# NITRATE REDUCTASE AND A NITRATE REDUCTASE INHIBITOR IN SOYBEAN LEAVES

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ABSTRACT

NITRATE REDUCTASE AND A NITRATE REDUCTASE INHIBITOR IN SOYBEAN LEAVES

By

Setsuko Omata Jolly

1) Two proteins having nitrate reductase activities have been isolated from young first leaves of soybeans and separated by DEAE-cellulose column chromatography. They have different mobilities when electrophoresed on polyacrylamide gels. These two enzymes have been designated NADH-nitrate reductase and NADPH-nitrate reductase because of their different affinities for the reduced pyridine nucleotides. NADPH-nitrate reductase was detected in extracts which contained no cysteine, whereas NADH-nitrate reductase was not found in the absence of cysteine. Both nitrate reductases utilize NADH, NADPH, $FADH_2$, reduced methylviologen and reduced benzylviologen as electron donors. Under optimum isolation conditions there was about three times as much NADH-nitrate reductase as NADPH-nitrate reductase in young soybean leaves. The NADH-nitrate reductase has a molecular weight of 330,000 as estimated by gel filtration, a pH optimum of 6.5 when using NADH as electron source, a Km for NADH of 8.1 μM, a Km for NADPH of 202 μM, and Km for nitrate of 110 μM. The NADPH-nitrate reductase has a molecular weight of 220,000, a pH optimum also of 6.5, a Km for NADH of 3.9 μM, a Km for NADPH of 1.5 μM, and a Km for

nitrate of 4,500 μM. The activity of the NADPH-nitrate reductase was enhanced by FAD when it was using reduced pyridine nucleotides but the activity of tne NADH-nitrate reductase was not. Potassium cyanide and sodium azide inhibited both nitrate reductases while potassium fluoride did not inhibit either. This NADPH-nitrate reductase was judged to be a new and a different protein from the NADH linked enzyme by numerous criteria. (A) The two enzymes were separated by chromatography and by electrophoresis. (B) Molecular weight, substrate affinities, and inhibitions were different. (C) No NADPH-phosphatase was present in the purified NADPH-nitrate reductase. This, combined with the fact that neither fluoride nor phosphate inhibits NADPH utilization by the NADPH-nitrate reductase, virtually eliminates the possibility that the assumed enzyme is a combination of NADH-nitrate reductase and a phosphatase.

2) It has long been known that leaf discs and plant extracts lose their ability to reduce nitrate if the plants are kept in the dark. This has been interpreted as a loss of nitrate reductase. However, single cells isolated by sucrose gradient centrifugation from leaves of soybeans which had been kept in the dark for 54 hours were able to reduce nitrate as rapidly as cells from leaves kept in the light. Furthermore, DEAE-cellulose column chromatography revealed that the nitrate reductase enzymes were still present in extracts from leaves kept in the dark. These observations suggested that an inhibitor of nitrate reductase might be formed in the dark. Such an inhibitor was isolated by DEAE-cellulose column chromatography from extracts of soybean leaves. This inhibitor was photoinactivated *in vitro*, but regained its activity in the dark even at 4°. When

isolation procedures were conducted rapidly, much less of the inhibitor was found in leaves recently exposed to light.

3) The nitrate reductase inhibitor was purified about 2,500-fold by DEAE-cellulose chromatography and Sephadex gel filtration. It is a heat-labile protein with an estimated molecular weight of 31,000 consisting of two subunits. The inhibitor was specific for the NADH-nitrate reductases from soybean, spinach, or pigweed leaves. No other enzyme was found to be sensitive to the inhibitor. It had no effect on the NADPH-nitrate reductase from soybeans or on the $FMNH_2$-nitrate reductase from *Escherichia coli*. It also has no effect on nitrite reductase, glutamate dehydrogenase or xanthine oxidase. The inhibitor did not progressively inactivate the nitrate reductase, did not release amino acids from the reductase or from other proteins, and its activity was not affected by protease inhibitors. Therefore, it does not act by proteolytic attack on the reductase. The inhibition was noncompetitive with respect to nitrate. The main action of the inhibitor seemed to be in the terminal nitrate reductase function and not in the diaphorase function of the enzyme complex.

It is postulated that a protein inhibitor of NADH-nitrate reductase limits the activity of the enzyme in leaves in the dark, but that the inhibitor is photoinactivated in the light to release the nitrate reductase activity.

# NITRATE REDUCTASE AND A NITRATE REDUCTASE INHIBITOR IN SOYBEAN LEAVES

By

Setsuko Omata Jolly

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DEDICATION

To Jim

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| | |
|---|---|
| CM | carboxymethyl |
| DCPIP | dichlorophenolindophenol |
| DEAE | diethylaminoethyl |
| DTT | dithiothreitol |
| EDTA | ethylenediamine tetraacetate |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| MES | 2-(N-morpholino)-ethanesulfonic acid |
| NR | nitrate reductase |
| PAL | phenylalanine ammonia lyase |
| PMS | phenazine methosulfate |
| PMSF | phenylmethylsulfonyl fluoride |
| PVP | polyvinylpolypyrrolidone |
| RNA | ribonucleic acid |
| RuDP | ribulose-1,5-diphosphate |
| SDS | sodium dodecyl sulfate |
| Tris | tris-(hydroxymethyl)-methylamine |

# INTRODUCTION

1) The literature about nitrate reductase is very extensive and only parts related to the contents of this dissertation are reviewed. This includes two major problems: the pyridine nucleotide specificity of nitrate reductase and the aspects of the control mechanism of nitrate reductase activity in the light and dark which seems to involve the presence of activators or inhibitors. Soybean leaves were used in this investigation, so the literature on nitrate reductase in this important crop plant is also examined.

Evans and Nason (1) first isolated nitrate reductase from soybean seedlings and found the nitrate reductase had a lower Km for NADPH than for NADH. However, about the same maximum activity was observed when either NADPH or NADH was used. In more recent studies of higher plant nitrate reductase, Hageman and co-workers (2,3,4,5) have shown that in general nitrate reductase almost exclusively utilizes NADH. For instance, spinach nitrate reductase will not utilize NADPH unless NADP-reductase and FMN are added to the preparation (3,6). However, they also found that nitrate reductase isolated from the leaves of soybean and several other plants could utilize both NADPH and NADH equally (2,4,5). Three possibilities existed. In some plants there could be two nitrate reductases, one for NADH and one for NADPH. Alternatively, nitrate reductase in these plants might be nonspecific for the pyridine nucleotide reductant (5). The

third possibility was the rapid conversion of NADPH to NADH by transhydrogenase or a phosphatase (7,8).

Nitrate reductase in soybean leaves has been studied in some depth by Hageman and co-workers (2,8). The amount of sulfhydryl agent in the extraction buffer influences the ratio of activity with NADH and NADPH. However, the presence of two enzymes could not be shown (2). Recently, Wells (7) and Wells and Hageman (8) explained the presence of NADPH-nitrate reductase activity in extracts of soybean and corn leaves on the basis of a rapid conversion of NADPH to NADH by a specific phosphatase. They were unable to separate their preparations into two nitrate reductases by either DEAE-cellulose column chromatography or by isoelectric focusing (7,8). Thus, they concluded that NADPH-nitrate reductase activity was an artifact due to the NADPH-phosphatase. This conclusion was supported by inhibition of the NADPH-nitrate reductase activity of their preparations by phosphatase inhibitors.

Evidence is presented here that it is possible to separate the nitrate reductase activity of soybean leaf extracts into a NADH-nitrate reductase and a NADPH-nitrate reductase activity by DEAE-cellulose column chromatography. The separation of these two enzymes of nitrate reductase was aided by the elucidation of a difference in their affinity for nitrate. The partially purified NADPH-nitrate reductase, isolated from the DEAE-cellulose column, is free of NADPH specific phosphatase activity. The properties of the two partially purified nitrate reductases are compared.

2) Green leaves lose nitrate reductase activity in the dark and rapidly regain it in the light (9-19). The mode of operation of light

in controlling nitrate reductase activity is very complex and not thoroughly understood. However, the data from many laboratories (9-19) clearly indicate that somehow light controls nitrate reduction. This control has been attributed to many causes: The utilization of the photosynthetic products as an energy source may regulate nitrate reductase activity (1,15,20,21,22,23). The stimulation of nitrate-uptake by light may increase and stabilize nitrate reductase activity (14,24,25). An influence of light upon the development of an active protein-synthesizing apparatus may increase nitrate reductase activity non-specifically (26,27). Gibberellic acid or kinetin may be important in the effect of light on nitrate reductase activity (28-31). The light may stimulate the synthesis of an effector for nitrate reductase (32). Also it is possible that a loss of activity in the dark might be from protein degradation (33), an inactivation of an active site of the enzyme, formation of an inhibitor, or binding with an inhibitory protein (25,34).

Recently, high molecular weight nitrate reductase inhibitors were isolated from maize roots by Pan and Marsh (107) and Wallace (36,37,38), and from rice roots by Kadam *et al.* (39). The inhibitors inhibited nitrate reductase prepared from leaves. Both inhibitors seemed to be very specific proteases for nitrate reductase. Evidence is presented in this dissertation that nitrate reductase exists in soybean leaves for 54 hours in the dark following a 5 day light-period, at a level as high as in leaves grown under continuous light. This is explained by the presence of a nitrate reductase inhibitor in soybean leaves. Based on these results, I would like to present one possible mechanism

to explain the rise and fall of nitrate reductase activity in the light and dark.

# LITERATURE REVIEW

## General Characteristics of Nitrate Reductase

Reduced nitrogen is supplied to soybean plants through either nitrogen fixation or nitrate reduction. Nitrogen fixation is normally initiated in soybeans 20 to 30 days after planting (40). Thus, initial nitrogen requirements must be met through utilization of nitrogen from the seed and nitrogen from the soil. Recent estimates indicate that some 25 to 30% (80-110 kg N per hectare per season) of the total plant nitrogen was supplied through the $N_2$-fixation process in soybeans as measured with the acetylene-reduction technique (40,41). A considerable portion (50-75%) of the reduced nitrogen in soybeans must be derived from nitrate through the nitrate reduction pathway (42,43,44).

A characteristic feature of nitrate metabolism in higher plants is its susceptibility to a range of environmental conditions. It has been established that light, drought, mineral, hormonal treatment, plant age, and genetic composition all influence the capacity for nitrate reduction (4). In most of these cases it appears that the control of nitrate reduction is mediated through a regulation of the enzyme, nitrate reductase (Reduced-NAD(P); nitrate oxidoreductase, E.C.1.6.6.2).

Nitrate reductase is the logical point to effect regulation of the input of reduced nitrogen for the plant because it is (a) the

first enzyme in the pathway, (b) the rate limiting step, (c) substrate inducible (9,10,14,15,24,62,63,64) and (d) a relatively unstable enzyme (9,10,50). Furthermore, the toxic effect of excess levels of nitrite and ammonium ions also indicate the desirability of regulating their production.

Nitrate reductase is a metallo-flavo protein containing molybdenum (46,47,48) and FAD (1,49,50,51) with active sulfhydryl groups (3,4,52). Nitrate reductase also exhibits the following partial activities: NAD(P)H dehydrogenase, as shown by the reduction of numerous electron acceptors such as cytochrome c (53,110) or dichlorophenolindophenol (54), and the reduction of nitrate by reduced benzyl or methyl viologens (21,55) or reduced flavin (3,51).

The molecular weight of nitrate reductase has been determined in several laboratories. For spinach nitrate reductase, Relimpio *et al.* (50) reported a molecular weight of 500,000, while Notton *et al.* (133) reported 240,000. Values of 19S (600,000) for wheat (193), 8S (about 230,000) for barley (53), and 160,000 for maize (5) indicate the probable range in green plants. The molecular weight of NADH-nitrate reductase from diatom *Thalassiosira pseudonana* was reported as 330,000 by Amy and Garrett (134). The molecular weight of NADPH-nitrate reductase from *Neurospora crassa* was estimated to be 228,000 by Garrett and Nason (81). The respiratory nitrate reductase from *Escherichia coli* had a molecular weight of 320,000 (84).

Nitrate reduction can occur in either leaves or roots; however, the bulk of the nitrate is reduced in plant foliage (4,41). Only low levels of nitrate reductase activity were observed in the extracts of roots from cauliflower, corn and sugarcane (9,10,135). Spencer (136)

was able to isolate nitrate reductase from the embryos of germinating wheat seed. Ferrari and Varner (137) have reported that nitrate reductase activity can be detected in extracts of barley aleurone cells which have been incubated with nitrate. Filner's studies with tobacco pith cultures clearly demonstrate the capacity of this tissue to utilize nitrate as a principal nitrogen source (138).

Nitrate reductase is known to be a substrate-nitrate inducible enzyme (9,10,14,15,24,62,63,64). Ammonia or nitrite does not induce the nitrate reductase in cauliflower (24) or corn seedlings (14). However, these ions were reported to induce the enzyme in radish cotyledons (65). Nitrite also induced nitrate reductase in rice seedlings (66) and bean seed cotyledons (67). Recently, Kaplant *et al.* (68) reported that nitrate induced the enzyme in darkness only upon addition of suitable sources of reducing equivalents which produce nitrite. However, nitrite itself brought about induction of the enzyme in darkness without supplementation of exogenous reducing factors. Therefore they suggested that nitrate reductase should be considered as a product-inducible enzyme rather than a substrate-inducible enzyme.

Additional characteristics of the enzyme have been published in reviews and publications (3,4,69,192).

## Subcellular Location of Nitrate Reductase

Studies of the intracellular location of nitrate reductase activity in green tissue have yielded conflicting results, but the consensus of opinion is that it is in the cytoplasm. Ritenour *et al.* (56) compared nonaqueous and aqueous techniques and concluded that

nitrite reductase is located within the chloroplast, but nitrate reductase is not. Nitrate reductase was stated to be an exochloroplastic enzyme (cytoplasmic), but the possibility was not eliminated that the enzyme might be associated with the external chloroplast membrane which was removed during aqueous and nonaqueous isolation.

Coup'e *et al.* (60), also using nonaqueous techniques, but using density gradient centrifugation separation rather than stepwise fractionation, found nearly 60% of the total nitrate reductase activity associated with the chloroplasts and concluded that nitrate reductase of barley was a chloroplastic enzyme. Also it has been reported to be localized within chloroplasts by Losada *et al.* (59).

Further indirect evidence for the cytoplasmic as opposed to the chloroplastic location of nitrate reductase is provided by the observation of Schrader *et al.* (57), who demonstrated that chloramphenicol inhibited the synthesis of nitrite reductase but not of nitrate reductase.

