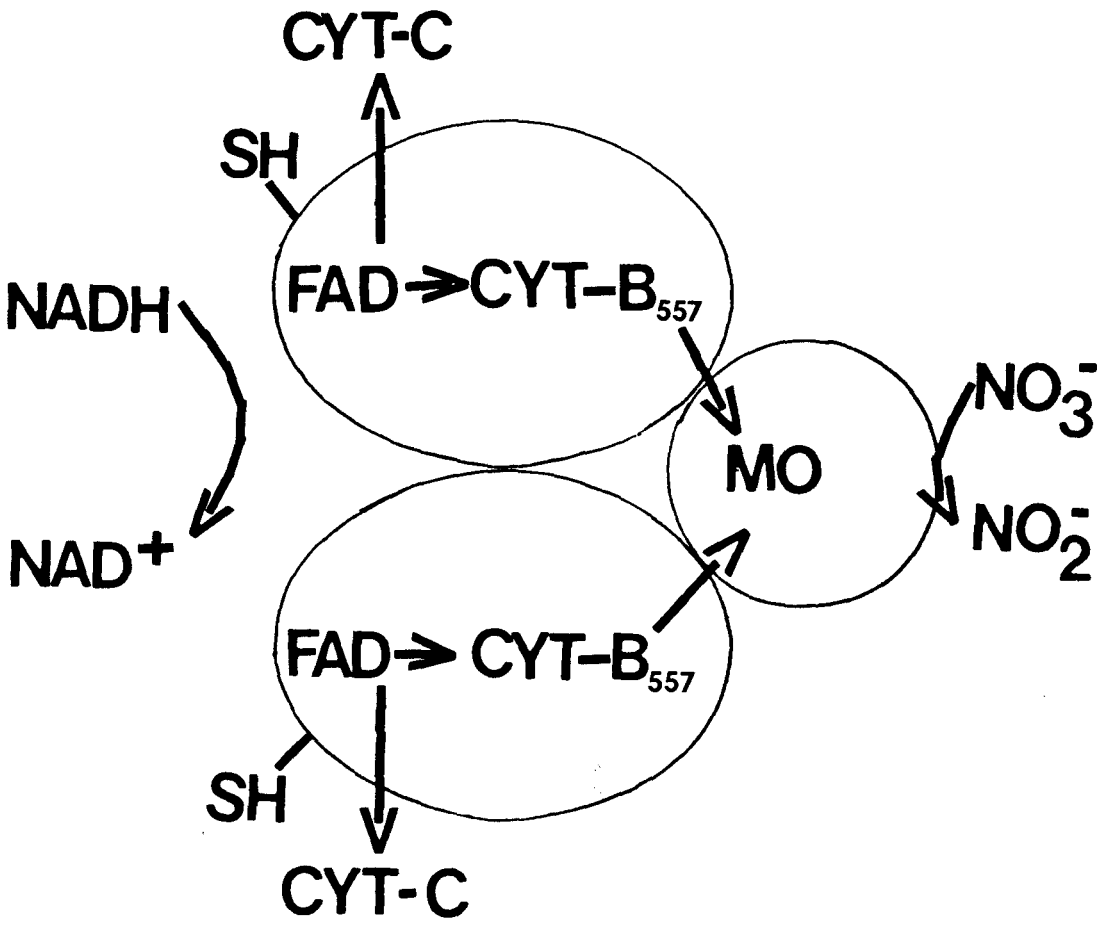

CYT-C
SH
FAD→CYT-B557
NADH
NAD+
MO
NO3-
NO2-
FAD→ CYT-B557
SH
CYT-C

Table 1

THE EFFECTS OF ABSOLUTE NITRATE AND GLUTAMINE CONCENTRATIONS ON THE AMOUNT OF NITRATE REDUCTASE ACTIVITY PRESENT IN WHOLE PLANTS

TREATMENT	ACTIVITY*
50 mM NITRATE	4.00
10 mM GLUTAMINE	0.10
50 mM NITRATE/ 50 mM GLUTAMINE	1.06
0 mM NITRATE/ 0 mM GLUTAMINE	0.11

*nmoles nitrate reduced/minute/mg protein. Plants were irrigated on day 7 with media described in Materials and Methods, and all activities determined from the 24 hr. harvest. All values statistically significant, $p < 0.05$.