Recently, Dalling *et al.* (58) concluded from their studies of the intracellular location of nitrate reductase, using isopycnic sucrose gradient centrifugation, that nitrate reductase activity was located in the cytosol. The low level of nitrate reductase activity associated with broken chloroplasts with certain extraction media was attributed to indiscriminate adsorption of nitrate reductase by the organelles. Their report did not substantiate an earlier paper by Lips and Avissar (61), who claimed that nitrate reductase and catalase (peroxisomal) activities were found in the same sedimentation fraction in sucrose gradient of young tobacco leaves. The latter authors claimed that nitrate reductase seemed to be located on the

membrane of the microbodies from which it was easily separated without apparently affecting the integrity of the organelle.

The intracellular location of nitrate reductase in non-green tissues has not been widely studied. Miflin (139) reported that only a small portion of the total nitrate reductase activity of the barley roots was associated with a particulate fraction. More recently he showed that this fraction with nitrate reductase could be separated from the mitochondria by sucrose density gradient centrifugation (140). However, Dalling *et al.* (141) studied the location of nitrate reductase in wheat roots, using isopycnic sucrose gradient centrifugation and reported that nitrate reductase activity was found only in the supernatant and it was not associated with any of the organelles.

## Electron Donors for Nitrate Reductase

Initial investigators of nitrate reductase from *Neurospora crassa* (49) and young soybean leaves (1) indicated that nitrate reductase could use either NADPH or NADH as an electron donor with a preference for NADPH. Subsequently, Beevers *et al.* (2) reported that nitrate reductase in extracts from 15 of 16 plant species had a specific or preferential requirement for NADH as cofactor and only soybeans could utilize either NADPH or NADH with equal efficiency in reducing nitrate. In all extracts, except those from soybeans, optimum nitrate reductase activity was observed at pH 7.5 when NADH was the cofactor. With preparations from soybeans, optimum activity was recorded at pH 6.25 with either NADH or NADPH. In other species, the trace of nitrate reductase activity with NADPH as cofactor was

maximum at pH 6.25 (2). Further investigations of nitrate reductase from higher plants (9,12,50,70) have also indicated that nitrate reductase from most of the plant species tested had a preferential specificity for NADH. Shrader *et al.* (3) reported that nitrate reductase from corn and marrow can utilize NADPH as electron donor only when it was supplied with NADP-reductase and exogenous FMN for enzymatic generation of $FMNH_2$.

Hageman and Hucklesby (5) classified higher plants as "species with NADH-nitrate reductase" or "species with NADH- and NADPH-nitrate reductase." In the latter group are included soybean leaves, foxtail leaves, leaves of certain corn genotypes and scutellum of corn. In addition to this, in 1968 Sims *et al.* (72) reported that *Lemna minor* plants exhibited a 20-fold increase in NADPH-specific nitrate reductase activity when sucrose was added to the culture medium. Shen (66) observed that the nitrate or nitrite induced nitrate reductase in rice seedlings could accept electrons more efficiently from NADH than NADPH. However, when the enzyme was induced by organic nitro-compounds, it could accept electrons more efficiently from NADPH.

Stoy (73) reported that riboflavin, photoreduced under anaerobic conditions, was more effective as an electron donor for nitrate reductase than was NADH. In 1965, Paneque *et al.* (51) reported that free $FMNH_2$ is the natural cofactor for nitrate reductase in higher plants. However, Schrader *et al.* (3) found that the concentration of $FMNH_2$ required to obtain half-maximal activity was 40- to 100-fold higher than for NADH. Since it seems unlikely that reduced flavin is present at millimolar concentrations *in vivo*, they suggested that NADH

is a more important electron donor than $FMNH_2$ for nitrate reductase in higher plants under physiological conditions.

## NADPH-Nitrate Reductase Activity as an Artifact

It is possible that nitrate reductase from all species is NADH-specific and NADPH-nitrate reductase activity is due to a transhydrogenase activity or a phosphatase activity present in the extract. Beevers *et al.* (2) and Tingey *et al.* (44) felt that these possibilities are not likely for soybean NADPH-nitrate reductase activity for the following reasons: (a) the constancy of the NADH to NADPH ratio during the purification procedure, (b) the addition of traces of NAD to reaction mixture containing excess NADPH produced no enhancement of nitrate reduction, (c) the pH optimum for leaf transhydrogenase is 8.8 (74), and (d) the phosphatase Km for NADPH varies between $1.5 \times 10^{-4}$M and $1.2 \times 10^{-3}$M (7,75) while nitrate reductase Km for NADPH is $8 \times 10^{-7}$M (2), which implies that NADPH will preferentially react with nitrate reductase rather than with the phosphatase.

Recently Wells (7) and Wells and Hageman (8) reinvestigated the pyridine nucleotide specificity of soybean and corn nitrate reductase. They could not separate NADH- and NADPH-nitrate reductase activities by either DEAE-cellulose column chromatography or column isoelectric focusing. Thus, they concluded that NADPH-nitrate reductase was an artifact due to the NADPH-specific phosphatase which was found in soybean and corn extracts. Although 90% of the phosphatase activity was separated by DEAE-cellulose column chromatography from nitrate reductase fraction, enough remained in their nitrate reductase fraction to catalyze the conversion of NADPH to NADH. Tingey (44)

disagreed with Wells and Hageman (7,8) from indirect experiments, namely the coupling of the oxidation of 6-phosphogluconate to nitrate reduction in soybean extract and the coincidence of an ozone effect on nitrate reductase and glucose-6-phosphate dehydrogenase.

Shen (66) observed that when nitrate reductase was induced by organic nitro-compounds in rice seedlings, it could accept electrons more efficiently from NADPH than NADH. No attempt was made to evaluate the role of phosphatase in nitrate reduction. He suggested that if there is only one nitrate reductase in rice seedlings, the higher rate of nitrate reduction with equimolar NADPH could not be explained by conversion of NADPH to NADH.

Nitrate Reductase from Soybean Leaves

Nitrate reductase obtained from soybean leaves appears to differ from nitrate reductase obtained from other plant species. This view is supported by (a) the divergence of pH optimum with NADH as cofactor, (b) the ability of the soybean enzyme to readily utilize NADPH and (c) FAD appears to be bound less tightly (2). The utilization of NADH and NADPH as electron donors has been studied in some depth with soybean leaf extracts.

The relative efficiencies of NADH or NADPH to function as cofactor for the nitrate reductase from soybean leaves was dependent on the enzyme extraction medium. Evans and Nason (1) did not use sulfhydryl reagents to extract nitrate reductase and got a 1:1 ratio of NADH- to NADPH-nitrate reductase activity. Beevers *et al.* (2) reported that NADH was twice as effective as NADPH for the nitrate reductase in extracts of soybeans prepared with a grinding medium containing 5 or

10 mM cysteine, and NADH or NADPH was equally effective when soybean extracts were prepared without cysteine. The addition of 5 or 10 mM cysteine increased the level of nitrate reductase activity 5.5-5.8-fold with NADH and 2.4-3.0-fold with NADPH. The cysteine level in extraction medium also changed the Km constants (2). When soybean leaves were homogenized with 5 mM cysteine the Km ($NO_3^-$) was 0.185 mM and without cysteine the Km ($NO_3^-$) was up to 9.5 mM with NADH as a cofactor. With NADPH as a cofactor the Km ($NO_3^-$) was 1.45 mM with cysteine and 3.7 mM without cysteine. Cysteine had only a slight effect upon Km for the reduced pyridine nucleotides. The Km (NADH) was slightly greater (2.21 µM vs 1.22 µM) in enzyme preparations derived from crude homogenate which had cysteine than in preparations without cysteine, while the Km (NADPH) of 0.18 µM was unaffected by cysteine levels during preparation.

## Source of Electron Donors for Nitrate Reductase *in vivo*

The question of the source of reduced pyridine nucleotides for nitrate reduction has been an enigma. In nonphotosynthetic tissue, because nitrate increases oxygen consumption (76), respiratory metabolism may supply the electron donors for nitrate reduction (4). Klepper (77) showed, when leaf discs from morning-glory leaves and corn scutellum were vacuum infiltrated with sugar phosphates (3-phosphoglyceraldehyde, glucose-6-phosphate and fructose-1,6-diphosphate), they produced more (up to 2-fold) nitrite in the dark than the control. The amount of nitrite formed was related to the concentration of the sugar phosphates added to the medium. He also found that the addition of phosphoglyceraldehyde and NAD to a 30,000xg supernatant of leaf

homogenate resulted in the reduction of nitrate to the same levels as with NADH alone. Therefore he concluded that the generation of NADH by the cytoplasmic NAD-dependent glyceraldehyde-3-phosphate dehydrogenase may be used in part for nitrate reduction in green tissue.

Evidence for the *in vivo* reduction of nitrate by an NADPH-dependent nitrate reductase has not been demonstrated. However, Tingey *et al.* (44) showed that nitrate reduction in soybean extract could be coupled to the oxidation of 6-phosphogluconate which is a well known oxidation by a NADPH-specific dehydrogenase. He also found that nitrate reduction in soybean foliage exposed to ozone for two hours was significantly depressed immediately following exposure, but nitrate reductase activity returned to or exceeded the control level after 24 hours (45). Glyceraldehyde-3-phosphate dehydrogenase activity was depressed for at least 72 hours following the ozone exposure, while glucose-6-phosphate dehydrogenase activity increased following ozone exposure with a time course similar to that for the restoration of nitrate reductase activity (44). Recently Fowler and Stepan-Sarkissian (78) investigated the possibility that the malic enzyme could contribute NADPH for nitrate assimilation in pea roots. During the induction of nitrate reductase by exposing pea roots to potassium nitrate, significant increases occurred in the activity of the pentose phosphate pathway dehydrogenases, but there was not a significant change in the malic enzyme. They concluded that the malic enzyme plays only a minor role in NADPH provision for nitrate assimilation.

In 1953, Evans *et al.* (1) showed the photochemical reduction of nitrate in a system containing grana, nitrate reductase and $KNO_3$. Currently, it is not clear whether the influence of light on nitrate metabolism resides in its effect on the generation of reductant. Also the cellular location of nitrate reductase is not certain. However, it is most likely that the photosynthetic product, NADPH, is used directly in the chloroplasts or indirectly after it is transferred to the cytoplasm via the triose phosphate/3-P-glycerate shuttle involving D-glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglyceraldehyde kinase (79). For the indirect transfer of NADPH to the cytoplasm, a NADPH-linked non-reversible D-glyceraldehyde-3-phosphate dehydrogenase may be utilized (80). For the transfer of reducing equivalents to the cytoplasm as NADH, a NAD-linked reversible D-glyceraldehyde-3-phosphate dehydrogenase might be used (79).

Structure of Nitrate Reductase

In 1965, Paneque *et al.* (51) reported that free $FMNH_2$ and $FADH_2$ are the natural cofactors for nitrate reduction in higher plants and that NAD(P)H-nitrate oxidoreductase is a mixture of two enzymes--NAD(P)H diaphorase which can use a variety of oxidized compounds (such as cytochrome c or dyes) as electron acceptor and nitrate reductase which they classified as $FMNH_2$ ($FADH_2$):nitrate oxidoreductase, or terminal nitrate reductase. However, the two enzymatic activities have not been physically separated. Recently, Garrett and Nason (81) purified nitrate reductase from *Neurospora crassa* and found that all activities known to be associated with this enzyme, i.e., NADPH-nitrate reductase, NADPH-cytochrome c reductase, $FADH_2$-nitrate reductase and

reduced methylviologen-nitrate reductase, were eluted together from DEAE-cellulose and Sephadex gel filtration columns and all activities kept a somewhat constant proportion even after 500-fold purification. Moreover, all activities were associated with a particular protein zone after polyacrylamide gel electrophoresis. They also succeeded in crystallizing the enzyme by an ammonium sulfate procedure (82), and concluded that assimilatory nitrate reductase in *Neurospora crassa* is a discreet enzyme system represented under physiological conditions by a single physical entity, made up perhaps of several tightly bound polypeptide chains.

Also respiratory nitrate reductases from bacteria have been highly purified and their properties have been studied (83,84,85,86). MacGregor *et al.* (83) purified nitrate reductase from *Escherichia coli* which appeared homogeneous by polyacrylamide gel electrophoresis. The molecular weight was 720,000-770,000. Electron micrographs showed it to be spherical. Sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated that the enzyme is composed of two different subunits with molecular weights of 142,000 and 58,000. The structure of nitrate reductase was composed of four large polypeptides, four small polypeptides, and four molecules of molybdenum per molecule of the enzyme. Riet and Planta (86) found three different subunits in nitrate reductase from *Klebsiella aerogenes* when it was solubilized from the membranes by deoxycholate. The molecular weight of the monomeric form of the enzyme was 260,000 and subunits were 117,000, 57,000 and 52,000, which were present in a 1:1:2 molar ratio. The smallest subunit appeared to have a structural function since nitrate

reductase which lacked this subunit was much more labile than the enzyme with it.

The nitrate reductases require molybdenum both for their formation and activity (87,88,89). Chemical tests have indicated that during the enzymatic reduction of nitrate to nitrite, the oxidation state change undergone by the molybdenum present in the enzyme from *Neurospora crassa* involved Mo(V) and Mo(VI) (90) and ESR spectroscopic studies have strongly suggested that the nitrate is directly bound to the Mo(V) center of the enzyme from *Micrococcus definitrificans* before reduction (91,92).

Subramanian and Sorger (93) observed that induction of *Neurospora crassa* by nitrate under molybdenum-deficient or tungstate-toxic conditions resulted in low NADPH-nitrate reductase and reduced benzylviologen-nitrate reductase activities, but the level of NADPH-cytochrome c reductase activity was the same as under normal conditions. They proposed that during molybdenum deficiency or tungstate toxicity, the component proteins of the nitrate reductase complex are all synthesized, but owing to the non-availability of molybdenum, the molybdenum-binding site on the enzyme is folded *in vivo* in such a way that it can no longer bind this metal *in vitro*.

A cell-free extract of the *Neurospora crassa* mutant *nit*-1, which specifically contains the NADPH-cytochrome c reductase but not the NADPH-nitrate reductase, could assemble NADPH-nitrate reductase *in vivo* with the assistance of molybdo-polypeptides from molybdo-enzymes (87,88,89,94,95).

The Role of Light in Nitrate Reduction

The level of nitrate reductase activity in leaf tissue varies diurnally (11,13,15,19) and is influenced by intensity of illumination (9,10,11,12,14). The mode of operation of light in controlling nitrate reductase activity is complex. However, the data from many laboratories (9,10,11,12,13,14,15,19) clearly indicate that somehow light controls nitrate reduction.

It was once commonly believed that NADPH was the preferred co-factor because of the initial work on nitrate reductase obtained from young soybean seedlings (1) and *Neurospora crassa* (49). Since the chloroplast is the prime source of NADPH, and since nitrate reduction can be coupled to light via NADP and a chloroplast preparation (1,59), it had been accepted (20) that photosynthetically generated NADPH was the electron donor for nitrate reduction in leaves. Ramiriz *et al.* (21) showed that nitrate can serve as a Hill oxidant. With time, this pathway did not appear to satisfy all experimental observations, as nitrate reductase from most green leaves was NADH- rather than NADPH-specific (2,9,12,50,70) and the enzyme appeared to be localized in the cytoplasm rather than in chloroplasts (57,58).

Kannangara and Woolhouse (15) found that when the excised leaves of *Perilla* containing nitrate were illuminated in a $CO_2$-free atmosphere no enzyme activity was detected. Thacker and Syrett (22) reported that nitrate reductase activity in *Chlamydomonas* decreased rapidly when $CO_2$-fixation was prevented by (a) darkening cultures, (b) aerating cultures with $CO_2$-free air, or (c) addition of DCMU (3-(3,4-dichlorophenol)-dimethylurea), and a small loss of nitrate reductase activity from darkened cells occurred when (a) acetate-adapted cells were

supplied with acetate, or (b) cells were allowed to accumulate carbon reserves by nitrogen starvation before darkening. These data (15,22,23) may suggest that the reductant utilized for nitrate reduction is generated with or from the carbon products formed during $CO_2$ fixation.

Hageman and Flesher (10) demonstrated that extracts prepared from green plants that had been exposed to increasing periods of darkness showed a progressive decrease in nitrate reducing ability which was restored on subsequent illumination. Since an unlimited amount of reduced pyridine nucleotide was always present in the assay medium, it was suggested that light had a role in the induction of nitrate reductase rather than in the generation of reduced pyridine nucleotides (9,10,23).

Beevers *et al.* (14) demonstrated that light was not an absolute requirement for the induction of nitrate reductase in green leaf tissue as long as sufficient nitrate was present in the induction medium. They recorded the effect of light and dark environments and the levels of nitrate in induction medium on the uptake of nitrate by excised corn seedlings as well as enzyme induction. In the dark, there was essentially a linear increase in nitrate content and enzyme induction in the tissue, with increased nitrate content of the induction media. In the light, the uptake of nitrate was greater (4-fold) than in the dark and proportional to the amount of nitrate supplied. Since nitrate is essential for the induction and the stabilization of nitrate reductase (24), they concluded that the effect of light on induction was attributed to enhanced nitrate uptake. A similar conclusion for the effect of light on nitrate utilization had been made

by Warburg and Negelein (25). This conclusion is supported by the observations of changes in permeability of cell membranes following illumination (96,97,98).

Dark-grown oat and barley seedlings accumulate large amounts of nitrate in darkness, but nitrate reductase activity does not increase above the low endogenous level until light is supplied (26,27,99). However, when nitrate reductase in dark-grown oat leaves was induced in light for 12 hours and then returned to darkness, the activity continued to increase for another 24 hours (99).

Travis *et al.* (26) found that light treatment was required before the enzyme could be induced in etiolated leaves and the capacity for nitrate reductase induction by nitrate was positively correlated with the level of cytoplasmic polyribosomes under a variety of experimental conditions. (a) Light-grown leaves contained high levels of polyribosomes and similarly high levels of nitrate reductase activity were induced. (b) The level of polyribosomes and the ability to form nitrate reductase activity rapidly decreased in light-grown leaves following transfer to an anaerobic environment in the dark. (c) After treatment of leaves in darkness under nitrogen to dissociate polyribosomes to monoribosomes, their ability to form nitrate reductase following transfer back to light again correlated with the level of reformed polyribosomes. (d) Etiolated leaves contained a low level of cytoplasmic polyribosomes, and nitrate reductase activity was induced following exposure to light only after a lag of two to four hours. During this lag period there was also a marked increase in the level of polyribosomes. Thus, they concluded that the apparent requirement of light for nitrate reductase induction in etiolated

leaves seems not to be specific, but rather an influence of light upon the development of an active protein-synthesizing apparatus was indicated. The specific effect of light on the protein-synthesizing apparatus may be related to the control of messenger RNA synthesis and the monoribosome to polyribosome transformation as suggested by data of Williams and Novelli (100). However, when excised light-grown leaves were transferred to darkness under $N_2$, polyribosome and soluble protein content were decreased, but when they were transferred to darkness under air polyribosome and protein content were at the same level as in leaves under continuous light. So, again it is difficult to explain the disappearance of nitrate reductase activity in the dark under air by the level of polyribosomes.

In the case of tobacco leaves, it was reported that the requirement of light for nitrate reductase induction could be replaced by providing appropriate concentrations of gibberellic acid and kinetin in the dark (29,30). Köhler (28) found that light seems to relate to the synthesis of gibberellin. Lips and Roth-Bejarano (29) suggested that light is necessary for nitrate reductase induction (or synthesis) in tobacco leaves because of its effect on the concentration of one or more endogenous growth regulators. This effect may be due to either a stimulation of synthesis or the retardation of breakdown of those hormones required for nitrate reductase synthesis. However, Gandhi and Naik (31) demonstrated that in the absence of light, gibberellic acid alone or in combination with kinetin could not restore enzyme synthesis in intact green or etiolated rice seedlings. They suggested that the results by Lips and Roth-Bejarano (29) and Roth-Bejarano and Lips (30) could be a manifestation of the residual

effect of light because the tobacco plants were grown for two months in light.

Ingle (32) induced nitrate reductase in radish cotyledon with [$^{3}$H]uridine plus [$^{14}$C]leucine and purified nitrate reductase 100-fold. He found that the incorporation of the radioactivity into nitrate reductase is negligible in comparison to the total amount of labeled protein, while nitrate reductase activity was highly increased. He concluded that the requirement of protein synthesis for nitrate reductase induction was not necessary directly for nitrate reductase protein *de novo* synthesis, but may involve the synthesis of an effector necessary for nitrate reductase activity.

Inactivation of the Enzyme in the Dark

The mechanism of inactivation of nitrate reductase and other enzymes in the dark is not yet known. Travis *et al.* (16) found that the disappearance of nitrate reductase activity in barley leaves during darkness was inhibited by cycloheximide, actinomycin D, and low temperature. Thus, protein synthesis was probably required for the disappearance of nitrate reductase activity in the dark. Mixing extracts from dark- and light-treated leaves gave additive activity which indicated the absence of excess inhibitors in the extracts. They suggested that a possible explanation for the loss of nitrate reductase activity in the dark might be the binding of an inhibitory protein, or the inactivation of an active site, or degradation of nitrate reductase.

The decay of phenylalanine ammonia lyase (PAL) activity also can be induced by transferring tissues from light to dark (164,165,166)

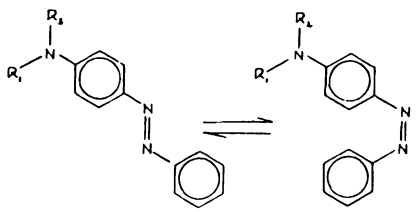
and the inactivation was prevented by cycloheximide (166,167,168). During rapid inactivation of PAL in darkness, radioactivity from $^{14}$C-amino acids incorporated into the enzyme prior to inactivation was lost (166). Therefore, the inactivation in this case may involve degradation of the enzyme, although complexing with the inhibitor protein cannot be ruled out as a mechanism of inactivation. Because turnover of the enzyme in darkness was much faster than that of the bulk of the soluble leaf protein, the requirement for a PAL-specific protease was suggested (169). However, Zucker (169,170) found that *Xanthium* leaf discs synthesized PAL in the dark as fast as in the light. To account for the rapid decay in enzyme activity in the presence of continuing synthesis, it must be assumed that inactivation of PAL increases greatly in the dark. Pulse labeling experiments verified this assumption (170). These experiments demonstrated that photoregulation of PAL synthesis did not occur; rather, the cycloheximide-sensitive inactivating system might be effected by conditions of illumination, inactivation being low or absent in the light but increasing greatly in the darkness. Zucker suggested that a reasonable explanation for these observations was that synthesis of the inactivator protein was repressed by a photosynthetic product.

## Model Systems of Photoregulation of Enzyme Activity

Most model systems are composed of enzymes that can be photosensitized by light-absorbing chromophores. Where the chromophores occur naturally the systems have potential for participation in photoregulated events *in vivo*. For example, pyridoxal phosphate, which has an absorption in the near uv, can bind to the epsilon amino group of

lysine in proteins and can serve as a chromophore in the photosensitization of a specific histidine residue in spinach leaf aldolase and other enzymes (171). The photoactivation of a purified bacterial urocanase by near uv was reported (172). Apparently no chromophore absorption could be detected in purified enzyme, but difference spectra between active and inactive enzyme were not presented. Perhaps photosensitivity may involve light absorption by amino acid residues activated by configurational strains imposed on them by the conformation of the enzyme (173). Participation of an FMN-containing amino acid oxidase in blue light stimulation of respiration is another example of a natural model system (174).

Erlanger and his colleagues have shown how systems normally insensitive to light (the enzymes chymotrypsin [172] and acetylcholinesterase [176] and the electroplax of the electric eel [177]) can be photoregulated by means of photochromic effector molecules. These molecules share a common *p*-phenylazophenyl group which, under the influence of light, undergoes a reversible configurational change to yield a *cis* or *trans* isomer. Their ability to induce photoregulation derives from differences in the biochemical activities of the two isomers.




Although the systems studied can serve as models for photoregulated processes found in nature, generation of the *cis* and *trans* isomers (and hence regulation) required bright sources of ultraviolet and visible light,

respectively. Ideally, one would prefer a system in which the *cis* isomer occurs in the dark.

Recently Bieth *et al.* (158) reported this type of model, in which levels of acetylcholinesterase activity can be made to respond to the presence or absence of sunlight. The photochromic molecule responsible for the properties of the system was N-*p*-phenylazophenylcarbamyl choline iodide (I). The two isomers of this molecule differed in their ability to inhibit acetylcholinesterase. Ki was found to be $1.6 \times 10^{-6}$M for the *trans* and $3.6 \times 10^{-6}$M for the *cis* isomer. The influence of sunlight on the acetylcholinesterase system was determined in the following way: A solution containing (I) ($2.5 \times 10^{-4}$M) and acetylcholinesterase ($2.3 \times 10^{-8}$M) in pH 7.0 assay buffer (0.02M sodium phosphate, 0.1M NaCl, 0.01M $MgCl_2$, $5 \times 10^{-5}$M EDTA, and 0.01% gelatin) was exposed to sunlight for 15 min at 30°. It reflected the ability of sunlight to convert the *trans* isomer to *cis* isomer with a concurrent decrease in the inhibition of acetylcholinesterase activity of about 12%. The system was reversible; placing it in the dark restored the inhibition activity to its former level. Under the conditions of this experiment, only 50% conversion of the *trans* to *cis* isomer occurred. They stated that changes in activity levels of as much as 30% could be induced when complete conversion to the *cis* isomer was effected by means of Spectroline B-100 ultraviolet lamp instead of the sunlight.

Regulation of enzyme activity by light-sensitive reversible effector molecules is an economical process, since there is no need for the organism to continually synthesize large quantities of new enzyme. We can find evidence of such effector molecules in nature.

One example might be abscisin (178) which induces dormancy in plants. It is known to be photochromic and only the *cis* isomer is biologically active. Another more complex substance is phytochrome (179,180), which is known to function by a photochromic mechanism in the regulation of many plant processes. The widespread occurrence in plants and animals of carotenoid substances, many of which can undergo photochromic alterations, suggests that their function may very well be intimately concerned with photoregulation (158).

Nitrate Reductase Inhibitors

Nitrate reductase is sensitive to inhibition by *p*-chloromercuribenzonate (1 μM to 1 mM) especially when NADH is the electron donor (1,3). Cysteine or glutathione protects the enzyme against this inhibitor. The enzyme is sensitive to reagents that react with metals; cyanide and azide are particularly effective, while organic chelating reagents give various degrees of inhibition (1). Atabrin inhibits marrow nitrate reductase at a concentration of 5 mM. Neither carbon monoxide nor fluoride (1 mM) inhibits soybean nitrate reductase (1).

Dirr *et al.* (102) found that the absence of active nitrate reductase in extracts of *Leucothoe* and *Rhododendron* (Ericaceae) leaves was due to a galloyl ester-like compound, similar in nature to tannic acid. It was heat stable, non-dialyzable and complexed with polyvinylpyrrolidone (PVP). This compound inhibited other enzymes, glutamate dehydrogenase and malate dehydrogenase, nonspecifically.

Dalling *et al.* (58) showed that a low activity of nitrate reductase in crude homogenate or the supernatant fraction of tobacco leaves was due to a heat-stable, small molecular weight inhibitor which was removed or bound by soluble or insoluble PVP.

NADH-nitrate reductase of the Berlin strain of *Chlorella vulgaris* Beijerinck is obtained in crude extracts largely in an inactive form (142,143) which can be activated by oxygen in the unfractionated extracts, or ferricyanide (145). Solomonson *et al.* (146) found that the cell extracts of the Berlin strain contained a component which can cause a rapid and reversible inactivation of the ferricyanide activated enzyme. The component was isolated from the cell extracts by ultrafiltration and called an ultrafiltrate Fraction X. The molecular weight of Fraction X was estimated to be around 1,000-10,000. Fraction X can reduce ferricyanide and DCPIP. The enzyme inactivated by Fraction X can be completely reactivated by ferricyanide. Solomonson *et al.* (146) suspected that an active component of Fraction X may be a redox substance with some of the properties of the ferricyanide-ferrocyanide system.

Nitrate reductase is an ubiquitous enzyme in almost all organs of higher plants (4), but its activity in extracts of roots is very low (103-104). The possibility of the presence of a phenolic natural inhibitor in the roots of apple trees has also been indicated (105, 106). The inhibitor could be readily eliminated from root extracts by dialysis against a buffer containing PVP.

Pan and Marsh (107) reported a protein-like macromolecule in maize root which inactivated nitrate reductase in leaf extracts. There appeared to be two inhibition reactions: one is rapid and

quickly inhibited the reductase activity when added directly to the reaction mixture; the other is slow acting and requires pre-incubation with the enzyme.

Kadam *et al.* (39) found an inhibitor of nitrate reductase in the roots of rice seedlings. The substance inhibited nitrate reductase activity in leaf extract when NADH or $FMNH_2$ were used as electron donors. However, when benzylviologen reduced by sodium dithionite was used as electron donor, the inhibitor was not effective. Pre-incubation with NADH protected the enzyme from the inhibitor, while cysteine and nitrate were without any effect. The inhibitor is thus probably acting at a site where NADH is donating electrons to nitrate reductase. Pre-incubation of the enzyme with NADH alone stimulated the reaction but the extent of stimulation was considerably diminished by adding the inhibitor along with NADH. So, NADH and the inhibitor showed exactly opposite effects on the enzyme. The inhibitor and NADH seemed to be competitive as negative and positive effectors on nitrate reductase. The inhibitor was non-dialyzable, heat labile and inactive at 0°. It was not detected in shoots of green or etiolated rice seedlings. It is unlikely that the inhibitor was a general proteolytic enzyme, alhtough the preincubation of the inhibitor with nitrate reductase at 25° was required for the inhibition activity.