Table 2

THE EFFECT OF ABSOLUTE NITRATE AND GLUTAMINE CONCENTRATIONS ON THE AMOUNT OF NITRATE REDUCTASE ACTIVITY PRESENT IN WHOLE PLANTS, EXTENDED

NITRATE, mM	GLUTAMINE, mM	ACTIVITY*
0	0	0.11
0	10	0.10
50	0	4.00
50	10	3.38
50	50	1.06
50	250	0.21
10	0	3.50
10	2	1.81
10	10	1.51
10	50	0.22
1	0	0.80
1	0.2	0.49
1	1	0.38
1	5	0.34

*nmoles nitrate reduced/minute/mg protein (Plants were irrigated on day 7 with media described in Materials and Methods and all activities determined from the 24 hour harvest). All values statistically significant, $p < 0.05$.

Figure 2. THE EFFECTS OF ABSOLUTE NITRATE AND GLUTAMINE CONCENTRATIONS ON THE AMOUNT OF NITRATE REDUCTASE ACTIVITY PRESENT IN WHOLE PLANTS. Squash plants were irrigated on day 7 with media described in Materials and Methods and all activities were determined from cotyledons harvested at 24 hours. Nitrate reductase activity is expressed in nmoles nitrate reduced/min./mg protein. ■ = 50 mM nitrate and ○ = 10 mM nitrate concentrations.

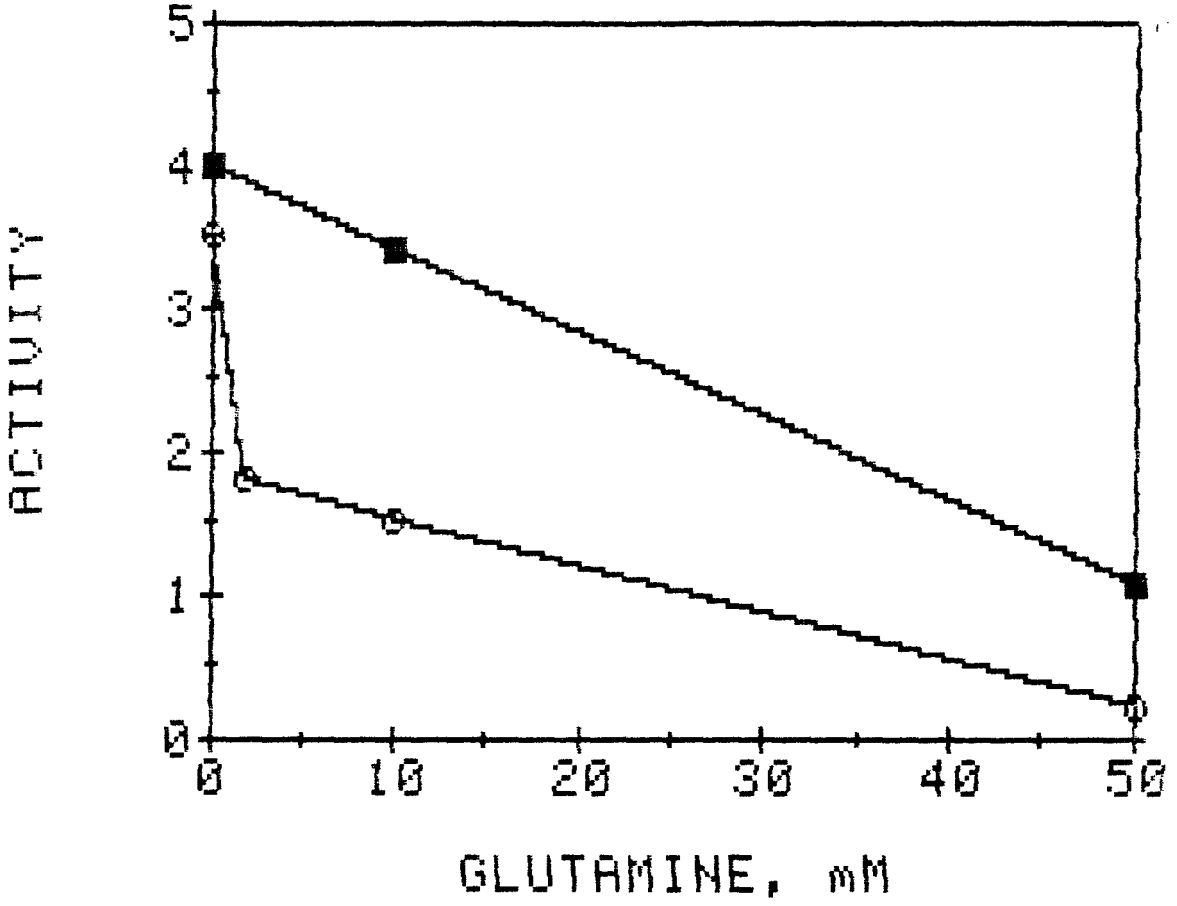

ACTIVITY
5
4
3
2
1
0
0
10
20
30
40
50
GLUTAMINE, mM

Figure 3. CELLULAR NITRATE CONCENTRATIONS PRESENT IN WHOLE PLANTS. Squash plants were irrigated on day 7 with media described in Materials and Methods and all activities were determined from cotyledons harvested at 24 hours. Cellular nitrate concentration is expressed in umoles nitrate/gram fresh weight tissue. ■ = 50 mM nitrate and ○ = 10 mM nitrate concentrations.