Wallace (36,37,38) studied in detail a nitrate reductase-inactivating enzyme from maize roots. It was largely precipitated by 40 to 55% ammonium sulfate. The inhibition effect was temperature and time dependent. It was purified 460-fold and its molecular weight was estimated to be approximately 44,000 by gel filtration. The

inactivating enzyme could be chromatographed on cation-exchange cellulose at pH 5.0, but was not retained on an anion exchange cellulose at pH 7.0. Inhibition by phenylmethyl sulphonyl fluoride and the degradation of casein by the inactivating enzyme fraction suggested that it is a protease with a serine active site. NAD(P)H-cytochrome c reductase component of nitrate reductase is the main site of action of the inactivating enzyme. When tested on the NADH-nitrate reductases from the maize root and scutella, NADH-cytochrome c reductase was inactivated at a greater rate than was the $FADH_2$-nitrate reductase component. With the *Neurospora* NADPH-nitrate reductase only the NADPH-cytochrome c reductase was inactivated. *p*-Chloromercuribenzonate at 50 μM, which gave almost complete inhibition of the NADH-cytochrome c reductase fraction of the maize nitrate reductase, had no marked effect on the action of the inactivating enzyme. A reversible inactivation of the maize nitrate reductase has been shown to occur during incubation with NAD(P)H. The maize root inactivating enzyme also inactivated the nitrate reductase in the pea leaf, but not the reductase from either *Pseudomonas denitrificans* or *Nitrobacter agilis*.

# MATERIALS AND METHODS

## Plant Materials

Soybeans (*Glycine max* L. Merr. variety Amsoy) were germinated in vermiculite in a dark room for three days and grown in a chamber with 2,000 ft. candles of continuous light (from fluorescence lamps supplemented with a few tungsten lamps) for five days at 28°. After the five day light-period, some plants were exposed to darkness for 54 hours, and others were kept continuously in the light. Nitrate reductase was induced by 50 mM $KNO_3$ added to the Hoagland's nutrient solution every day after the 4th day of light. The young first leaves were used for enzyme and inhibitor preparations.

Spinach (*Spinacia oleracea* L., variety Long Standing Bloomsdale) was grown in soil during a 12 hour photoperiod with 24° day and 16° night temperatures. Plants were watered every other day with the Hoagland's nutrient solution and nitrate reductase was induced with 50 mM $KNO_3$. When commercial spinach leaves were used, they were dipped in 50 mM $KNO_3$ in Hoagland's nutrient solution and illuminated for 9 hours before use for enzyme preparations.

## Chemicals

All chemicals were from Sigma Chemical Company, except the following: Blue Dextran was from Pharmacia, acrylamide gel electrophoresis chemicals from Canalco, $KNO_3$ from Baker, and SDS from

Mallinckrodt, DEAE-cellulose (DE 52) was obtained from Whatman, and Sephadex and DEAE-sephadex A-50 were from Pharmacia. Superoxide dismutase was from Worthington Biochemical Corp.

NAD(P)H-Nitrate Reductase Assay

Nitrate reductase (E.C.1.6.6.2) was measured in a final volume of 1.0 ml containing 25 µmoles potassium phosphate at pH 6.5, 10 µmoles $KNO_3$ for NADH-nitrate reductase assays or 80 µmoles $KNO_3$ for NADPH-nitrate reductase assays, 0.02 µmole FAD and enzyme. The reaction was initiated by adding 0.2 µmole of NADH or NADPH and after 15 min at 30° stopped by addition of 0.1 ml of 1M zinc acetate (5,58). After centrifugation for 2 min at 1,000xg, a 1 ml aliquot was assayed for nitrite by addition of 0.5 ml of sulfanilamide reagent (1% w/v in 1.5N HCl) followed by 0.5 ml of N-(1-naphthyl)ethylenediamine reagent (0.02% w/v). The color developed at room temperature for 30 min prior to reading at 540 nm by a Gilford Spectrophotometer model 2400-S (5).

For a continuous spectrophotometric assay, the decrease of NAD(P)H was recorded at 340 nm. The complete reaction mixture (3 ml) except $KNO_3$ was incubated for 5 min at 30° and the reaction was started by adding 0.3 ml of 0.1M or 0.24 ml of 1M $KNO_3$ for NADH-nitrate reductase and NADPH-nitrate reductase assays, respectively. Endogenous NAD(P)H oxidation was subtracted in each reaction.

One unit of nitrate reductase is the formation of one µmole of nitrite per min.

## $FADH_2$-, Reduced Methylviologen-, and Reduced Benzylviologen-Nitrate Reductase Assays

Essentially the same reaction mixture was used as for the NAD(P)H-nitrate reductase assays except the pyridine nucleotide was replaced by 0.2 µmole of FAD or 0.7 mg of benzylviologen or 0.45 mg of methylviologen together with 0.8 mg sodium dithionite in 0.1 ml of 95 mM sodium bicarbonate (3,21,51,55,109). These reactions were stopped by shaking vigorously for about 30 seconds with a Vortex-genie mixer to oxidize the excess sodium dithionite and then 0.1 ml of 1M zinc acetate was added.

## *In vivo* Assay of Nitrate Reductase (5)

Freshly harvested soybean leaves were washed, blotted dry and cut into 10 mm diameter discs. Ten leaf discs were then placed into a 10 ml flask containing a cold infiltration medium of 0.2M $KNO_3$ and 1 mM potassium phosphate at pH 6.5. For infiltration the flasks were evacuated by a water aspirator in a desiccator and then the vacuum released; the process was repeated three times. The systems were incubated in a 30° water bath in the dark and 0.2 ml aliquots of the medium were removed at time intervals for determination of nitrite colorimetrically (5).

## NAD(P)H-DCPIP Reductase Assay

This activity was assayed spectrophotometrically using dichlorophenolindophenol (DCPIP) as the electron acceptor. Cuvettes contained 1000 µmoles potassium phosphate at pH 6.5, 0.15 µmole DCPIP, 0.67 µmole NADH or NADPH and an appropriate amount of enzyme and $H_2O$ to a final volume of 3 ml. The reduction of the dye was followed as the

decrease in absorbance at 600 nm with a recording spectrophotometer (3).

NAD(P)H-Cytochrome c Reductase Assay

Cytochrome c reductase activity was determined by observing the increase in absorbance at 550 nm with a recording Gilford spectrophotometer in a 1 ml reaction mixture containing 25 μmoles potassium phosphate at pH 6.5, 0.02 μmole FAD, 0.5 mg cytochrome c, 0.2 μmole NAD(P)H and an appropriate amount of enzyme (110).

Assay of the Nitrate Reductase Inhibitor

Unless stated otherwise, an aliquot of the nitrate reductase inhibitor was added to a standard NADH-nitrate reductase reaction mixture which contained 25 μmoles potassium phosphate at pH 6.5, 10 μmoles $KNO_3$, and a known amount of NADH-nitrate reductase from soybean leaves. Without preincubation, the reaction was started by adding 0.2 μmole NADH. Reaction was 30 min at 30°. As a control the buffer solution for the inhibitor was added to the reaction mixture instead of the inhibitor. Inhibition is expressed in terms of the decrease in nitrite production caused by the inhibitor; percent inhibition is 100 $\left(\frac{\text{control rate - inhibited rate}}{\text{control rate}}\right)$. When possible, the amount of the inhibitor was adjusted to around 50% inhibition of nitrite production where the relation between the amount of the inhibitor and the inhibition rate is approximately linear.

NADPH-Phosphatase Assay

This was measured in the same reaction mixture as for the NAD(P)H-nitrate reductase assay. The reaction was stopped after 15

min at 30° by boiling for 1 min. The amount of NAD plus NADH was measured by cycling assay with PMS, thiazolyl blue, ethanol and yeast alcohol dehydrogenase. The increase in absorbance at 570 nm was recorded at 30° and the total amount of NAD(H) was calculated as described in reference 111.

Nitrite Reductase Assay

The activity was followed by measuring colorimetrically the rate of disappearance of nitrite (71,112). Enzyme preparation was added to a solution containing 25 µmoles potassium phosphate at pH 7.5, 2 µmoles $NaNO_2$ and 0.75 µmole methylviologen in a final volume of 1 ml. The reaction was started by addition of 0.15 ml of sodium dithionite (25 mg in 1 ml of 0.29 M sodium bicarbonate). After 15 min incubation at 30°, the reaction was stopped by agitation of the reaction mixture with a Vortex mixer to oxidize the dithionite. The reaction mixture (0.01 ml) was diluted to 1 ml with water and assayed for nitrite as described in NAD(P)H-nitrate reductase assay (71,112).

Glutamate Dehydrogenase Assay

The activity was measured as the amination of α-ketoglutarate (113,114) at 30° in a cuvette with a 1-cm light path. The reaction mixture contained 200 µmoles potassium phosphate at pH 8.0, 400 µmoles $(NH_4)_2SO_4$, 0.4 µmole NADH and an apparopriate amount of enzyme. After preincubation for 2.5 min, the reaction was started by adding 7 µmoles potassium-α-ketoglutarate. Total volume was 3 ml. The change in absorbance at 340 nm in the initial minutes with the

complete reaction mixture, corrected for the change in the absence of α-ketoglutarate, was taken as the rate of the reaction.

Glycolate Oxidase Assay

This was assayed anaerobically as DCPIP reduction (115,116). To a Thumberg Beckman cuvette (1 cm light path) were added 100 μmoles pyrophosphate at pH 8.5, 0.37 μmole DCPIP in the buffer, 0.25 μmole FMN and enzyme plus water to give a total volume of 2.5 ml, and in side arm 0.1 ml of 0.125M sodium glycolate was added. The cuvette was then evacuated and flushed 10 times with $N_2$. Dye reduction at 30° was measured at 600 nm with a Gilford spectrophotometer.

NAD-Malate Dehydrogenase Assay

The activity was measured spectrophotometrically by following the oxidation of NADH at 340 nm with oxalacetate (117,118). The assay mixture contained 67 μmoles HEPES buffer (pH 7.4), 0.126 μmole $NADH_2$, 0.4 μmole oxalacetate and enzyme plus water to give a total volume of 1 ml in the cuvette.

NAD-Lactate Dehydrogenase Assay

The activity was measured spectrophotometrically by following the oxidation of NADH with sodium pyruvate (119). The reaction mixture contained 34 μmoles potassium phosphate at pH 7.4, 0.126 μmole NADH, 0.3 μmole sodium pyruvate and enzyme plus water to give a total volume of 1 ml in the cuvette.

α-Glycerol Phosphate Dehydrogenase Assay

The activity was measured by following the oxidation of NADH (122). The reaction mixture contained 50 μmoles triethanolamine-HCl

buffer at pH 7.5, 0.1 µmole dihydroxyacetone phosphate and 0.135 µmole NADH and enzyme plus water to give a total volume of 1 ml in the cuvette.

Acid Phosphatase Assay

The enzyme was assayed by measuring the amount of *p*-nitrophenyl-phosphate hydrolyzed to *p*-nitrophenol which absorbs at 410 nm (123, 124). Ten microliters of enzyme was added to 0.2 ml *p*-nitrophenyl-phosphate solution containing 0.4% disodium salt (Sigma's 70%) and an equal volume of 0.1M potassium acetate at pH 5. After 10 min incubation at 35° the reaction was stopped by adding 2 ml of 0.02M sodium hydroxide, and the color was read at 410 nm.

Xanthine Oxidase Assay

The activity was measured spectrophotometrically by following the formation of urate from hypoxanthine at 290 nm. The reaction mixture contained 30 µmoles potassium phosphate, 0.45 µmole hypoxanthine and enzyme plus water to give a total volume of 1 ml in the cuvette (120,121).

Protease and Peptidase Assays

The hydrolysis of α-benzoyl-L-arginine-*p*-nitroanilide was measured by the procedure used by Kruger (188), while the substrate L-leucine-*p*-nitroanilide was tested by the method of Beevers (189). In the assay for protease activity 1.0 ml of casein solution or partially purified NADH-nitrate reductase was incubated with a sample of the nitrate reductase inhibitor at 30°. Aliquots of 0.5 ml were removed at certain times (0, 15, 30 min) and treated with an equal

volume of 10% trichloroacetic acid to precipitate protein. After centrifugation, 0.1 ml of aliquot was added to a ninhydrin-citrate-glycerol mixture that consisted of 0.5 ml of 1% ninhydrin solution in 0.5M citrate buffer at pH 5.5, 1.2 ml of glycerol which had been passed through a column of Amberlite MB3, and 0.2 ml of 0.5M citrate buffer at pH 5.5. The mixture was heated in a boiling water bath for 12 min. After cooling, the color was read at 570 nm within 1 hour. Tryptophan was used as a standard amino acid solution (190).

Protein Assay

Protein was estimated by the procedure of Lowry *et al.* (132).

Isolation of NADH- and NADPH-Nitrate Reductase

Twenty grams of soybean leaves were homogenized by a Waring blender for 2 min in 50 ml of a buffer containing 25 mM potassium phosphate at pH 7.5, 1 mM EDTA, 0.2% insoluble PVP and amounts of cysteine varying between 0 and 20 mM. The homogenate was filtered through four layers of cheesecloth, then one layer of Miracloth, and then centrifuged for 10 min at 37,500xg. The supernatant was put directly on a DEAE-cellulose (Whatman DE-52) column (2 x 21 cm) which had been equilibrated with a buffer containing 10 mM potassium phosphate at pH 7.0, 1 mM EDTA, and amounts of cysteine varying between 0 and 10 mM. After washing the column with 250 ml of equilibration buffer, it was developed with a linear NaCl gradient obtained from mixing 375 ml of equilibration buffer and 375 ml of 0.5M NaCl in equilibration buffer. Ten milliliter fractions were collected. After determining the peaks of NADH-nitrate reductase and NADPH-nitrate

reductase, each peak fraction was pooled and then the protein was precipitated by 50% saturated ammonium sulfate. The precipitate was collected by centrifugation and dissolved in a small volume of buffer containing 10 mM potassium phosphate at pH 7.0, 1 mM EDTA, 5 mM cysteine and 0.5M sorbitol. This enzyme preparation was passed through a Sephadex G-25 column (2 x 18 cm) which had been equilibrated with the same buffer. The nitrate reductase was collected in the void volume and frozen in liquid nitrogen. The frozen nitrate reductase was stable when stored at -18°.

Isolation of Nitrite Reductase and Glutamate Dehydrogenase

Commercial spinach was dipped in the Hoagland's nutrient solution and 50 mM $KNO_3$ and illuminated for 9 hours. The treated spinach or soybean leaves from plants grown in a growth chamber were ground by a Waring blender for 90 sec in 2.5 volume of a solution containing 25 mM potassium phosphate at pH 8.0, 1 mM EDTA, 1 mM cysteine and 0.2% insoluble PVP. The homogenate was passed through four layers of cheesecloth and then one layer of Miracloth, and then centrifuged for 20 min at 20,000xg. To the supernatant was added 35% cold acetone, and the precipitate was discarded after centrifugation. The supernatant was brought to 75% acetone, and the precipitate was collected by centrifugation and dissolved in a small amount of 10 mM potassium phosphate at pH 8.0 with 1 mM DTT (125). The suspension was dialyzed overnight against the same buffer. The dialyzed enzyme suspension was centrifuged and the supernatant was applied to a DEAE-cellulose column which had been equilibrated by the above buffer (71). The column was developed with a linear gradient from 0 to 0.4M NaCl.

Glutamate dehydrogenase was eluted at about 0.06M NaCl and nitrite reductase at about 0.135M NaCl.

Isolation of Glycolate Oxidase

This enzyme was partially purified from spinach leaves. First the pH of the leaf extract was lowered to 5.4 by addition of 10% acetic acid, and the precipitate was removed by centrifugation. Then the supernatant was fractionated by ammonium sulfate. The precipitate between 20-30% saturated ammonium sulfate was collected by centrifugation and dissolved in 20 mM potassium phosphate at pH 8.0. The enzyme solution was dialyzed against the same buffer overnight (126).

Preparation of Malate Dehydrogenase

The peroxisomes of spinach leaves were separated in a sucrose gradient by a zonal centrifugation (118). The protein in the peroxisomal fraction was concentrated by precipitation with 60% ammonium sulfate. The precipitate was desalted by a small Sephadex G-25 column and used for malate dehydrogenase activity (117).

Preparation of Glycerol Phosphate Dehydrogenase

The organelles of rat liver were separated in a sucrose gradient by a zonal centrifugation and the peroxisomal fraction was directly used for glycerol phosphate dehydrogenase assay (122).

Sucrose Density Gradient Centrifugation

Soybean leaves (12.5 g) were ground with a mortar and pestle in 17.5 ml of a buffer containing 25 mM potassium phosphate at pH 8.2,

1 mM EDTA, 10 mM cysteine, 25% sucrose and 0.1% soluble PVP. Discontinuous sucrose gradients were prepared at 4° by layering into 50 ml Beckman cellulose nitrate tubes (2.2 x 8.6 cm) 8 ml of 2.3M sucrose followed by 7.8 ml of 1.75M, 7 ml of 1.7M, and 5 ml of 1.6, 1.4, and 1.3M sucrose solutions. The sucrose solutions were prepared in 25 mM potassium phosphate at pH 7.0, 1 mM EDTA and 5 mM cysteine. Twenty milliliters of the leaf homogenate that had been filtered through four layers of cheesecloth and then one layer of Miracloth was then layered on the top of the gradient, and centrifuged at 3° for 3 hours at 25,000 rev/min (44,700xg to 106,900xg) in a Spinco SW 25.2 rotor (118). The gradients were fractionated by puncturing the bottom of the tube and collecting 2.5 ml aliquots.

Gel Filtration and Molecular Weight Estimation

The molecular weight of nitrate reductase was estimated by Sephadex G-200 gel filtration. The column (1.5 x 85 cm) was equilibrated with 25 mM potassium phosphate at pH 7.0, 1 mM EDTA, 5 mM cysteine and 0.5M sorbitol. One and five-tenths milliliter of sample was applied to the top of the column with 10% glycerol. The flow rate was 6 ml per hour. Two milliliter fractions were collected. The column was calibrated with blue dextran, purified spinach RuDP-carboxylase (560,000 D), catalase (240,000 D), yeast alcohol dehydrogenase (150,000 D) and bovine serum albumin (68,000 D).

The molecular weight of the nitrate reductase inhibitor was estimated by Sephadex G-100 gel filtration. The column (2.5 x 90 cm) was equilibrated with 25 mM potassium phosphate at pH 7.0, 1 mM EDTA and 1 mM cysteine. A 3 ml sample was applied to the bottom of the column. The flow rate was 40 ml per hour. Eight milliliter fractions

were collected. The column was calibrated with blue dextran, bovine serum albumin (68,000 D), ovalbumin (45,000 D), carbonic anhydrase (29,000 D) and cytochrome c (12,400 D).

Polyacrylamide-Gel Electrophoresis

The purity of the two nitrate reductases was estimated by electrophoresis on 5% polyacrylamide gels according to the procedure of Davis (127). The current used was 3 mA per tube (5 mm diameter). The gels were stained with coomassie brilliant blue, and unreacted dye was removed by destaining with 7.5% acetic acid and 5% methanol and about 1g Amberlit MB-3. Each gel was then monitored at 550 nm with a Gilford linear scanner.

The purity of the inhibitor was estimated by gel electrophoresis according to the modification of the procedure of Davis (127) and Ornstein (128). Gels were made from 4% (w/v) acrylamide, 0.135% (w/v) methylene bisacrylamide, 0.03% (w/v) N,N,N',N'-tetramethylethylene diamide, 0.4% (w/v) ammonium persulfate, and 1 mM DTT in 0.1M potassium phosphate at pH 7.5. Elution buffer was 0.1M potassium phosphate at pH 7.5 and 50 mM glycine. The current used was 8 mA per tube (5 mm diameter). Some gels were stained for protein and destained by the same method as above. Other gels were cut into 4 mm slices. Each slice was immersed in the NADH-nitrate reductase reaction mixture minus FAD, NADH and enzyme, and then kept in the dark at 4° to extract the protein from gel. After two days, gel slices were removed from the reaction mixture and NADH-nitrate reductase and NADH were added. The reaction was run for 30 min at 30° and the decrease in nitrite production by the extract was determined.

For urea-gel electrophoresis, essentially the same procedure was used as above except gels and samples contained 8M urea.

For SDS-polyacrylamide gel electrophoresis, 0.1% SDS was added in 7.5% acrylamide gels and elution buffer (pH 7.1) (129,130). The protein was incubated at 100° for 10 min in 10 mM sodium phosphate at pH 7.1, 1% SDS, 1% mercaptoethanol and 10% glycerol (129,130). Eight milliamps current was applied to each gel. As reference proteins catalase (subunits of 60,000 D). ovalbumin (45,000 D), carbonic anhydrase (29,000 D) and lysozyme (14,300 D) were used (5 µg each protein/tube). The mobility of the protein was calculated by the method of Weber and Osborn (131).

## Photoinactivation and Recovery of the Inhibitor

The reaction mixture for the NADH-nitrate reductase assay minus the enzyme and NADH was mixed in small 10 ml test tubes. After addition of the inhibitor they were placed under a Sun-umbrella (about 2,000 ft. candle) at 0°. For the dark pretreatment, test tubes were covered by aluminum foil. After two hours, nitrate reductase and NADH were added to the reaction mixture and nitrite formation was measured. To observe the recovery of the photoinactivated inhibitor, the system was kept in the dark room at 4° for 24 hours after two hours' illumination. A control, minus inhibitor, was run in each case.

# RESULTS

## Separation of NADH- and NADPH-Nitrate Reductase

As previous investigators have found (2,5), levels of nitrate reductase in extracts of soybean leaves were influenced by the concentration of sulfhydryl reducing agent in the extraction buffer and the length of time required to make the enzyme preparation. The activity was more unstable prior to chromatography on DEAE-cellulose and large losses of activity were found if chromatography was not performed as soon as possible. Therefore, the crude extract was put directly on the DEAE-cellulose column without prior treatment except to remove most of the particulate materials by filtration and centrifugation. This initial rapid partial purification seemed to be necessary to obtain good preparations of both enzymes, and represents a difference in my procedure from all previous investigators.

Despite previously reported failure to achieve a separation of NADH- and NADPH-nitrate reductase activities (2,7,8), by my procedure two peaks of nitrate reductase activity, when assayed with 10 mM $KNO_3$, were eluted from the DEAE-cellulose column by a linear NaCl gradient (Figure 1). When it was found that NADPH-nitrate reductase had a much higher Km constant for $NO_3^-$ than NADH-nitrate reductase, two nitrate reductase peaks were shown more clearly. The leading or first peak of nitrate reductase had higher activity with NADH than NADPH and a lower Km for $NO_3^-$, so that assays with 10 mM $KNO_3$ and

Figure 1. Separation of two nitrate reductases from soybean leaves by DEAE-cellulose column chromatography. First leaves from soybean plants (20 g) were ground in homogenizing buffer with 10 mM cysteine. The column was eluted with a solution containing 10 mM potassium phosphate at pH 7.0, 1 mM EDTA and 5 mM cysteine, and increasing NaCl concentration. Aliquots from 10 ml fractions (0.1 ml) were assayed for nitrate reductase activity. (A) Profile for protein elution (———) and NaCl gradient (-----). (B) Nitrate reductase activity assayed with 10 mM $KNO_3$ and NADH (———) or $FADH_2$ (-----). (C) Nitrate reductase activity assayed with 80 mM $KNO_3$ and NADPH (———) or $FADH_2$ (-----). (D) In this experiment, although 5 mM cysteine was used in the grinding medium, but only 1 mM cysteine was used in the elution buffer, nitrate reductase activity was assayed in 10 mM $KNO_3$ with NADH (———) and in 80 mM $KNO_3$ with NADPH (-----).

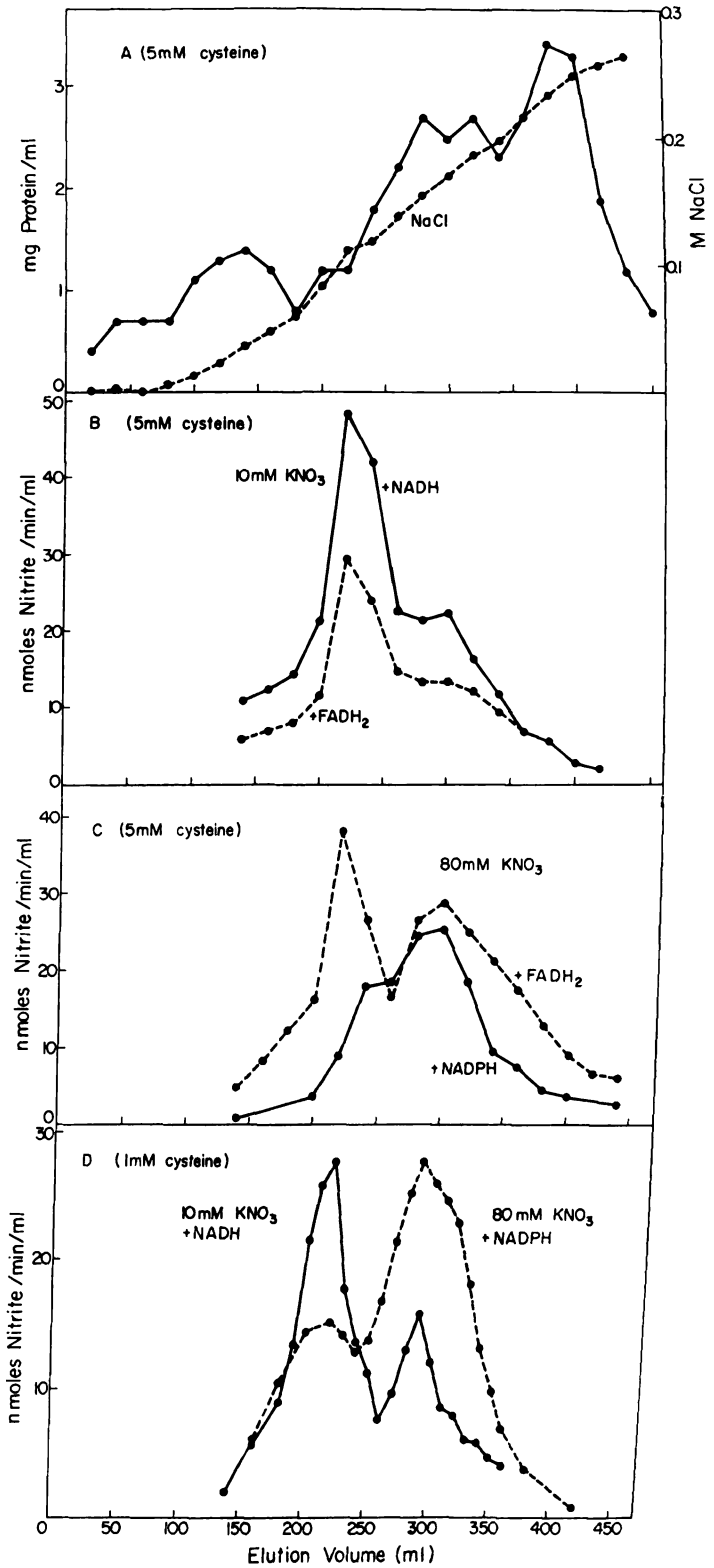

A (5mM cysteine)
mg Protein/ml
M NaCl
NaCl
B (5mM cysteine)
nmoles Nitrite /min/ml
10mM KNO3
+NADH
+FADH2
C (5mM cysteine)
80mM KNO3
+FADH2
+NADPH
D (1mM cysteine)
10mM KNO3
+NADH
80 mM KNO3
+NADPH
Elution Volume (ml)

Figure 1

NADH maximized activity in this area (Figure 1B). The second peak of nitrate reductase had a higher Km of $NO_3^-$ so that 80 mM $KNO_3$ in the assay was required to maximize its activity (Figure 1C). The second peak of nitrate reductase utilized NADPH as electron donor better than NADH. Thus the first peak was designated as being NADH-nitrate reductase and the second peak as being NADPH-nitrate reductase, although the latter will also use NADH to a considerable extent. When chemically reduced FAD was used instead of NADH and NADPH, the two nitrate reductase fractions were also observed (Figure 1B, 1C). Higher activity with $FADH_2$ was observed with 80 mM nitrate than with 10 mM nitrate even in the NADH-nitrate reductase fraction, because of a higher Km for $NO_3^-$ with $FADH_2$ than with NAD(P)H, as will be shown later. As shown in Figure 1C, with 80 mM $KNO_3$ the first peak (mainly NADH-nitrate reductase) and the second peak (mainly NADPH-nitrate reductase) were more active with $FADH_2$. NADH-nitrate reductase was eluted from the DEAE-cellulose column at about 0.11M NaCl and NADPH-nitrate reductase was eluted at about 0.17M NaCl.