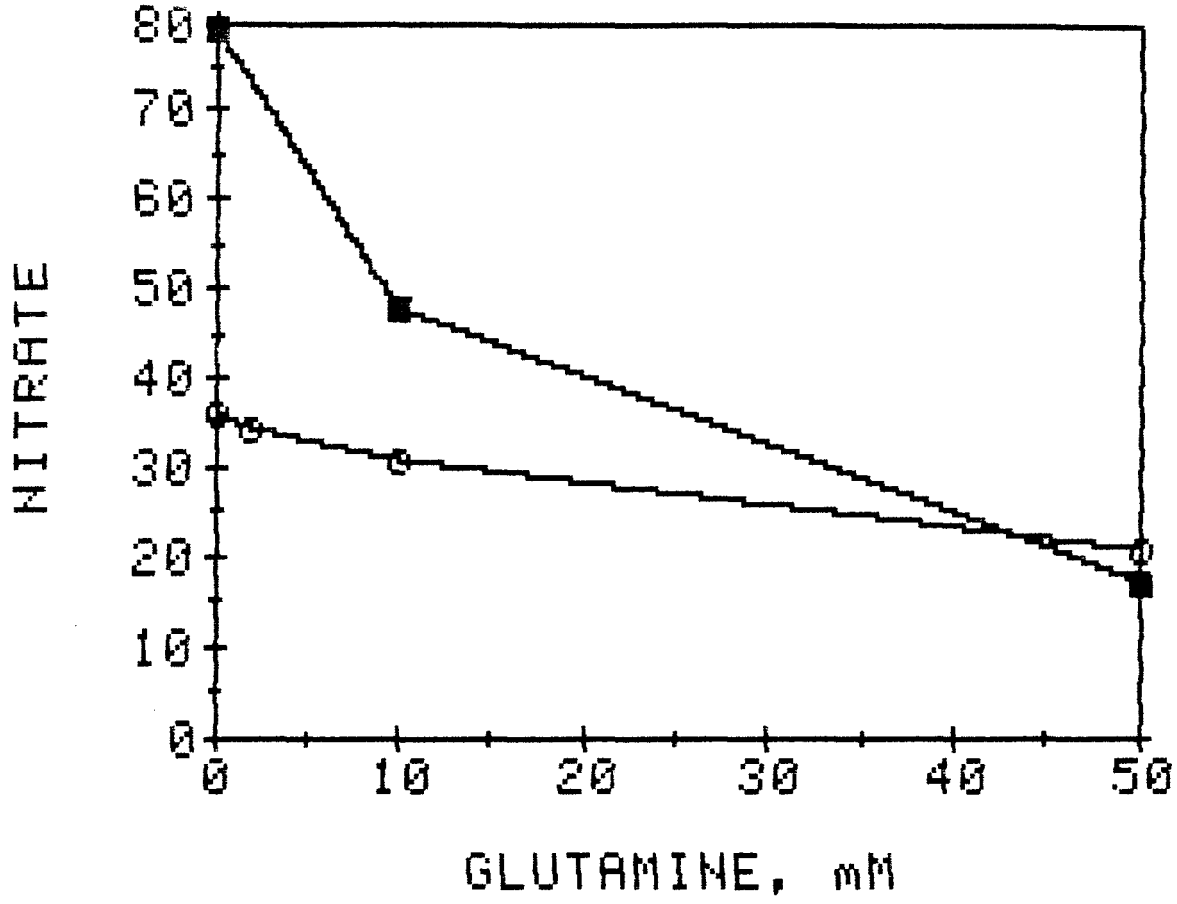

NITRATE
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
GLUTAMINE, mM

Figure 4. 7.5% TOTAL SQUASH PROTEIN SDS-PAGE. The gel was prepared as per Laemlli (50). Cotyledons were harvested at 24 hours following irrigation and samples were prepared as described in Materials and Methods. The gel was run at 60mA and stained with Coomassie blue. The samples are as follows:

Lane 1 = Molecular weight markers

Lane 2 = 0 mM nitrate/0 mM glutamine, 20 ul

Lane 3 = 0 mM nitrate/0 mM glutamine, 10 ul

Lane 4 = 50 mM nitrate/0 mM glutamine, 20 ul

Lane 5 = 50 mM nitrate/0 mM glutamine, 10 ul

Lane 6 = 50 mM nitrate/10 mM glutamine, 20 ul

Lane 7 = 50 mM nitrate/10 mM glutamine, 10 ul

Lane 8 = 50 mM nitrate/50 mM glutamine, 20 ul

Lane 9 = 50 mM nitrate/50 mM glutamine, 10 ul

Lane 10 = 50 mM nitrate/250 mM glutamine, 20 ul

Lane 11 = 50 mM nitrate/250 mM glutamine, 10 ul

Lane 12 = 0 mM nitrate/250 mM glutamine, 20 ul

Lane 13 = 0 mM nitrate/250 mM glutamine, 10 ul

Lane 14 = purified nitrate reductase, 20 ul

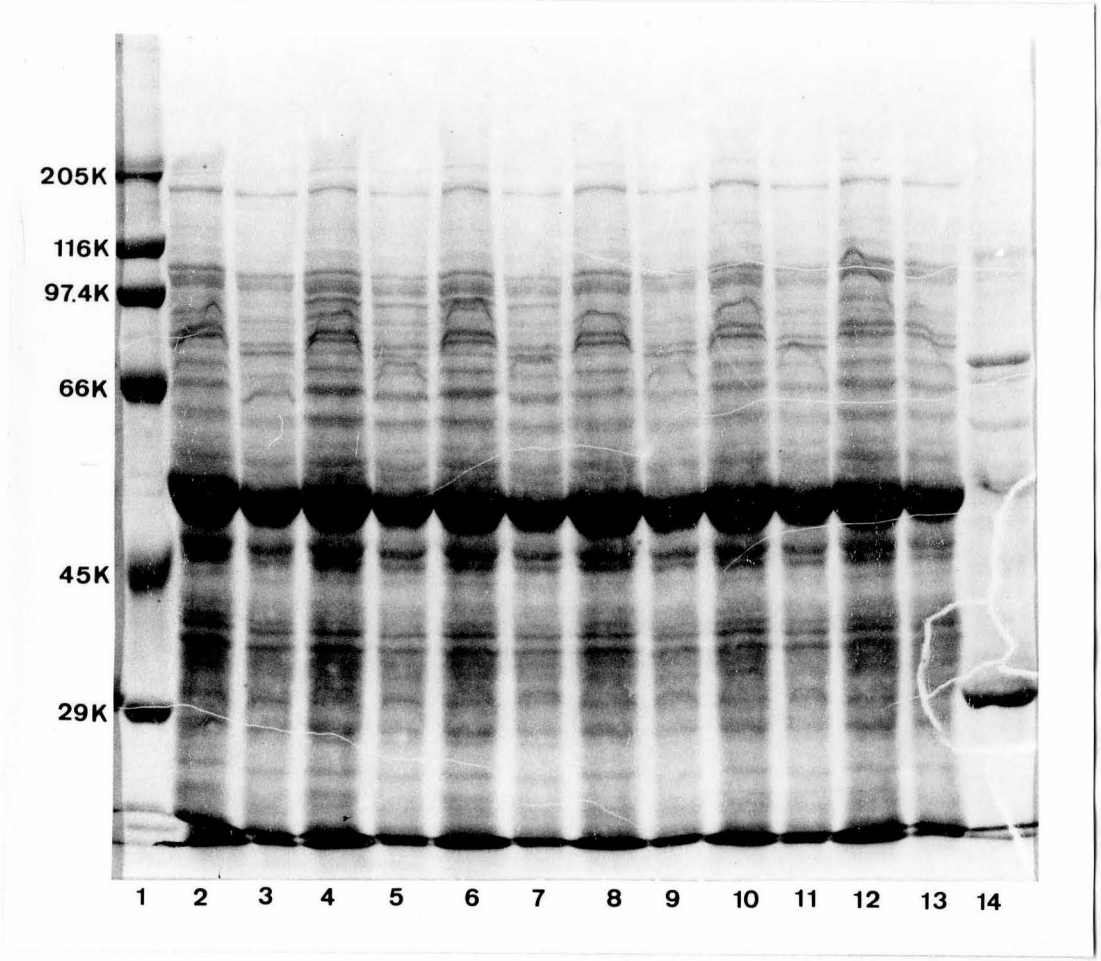

205K
116K
97.4K
66K
45K
29K
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Figure 5. WESTERN BLOT. The blot was prepared as described in Materials and Methods. Samples were transferred from the gel onto a nitrocellose membrane and screened with nitrate reductase antibodies. The samples are as follows:

Lane 1 = 0 mM nitrate/0 mM glutamine, 20 ul

Lane 2 = 0 mM nitrate/0 mM glutamine, 10 ul

Lane 3 = 50 mM nitrate/0 mM glutamine, 20 ul

Lane 4 = 50 mM nitrate/0 mM glutamine, 10 ul

Lane 5 = 50 mM nitrate/10 mM glutamine, 20 ul

Lane 6 = 50 mM nitrate/10 mM glutamine, 10 ul

Lane 7 = 50 mM nitrate/50 mM glutamine, 20 ul

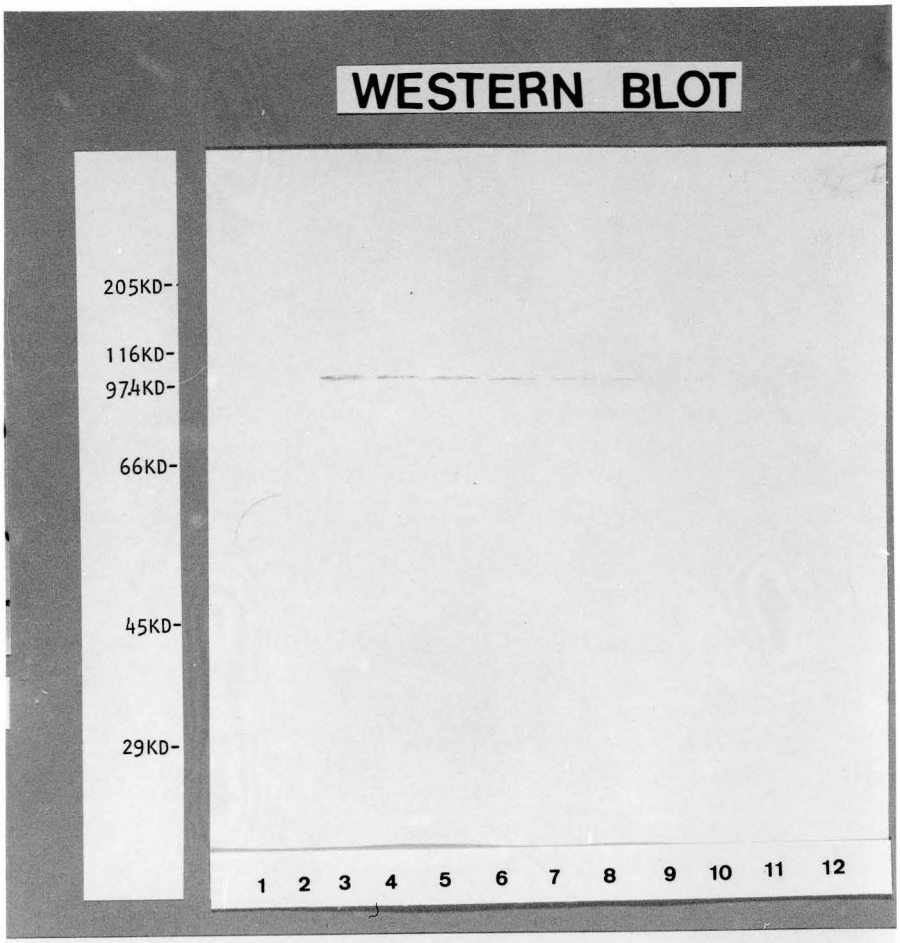
Lane 8 = 50 mM nitrate/50 mM glutamine, 10 ul