From Figures 1B and 1C it appears that the NADH-nitrate reductase fraction was about twice as active as the NADPH-nitrate reductase, when the enzyme was extracted and eluted in buffers containing 10 mM and 5 mM cysteine, respectively, which is known to protect NADH-nitrate reductase (2,5). When the nitrate reductases were extracted in a buffer containing 5 mM cysteine and separated on a DEAE-cellulose column with an elution buffer containing only 1 mM cysteine, surviving activities of the two peaks of nitrate reductase were about equal (Figure 1D). The difference is attributed to a loss

of about 50% of the NADH-nitrate reductase peak in 1 mM cysteine, whereas the NADPH-nitrate reductase activity was almost the same as in Figure 1C with 5 mM cysteine. When 20 mM cysteine and 10 mM cysteine were used for extraction and elution buffers, respectively, the level of NADH-nitrate reductase peak was three times higher than in Figure 1D with 1 mM cysteine, while the level of NADPH-nitrate reductase peak still stayed almost the same (data not shown).

When cysteine was omitted from the grinding medium and the elution buffer for DEAE-cellulose column, only one peak was eluted from the column. The elution position of this single peak was shifted to a higher concentration of salt (about 0.26M NaCl). The characteristics of this peak were similar to those of the NADPH-nitrate reductase eluted from the DEAE-cellulose column when 5 mM cysteine had been used in the extraction and 1 mM cysteine in the elution buffer. The higher salt concentration required to elute this NADPH-nitrate reductase may have been caused by altered protein concentration in the extracts prepared without the presence of sulfhydryl reducing agents.

Thus it is possible to separate the two nitrate reductases by DEAE-cellulose column chromatography. Further it is possible by not using cysteine during purification to isolate only the NADPH-nitrate reductase. To isolate NADH-nitrate reductase one must use 20 mM and 10 mM cysteine for extraction and elution buffers, respectively, and collect the most active fraction.

Considerable loss of activity was always experienced during purification and separation of nitrate reductase (Table 1). After the separation of the two enzymes, the best way to keep activity for

Table 1

Summary for Partial Purification of NADH-Nitrate Reductase and of NADPH-Nitrate Reductase

The results are from 20g of young soybean leaves ground in buffer with 10 mM cysteine. The elution buffer for the DEAE-cellulose column chromatography contained 5 mM cysteine.

| Stage of Procedure | NADH-NITRATE REDUCTASE | | | | NADPH-NITRATE REDUCTASE | | | |
|---|---|---|---|---|---|---|---|---|
| | $NO_2^-$ Production | Total Protein | Specific Activity | Recovery | $NO_2^-$ Production | Total Protein | Specific Activity | Recovery |
| | nmoles/min | mg | nmoles/min/mg protein | % | nmoles/min | mg | nmoles/min/mg protein | % |
| Homogenate | 5,900 | 650 | 9.1 | 100 | 2,150 | 650 | 3.3 | 100 |
| Centrifuged extract | 4,450 | 576 | 7.7 | 75 | 1,920 | 576 | 3.3 | 89 |
| Pooled active fractions from DEAE-cellulose column | 1,920 | 103 | 18.6 | 33 | 570 | 84 | 6.8 | 27 |
| 0-50% ammonium sulfate fraction | 1,050 | 43 | 24.4 | 18 | 459 | 45 | 10.2 | 21 |
| Sephadex G-25 fraction | 850 | 39 | 21.8 | 14 | 430 | 41 | 10.5 | 20 |

further investigation was to concentrate the pooled DEAE-cellulose fraction by mixing them with an equal volume of cold saturated ammonium sulfate solution, collecting the precipitate by centrifugation, and dissolving in a buffer containing 25 mM potassium phosphate at pH 7.0, 1 mM EDTA, 0.5M sorbitol and 5 mM cysteine. Residual ammonium sulfate was removed from these preparations by passing through Sephadex G-25 column that had been equilibrated with the same buffer. Preparations of each nitrate reductase could now be stored at -18° (after freezing by liquid nitrogen) for several months without extensive loss of activity.

The total and specific activities for the two nitrate reductase assays in the above isolation procedure, as presented in Table 1, represent only a 3-fold purification. Actual purification is probably somewhat more because the activity in the homogenate was high due to cross activity of the two enzymes. Also, the activity of NADPH-nitrate reductase in the homogenate would be overestimated due to the high levels of NADPH phosphatase in soybean leaf extracts (7,8). Therefore, the actual fold purification and percentage of recovery of NADPH-nitrate reductase may be higher than is shown in Table 1.

Further attempts to purify the preparations by Sephadex G-200 gel filtration resulted in a substantial decrease of total protein in the peak with nitrate reductase activity, but only about 10% of the nitrate reductase activity put on the column was recovered so that there was no increase in specific activity. Therefore, this technique could not be used as a preparative procedure.

Cofactor Requirements: For the NADH- and NADPH-Nitrate Reductase

Preliminary investigation of the optimal assay conditions of the two nitrate reductases indicated that NADH-nitrate reductase and NADPH-nitrate reductase had maximum activity when assayed with 10 mM $KNO_3$ and 80 mM $KNO_3$, respectively. The activities of NADH- and NADPH-nitrate reductases, which were purified by DEAE-cellulose column chromatography with high cysteine concentration and without cysteine, respectively, were measured at 10 mM $KNO_3$ and 80 mM $KNO_3$ for five different electron donors (Table 2). Both enzymes utilized NADH and NADPH. At the optimum $KNO_3$ concentration of 10 mM, the NADH-nitrate reductase was twice as active with NADH as with NADPH and at 80 mM $KNO_3$ these activities were inhibited about 50%. NADPH-nitrate reductase was more active with NADPH than with NADH and was more active with 80 mM $KNO_3$ than with 10 mM $KNO_3$. Both enzymes used reduced benzylviologen, methylviologen and FAD as electron donors. The use of reduced methylviologen gave the highest activity for both of the nitrate reductases. Whereas NADH-nitrate reductase was inhibited by 80 mM $KNO_3$ with NADH or NADPH, its activity with the other three electron donors was not inhibited at the higher substrate level. Activity of NADPH-nitrate reductase with all electron donors was higher at 80 mM than 10 mM $KNO_3$.

The requirement of the partially purified nitrate reductases for flavin nucleotide was further investigated (Figure 2). FAD or FMN did not stimulate the partially purified NADH-nitrate reductase. In fact, at concentrations in excess of 10 μM both FAD and FMN were inhibitory to NADH-nitrate reductase. On the other hand,

Table 2

Relative Rates of the NADH-Nitrate Reductase and NADPH-Nitrate Reductase with Different Electron Donors at Two Concentrations of $KNO_3$

The enzymes were the peak fractions from DEAE-cellulose column chromatographic separation.

| | Electron Donor | ACTIVITY | |
|---|---|---|---|
| | | 10 mM $KNO_3$ | 80 mM $KNO_3$ |
| | | nmoles $NO_2^-$ produced/10 min | |
| NADH-nitrate reductase | NADH | 18.3 | 10.7 |
| | NADPH | 10.3 | 4.2 |
| | Reduced benzylviologen | 13.3 | 12.8 |
| | Reduced methylviologen | 35.5 | 34.7 |
| | $FADH_2$ | 14.5 | 21.3 |
| NADPH-nitrate reductase | NADH | 5.2 | 7.8 |
| | NADPH | 7.5 | 11.2 |
| | Reduced benzylviologen | 2.5 | 13.2 |
| | Reduced methylviologen | 15.8 | 43.8 |
| | $FADH_2$ | 10.8 | 25.6 |

Figure 2. Flavin nucleotide requirement of NADH0 and NADPH-nitrate reductases. Procedures were the standard assays for 10 min at 30° of NADH-nitrate reductase (-----) and NADPH-nitrate reductase (———) described in Materials and Methods with the exception of variable final concentrations of FAD (——— or ———) or FMN (----- or -----) indicated.

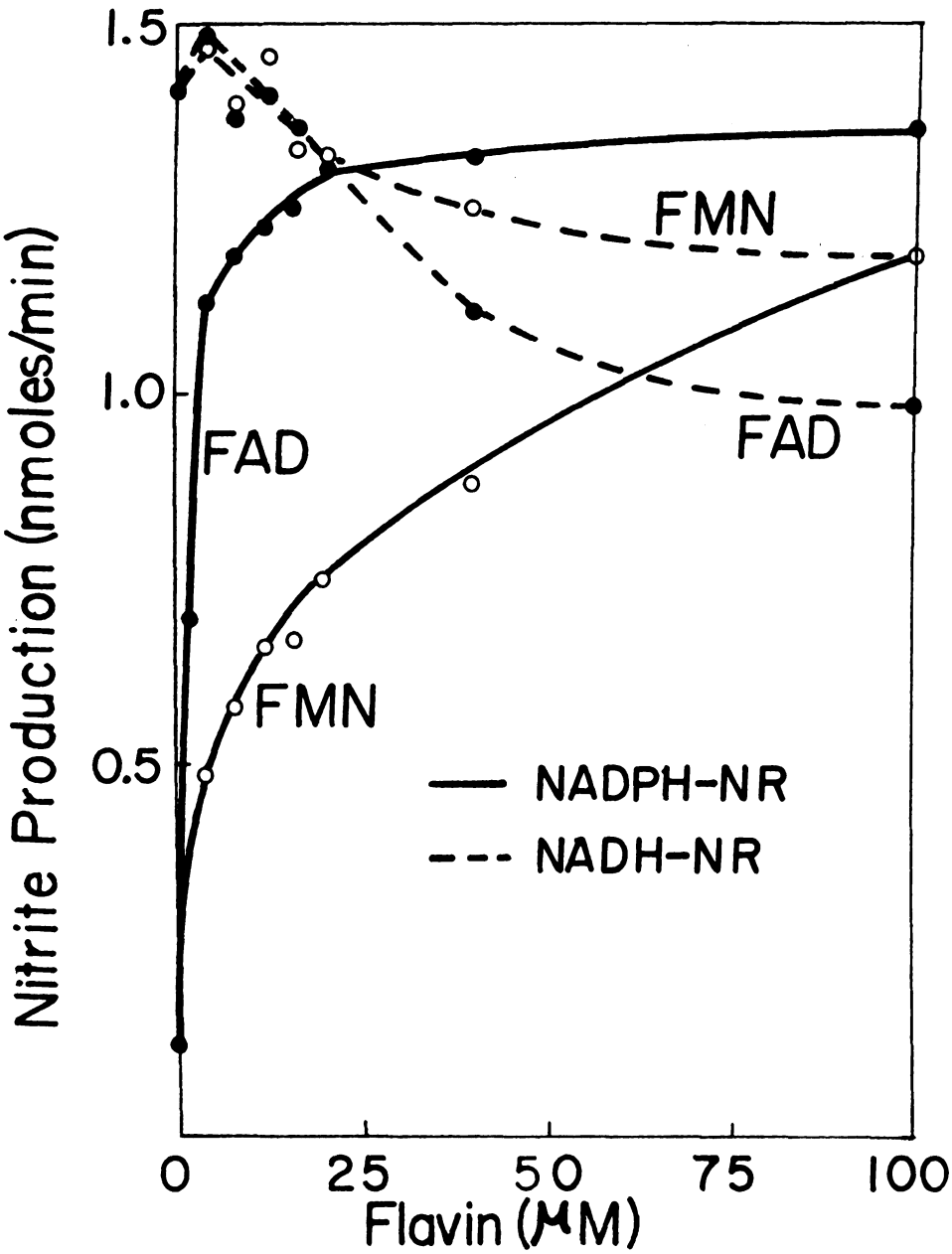

1.5
1.0
0.5
Nitrite Production (nmoles/min)
FMN
FAD
FAD
FMN
NADPH-NR
NADH-NR
0
25
50
75
100
Flavin (μM)

Figure 2

NADPH-nitrate reductase was stimulated by both FAD and FMN. Using NADPH with NADPH-nitrate reductase, Km constants for FAD were about 2 μM and for FMN about 10 μM. Above 100 μM both FAD and FMN also began to inhibit NADPH-nitrate reductase. Stimulation of NADPH-nitrate reductase by FAD was greater than with FMN. Therefore, as described in the methods section, 20 μM FAD was added to all nitrate reductase assays, particularly when NADPH was used as an electron donor.

pH Optima

The pH optimum in 25 mM potassium phosphate buffer for NADH-nitrate reductase with NADH was at 6.5 (Figure 3), but with NADPH the optimum was more acidic (pH 6.0) as occurs with other reductases which primarily utilize NADH. The pH optimum for NADPH-nitrate reductase with both NADPH and NADH was also at pH 6.5. The partially purified NADPH-nitrate reductase could have contained an acid phosphatase, which might have been responsible for the activity of this enzyme with NADPH as speculated by Wells (7) and Wells and Hageman (8). If this were the case, then the activity might have been partially inhibited by the phosphate buffer which is known to be an inhibitor of phosphatases (7,8,147,148). Therefore, the pH optimum of the NADPH-nitrate reductase with NADPH was assayed in 25 mM MES in the pH range 5.8 to 6.6. The pH optimum was found to be 6.2. Although this slight shift in pH optimum occurred in MES buffer, the activity of NADPH-nitrate reductase at the optimum pH was the same as that found in phosphate buffer at pH 6.5.

Figure 3. Effect of pH on nitrate reductases. Preparations of NADH-nitrate reductase (NADH-NR) on the left side and NADPH-nitrate reductase (NADPH-NR) on the right side were made as outlined in Table 1. They were assayed in 25 mM phosphate buffer with 10 mM $KNO_3$ for the NADH-nitrate reductase and 80 mM $KNO_3$ for the NADPH-nitrate reductase. Assays were with 2 x $10^{-5}$M NADH (———) or NADPH (-----).