Lane 9 = 50 mM nitrate/250 mM glutamine, 20 ul

Lane 10 = 50 mM nitrate/250 mM glutamine, 10 ul

Lane 11 = 0 mM nitrate/250 mM glutamine, 20 ul

Lane 12 = 0 mM nitrate/250 mM glutamine, 10 ul



Lane	1	2	3	4	5	6	7	8	9	10	11	12
Nitrate mM	0	0	50	50	50	50	50	50	50	50	0	0
Glutamine mM	0	0	0	0	10	10	50	50	250	250	250	250
ul Sample	20	10	20	10	20	10	20	10	20	10	20	10

Table 3

THE EFFECT OF ABSOLUTE NITRATE AND AMINO ACID CONCENTRATIONS ON THE AMOUNT OF NITRATE REDUCTASE ACTIVITY PRESENT IN WHOLE PLANTS

	% ACTIVITY*	
AMINO ACID	10mM	50mM
NONE	100.0	100.0
GLYCINE	65.9	38.2
ARGININE	52.1	20.3
ASPARAGINE	69.6	23.9
PHENYLALANINE	63.1	30.9
GLUTAMINE	47.5	12.4

*100% activity equals 2.17 nmoles nitrate reduced/minute/mg protein (Plants were irrigated on day 7 with media described in Materials and Methods and all activities determined from the 24 hour harvest). All values statistically significant, $p < 0.05$.

Table 4

CELLULAR NITRATE CONCENTRATIONS PRESENT IN WHOLE PLANTS[a]

	% NITRATE PRESENT IN LEAF[b]	
AMINO ACID	10mM	50mM
NONE	100.0	100.0
GLYCINE	79.1	70.3
ARGININE	63.0	66.6
ASPARAGINE	76.5	60.6
PHENYLALANINE	96.1	79.5
GLUTAMINE	77.4	54.7

[a]Constant 10 mM nitrate present for the growth of all plants.
[b]100% activity equals 43.37 umoles nitrate/g leaf (Plants were irrigated on day 7 with media described in Materials and Methods and all activities determined from the 24 hour harvest). All values statistically significant, $p < 0.05$.

Figure 6. THE EFFECTS OF ABSOLUTE NITRATE AND AMMONIUM CONCENTRATIONS ON THE AMOUNT OF NITRATE REDUCTASE ACTIVITY PRESENT IN WHOLE PLANTS. Squash plants were irrigated on day 7 with media described in Materials and Methods and all activities were determined from cotyledons harvested at both 20 and 24 hours. Nitrate reductase activity is expressed in nmoles nitrate reduced/ min/mg protein. ■ = 20 hour harvest and O = 24 hour harvest.

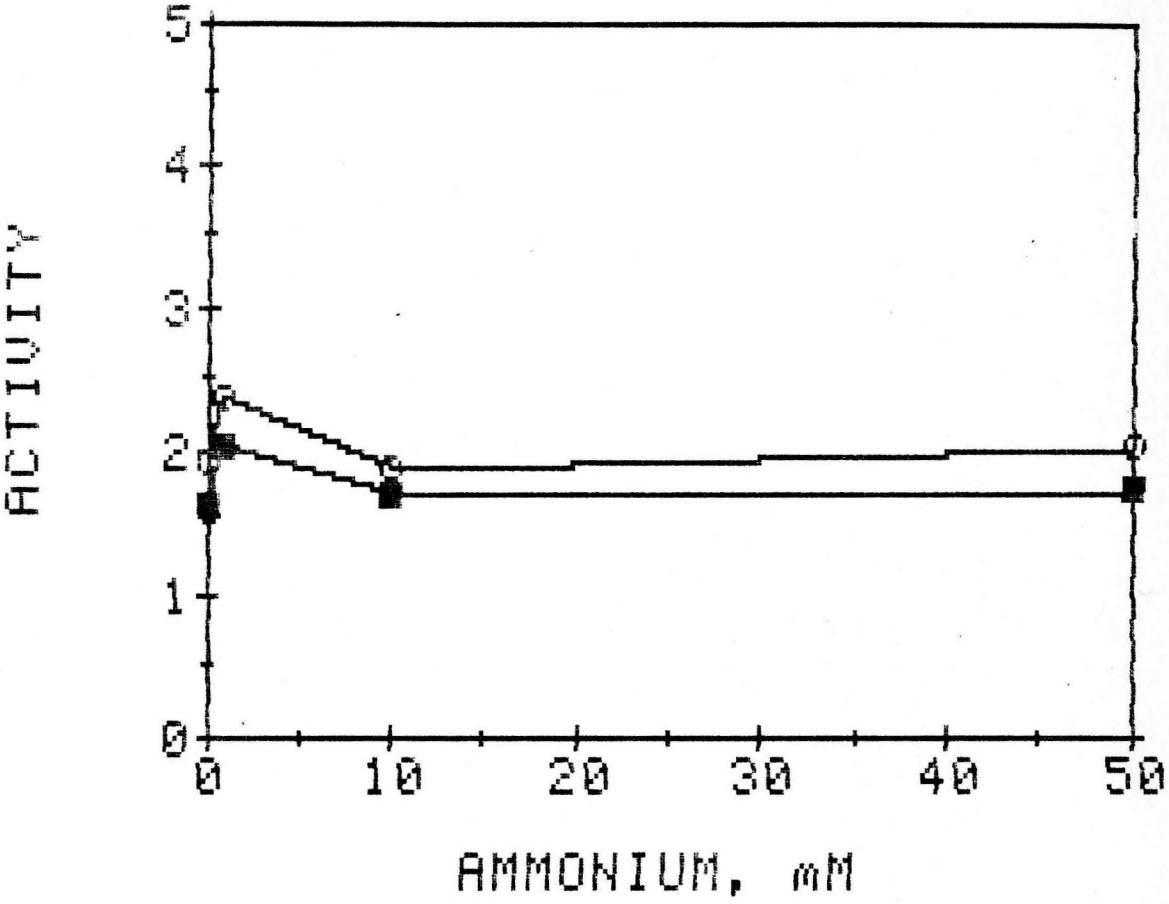

ACTIVITY
0
1
2
3
4
5
0
10
20
30
40
50
AMMONIUM, mM

Table 5

THE EFFECT OF ABSOLUTE NITRATE AND AMMONIUM CONCENTRATIONS ON THE AMOUNT OF NITRATE REDUCTASE ACTIVITY PRESENT IN WHOLE PLANTS[a]

	% ACTIVITY	
AMMONIUM	20 HOURS[b]	24 HOURS[c]
0 mM	100.0	100.0
10 mM	99.0	105.6
50 mM	106.8	107.5

[a]Constant 10mM nitrate present for the growth of all plants.
[b]100% activity equals 1.90 nmoles nitrate reduced/minute/mg protein.
[c]100% activity equals 1.61 nmoles nitrate reduced/minute/mg protein.
(Plants were irrigated on day 7 with media described in Materials and Methods and activities determnined at both the 20 and 24 hour harvests). All values satistically significant, $p < 0.05$.

Table 6

CELLULAR NITRATE CONCENTRATIONS PRESENT IN WHOLE PLANTS[a]

AMMONIUM	% NITRATE 20 HOURS[b]	24 HOURS[c]
0 mM	100.0	100.0
10 mM	74.5	74.6
50 mM	88.4	95.3

[a]Constamt 10 mM nitrate present for the growth of all plants.
[b]100% activity equals 24.65 umoles nitrate/g leaf.
[c]100% activity equals 27.12 umoles nitrate/g leaf.
(Plants were irrigated on day 7 with media described in Materials and Methods and activities determined at both the 20 and 24 hour harvests). All values statistically significant, $p < 0.05$.

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The final copies have been examined by the director of the thesis and the signiture which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Biology.

11/2/87
Date

John Smarrelli Jr.
Director's Signiture