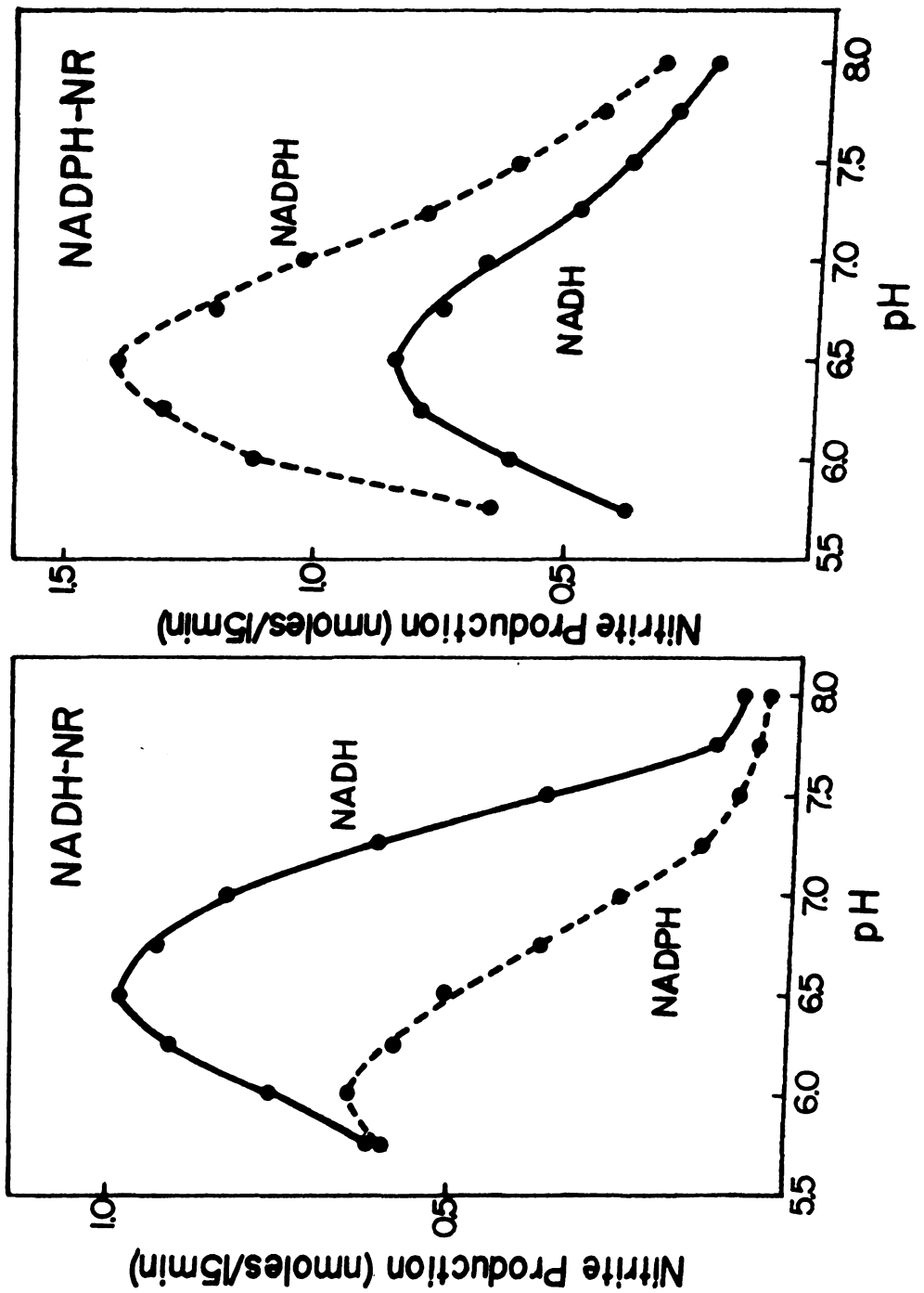

NADPH-NR
NADPH
NADH
Nitrite Production (nmoles/15min)
1.5
1.0
0.5
5.5
6.0
6.5
7.0
7.5
8.0
pH
NADH-NR
NADH
NADPH
Nitrite Production (nmoles/15min)
1.0
0.5
5.5
6.0
6.5
7.0
7.5
8.0
pH

Figure 3

NADPH Phosphatase Activity in the Nitrate Reductase Preparations

This phosphatase was very active in the extract of the soybean leaves, but it was separated from the nitrate reductases by the DEAE-cellulose column chromatography. No NADPH phosphatase was found in the nitrate reductase preparations from the DEAE-cellulose columns. Three different preparations each of NADH- and NADPH-nitrate reductase were tested for the formation of NAD(H) resulting from NADPH phosphatase activity during the nitrate reductase assay. No NAD(H) was found in any of these reaction mixtures, even those which had been incubated for 15 min at 30° before stopping the reaction by boiling for 1 min. The background level of detection in the cycling assay, which was used, was 0.02 nmole of NAD and/or NADH (111). Thus, in identical reaction mixtures both using NADPH as the electron donor, one was assayed for nitrate reductase activity and the other was assayed for NAD(H) formation during the reaction. Under these conditions, using a preparation of NADPH-nitrate reductase, 0.5 nmole of nitrite was formed per min during a 15 min reaction, and less than 0.02 nmole of NAD(H) was formed per min. When NADH was added to the reaction mixture which had been boiled, it was possible to detect as much as 95% to 99% of the amount added. So, it appears that the assay of NAD(H) by a cycling procedure was not inhibited in the boiled reaction mixture, but that NAD(H) had not been formed by the action of a NADPH phosphatase during the nitrate reductase reaction. It was concluded that the NADPH-nitrate reductase activity of the fraction could not be attributed to a NADPH phosphatase which could convert it to NADH for the NADH-nitrate reductase.

Kinetic Parameters

For kinetic studies both nitrate reductases were purified by Sephadex G-200 gel filtration following the DEAE-cellulose column chromatography and ammonium sulfate fractionation. The kinetic constants associated with the electron donors (NADH, NADPH, $FADH_2$) and with $KNO_3$ as an electron acceptor are summarized in Table 3. The $Km(NO_3^-)$ for NADH-nitrate reductase with NADH at 0.11 mM was significantly less than $Km(NO_3^-)$ for NADPH-nitrate reductase with NADPH at 4.5 mM (the Lineweaver-Burk plots are shown in Figure 4). However, the Km(NADH) for NADH-nitrate reductase at 8.1 μM was greater than the Km(NADPH) value of 1.5 μM for NADPH-nitrate reductase (Table 3). The $Km(NO_3^-)$ for NADH-nitrate reductase with NADPH was lower than with NADH; however, the Vmax in the latter case was much higher than in the former case. $Km(NO_3^-)$ values with $FADH_2$ were much higher and too large to be of physiological significance. It should be emphasized here that Km values of $NO_3^-$ for NADH-nitrate reductase are about 40-fold less than $Km(NO_3^-)$ for NADPH-nitrate reductase, so that in the presence of limiting nitrate, the NADH-nitrate reductase in the plant may be the more physiologically active enzyme.

Inhibitors

Of the many known inhibitors of nitrate reductase (1,3), only three were tested (Table 4). NADH-nitrate reductase was inhibited more by KCN and $NaN_3$ than NADPH-nitrate reductase. $NaN_3$ was a slightly more potent inhibitor of both nitrate reductases than KCN. KF at 10 mM which is known as an inhibitor of phosphatase (7,8,149,150) did not inhibit either nitrate reductase.

Table 3

Substrate Affinities of NADH- and NADPH-Nitrate Reductases

| | NADH-Nitrate Reductase | NADPH-Nitrate Reductase |
|---|---|---|
| Km($KNO_3$) with NADH as electron donor | 0.11 mM | 5.8 mM |
| Km($KNO_3$) with NADPH as electron donor | 0.05 mM | 4.5 mM |
| Km($KNO_3$) with $FADH_2$ as electron donor | 2.4 mM | 14.6 mM |
| Km(NADH) with $KNO_3$ as electron acceptor | 8.1 µM | 3.9 µM |
| Km(NADPH) with $KNO_3$ as electron acceptor | 202 µM | 1.5 µM |
| Km($FADH_2$) with $KNO_3$ as electron acceptor | 42 µM | 24 µM |

Figure 4. Double reciprocal kinetic plots for $NO_3^-$ of NADH- and NADPH-nitrate reductases. NADH-nitrate reductase and NADPH-nitrate reductase were purified through Sephadex G-200 gel filtration following DEAE-cellulose column chromatography and ammonium sulfate fractionation. The standard assay procedure as described in Materials and Methods was used with the exception of the final concentrations of $KNO_3$. The reaction was for 30 min at 30°. v is expressed as nmoles nitrite produced per 30 min and [S] is referred to the millimolar concentration of $KNO_3$.

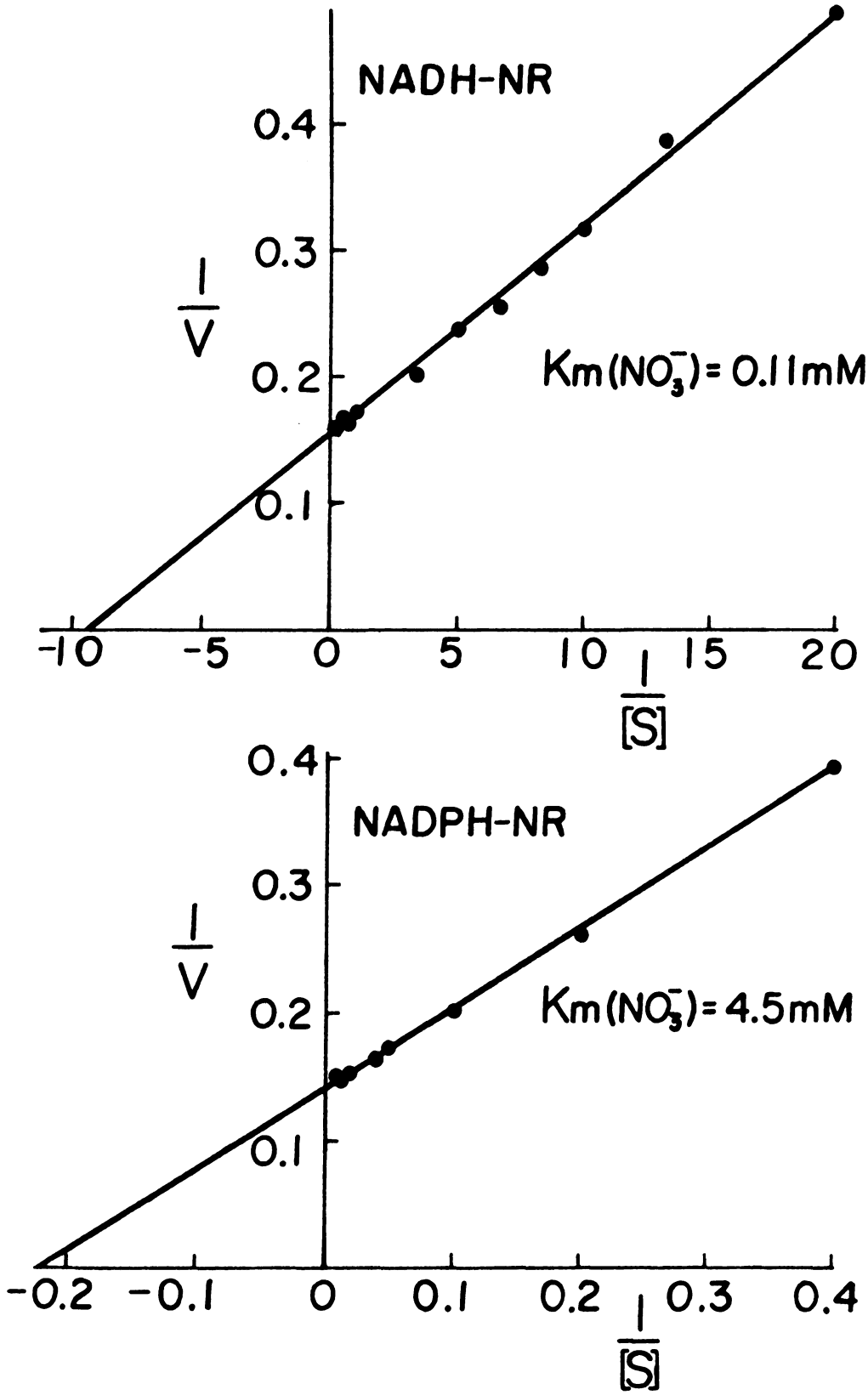

NADH-NR
0.4
0.3
0.2
0.1
1/V
Km(NO3-)= 0.11mM
-10
-5
0
5
10
15
20
1/[S]
NADPH-NR
0.4
0.3
0.2
0.1
1/V
Km(NO3-)= 4.5mM
-0.2
-0.1
0
0.1
0.2
0.3
0.4
1/[S]

Figure 4

Table 4

Inhibition of Nitrate Reductases

NADH- and NADPH-nitrate reductases were assayed under their respective optimum conditions. The enzymes were not preincubated with the inhibitor before the reactions were started. Uninhibited activities were 1.53 milliunits of NADH-nitrate reductase and 1.71 milliunits of NADPH-nitrate reductase.

| Inhibitor | Final Concentration | NADH-Nitrate Reductase | NADPH-Nitrate Reductase |
|---|---|---|---|
| | M | percent inhibition | |
| KCN | $10^{-4}$ | 69 | 8 |
| | $10^{-3}$ | 95 | 47 |
| | $10^{-2}$ | 100 | 91 |
| $NaN_3$ | $10^{-4}$ | 92 | 53 |
| | $10^{-3}$ | 98 | 93 |
| KF | $10^{-3}$ | 0 | 0 |
| | $10^{-2}$ | 0 | 0 |

Molecular Weight of the Two Nitrate Reductases

The molecular weights were estimated to be 220,000 for NADPH-nitrate reductase and 330,000 for NADH-nitrate reductase, as determined by a calibrated Sephadex G-200 column (151). These data are shown as a combined graph of two determinations in Figure 5.

In consideration of this difference in molecular weight, it should be possible to separate the two enzymes by a single passage through a Sephadex G-200 column. When such a separation was attempted, only partial resolution was achieved. The resolution may have been better than it appeared because of cross reaction of the two nitrate reductases for the two reduced pyridine nucleotides. Due to lack of a highly concentrated, stable and highly active nitrate reductase preparation, the small amount of highly purified nitrate reductases which could be obtained by these procedures preclude an extensive investigation of the physical and chemical properties of the two nitrate reductases at this level of purification.

Purity of the Two Nitrate Reductases

The nitrate reductases, which had been separated on a DEAE-cellulose column and further purified by Sephadex G-200 gel filtration and concentrated by 50% ammonium sulfate, were subjected to polyacrylamide gel electrophoresis. The gels were stained for protein with coomassie brilliant blue. After destaining, the gels were scanned at 550 nm with Gilford linear transport spectrophotometer. The scan and representative drawing are shown in Figure 6. The NADPH-nitrate reductase preparation had only a single protein component with an $R_f$ of 0.48 relative to the tracking dye. The NADH-nitrate

Figure 5. Molecular weight determination of the nitrate reductases by Sephadex G-200 gel filtration. Each nitrate reductase in Figure 1-D was precipitated by 50% ammonium sulfate and redissolved in 1.5 ml of 25 mM potassium phosphate, 1 mM EDTA, 5 mM cysteine and 0.5M sorbitol. The Sephadex G-200 column (1.5 x 85 cm) had been equilibrated with the same buffer. Each nitrate reductase was run separately through the column.

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

Vt: total volume of the gel bed
Ve: elution volume for protein
Vo: void volume (dead volume)

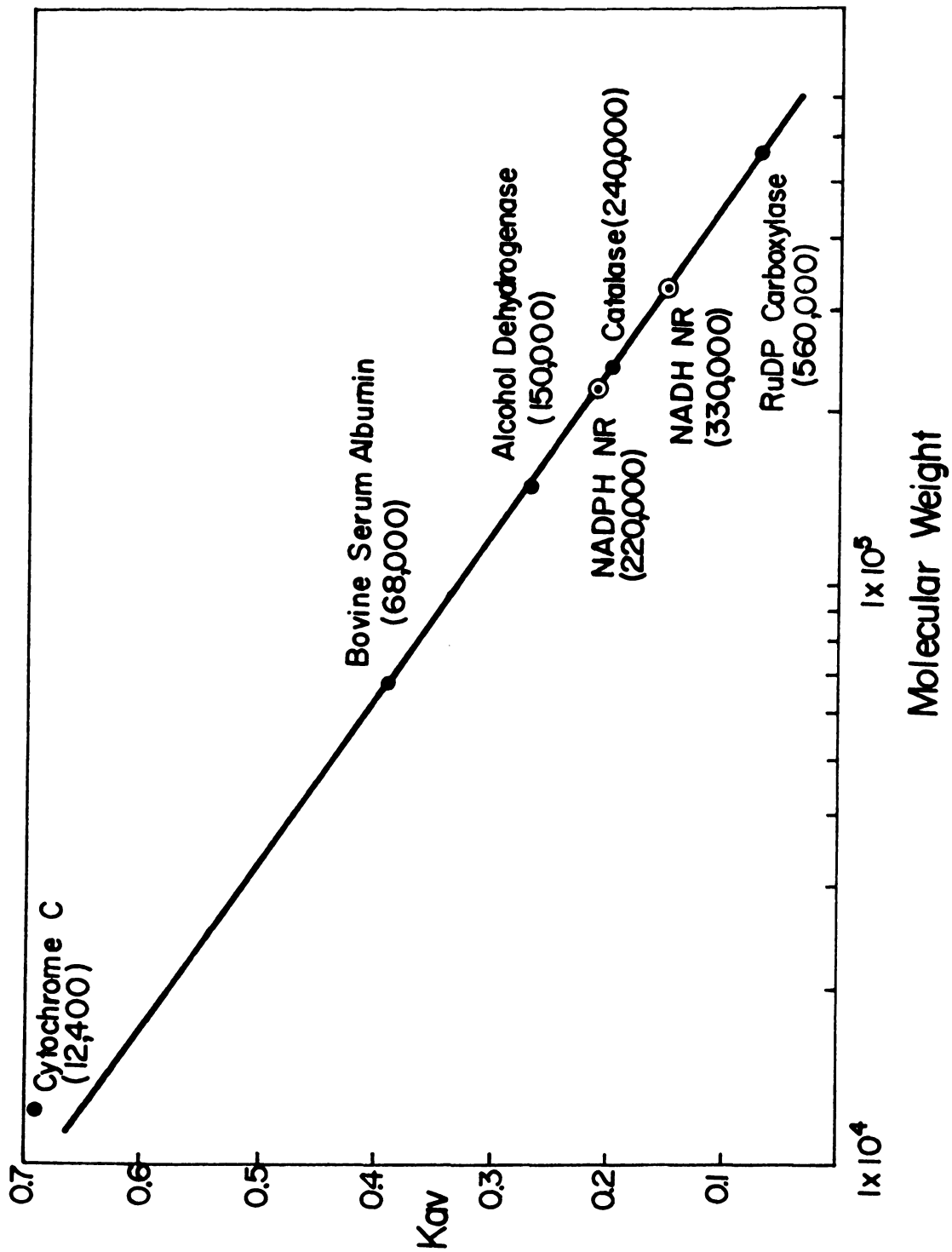

Cytochrome C
(12,400)
Bovine Serum Albumin
(68,000)
Alcohol Dehydrogenase
(150,000)
NADPH NR
(220,000)
Catalase(240,000)
NADH NR
(330,000)
RuDP Carboxylase
(560,000)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Kav
1x10⁴
1x10⁵
Molecular Weight

Figure 5

Figure 6. Polyacrylamide gel electrophoresis of NADH- and NADPH-nitrate reductase. With each of the two nitrate reductases, the fraction in the tube from the Sephadex G-200 gel filtration having the highest activity was applied to gel electrophoresis (pH 7.5) along with 10% glycerol and bromphenol blue. Amounts of protein applied were 160 µg of the NADH enzyme and 120 µg of the NADPH enzyme.

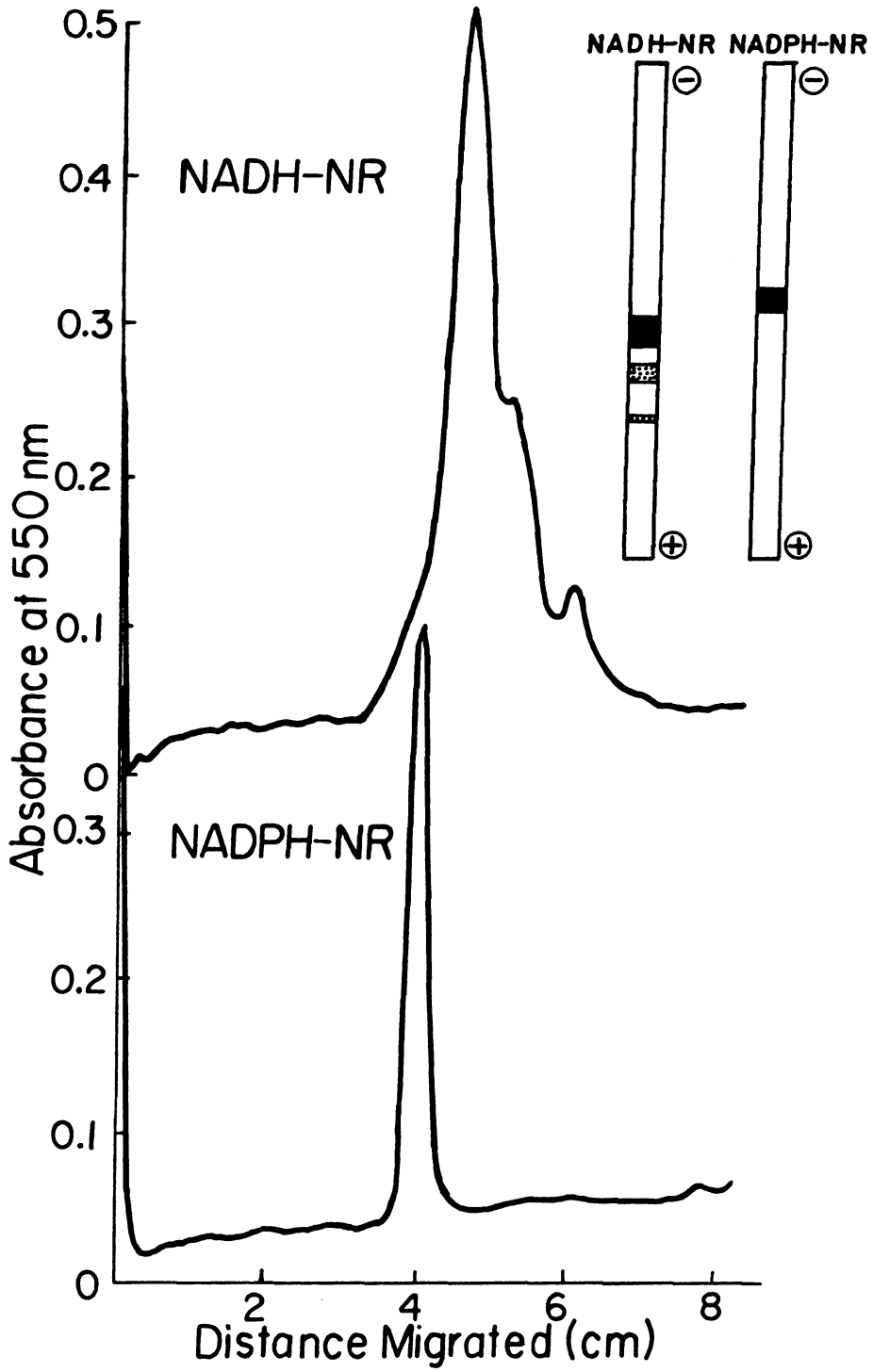

NADH-NR NADPH-NR
NADH-NR
NADPH-NR
Absorbance at 550 nm
0.5
0.4
0.3
0.2
0.1
0
0.3
0.2
0.1
0
2
4
6
8
Distance Migrated (cm)

Figure 6

reductase preparation had one major band at an $R_f$ of 0.54 and two minor bands at $R_f$ values of 0.62 and 0.71. Thus the two nitrate reductase enzymes were different and could be separated by electrophoresis. There was no cross contamination between the two nitrate reductase preparations. The NADPH-nitrate reductase appeared to be nearly pure after the Sephadex G-200 column purification step.

## Nitrate Reductase Activity in Light-Grown and Dark-Grown Soybean Leaves

After two to three days for germination, soybean plants were grown under continuous light for five days. After a five-day light-period, part of the plants were transferred into a dark room. Nitrate and Hoagland's nutrient solution were added every day after the 4th day of light. Total nitrate reductase activity was monitored by an *in vivo* assay during the dark period and in the following light-period (Figure 7). During the first 12 hours in the dark, about 75% of the activity disappeared. After 54 hours of darkness, plants were returned to the light. As reported before (9-12,14-18), leaves regained nitrate reductase activity in the light rapidly, when compared to the loss of activity in the dark. Data for the leaf material after 54 hours in the dark are presented in Figure 8 for comparison with plants kept in the light. By this method soybean leaves devoid of nitrate reductase activity by the *in vivo* assay could be obtained in 54 hours and used for further studies.

Soybean leaves grown under continuous light (light grown) or exposed to darkness for 54 hours (dark grown) were gently homogenized and subcellular organelles were separated by isopycnic sucrose gradient centrifugation (Figure 9). The supernatant fraction at the

Figure 7. Loss of total *in vivo* nitrate reductase activity in the dark and its restoration in the light. After germination, soybean plants were grown under continuous light at 28° in vermiculite with Hoagland solution and 0.05M $KNO_3$. After five days, plants were transferred to a dark room still with Hoagland solution and nitrate. Nitrate reductase activity was measured by the *in vivo* assay procedure during dark-period and the subsequent light-period.

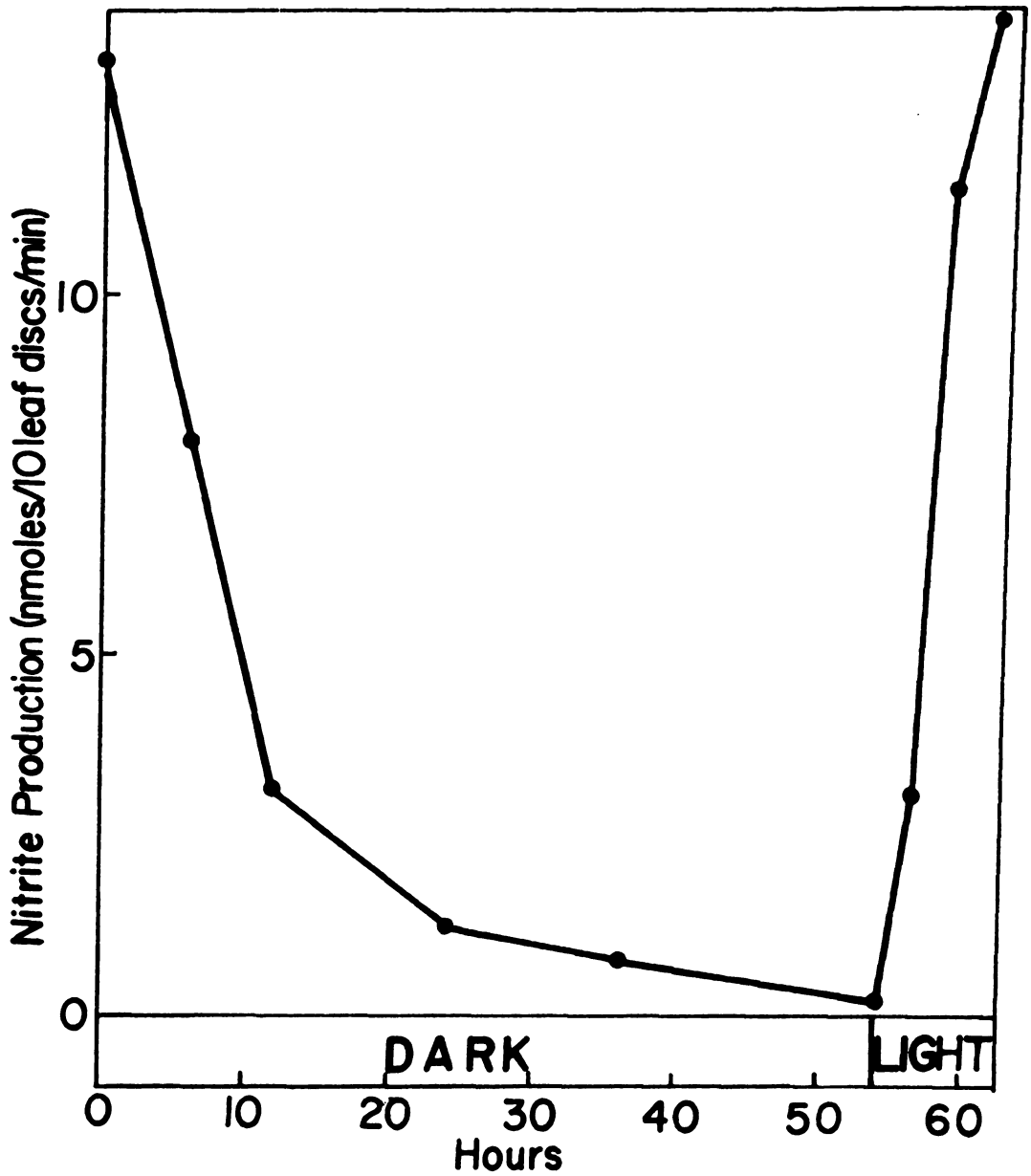

Nitrite Production (nmoles/10 leaf discs/min)
10
5
0
DARK
LIGHT
0
10
20
30
40
50
60
Hours

Figure 7

Figure 8. Total nitrate reductase activity by the *in vivo* assay for soybean leaves from plants grown with Hoagland solution and 0.05M $KNO_3$ in the light for 5 days and then for an additional 54 hours in the light or dark.

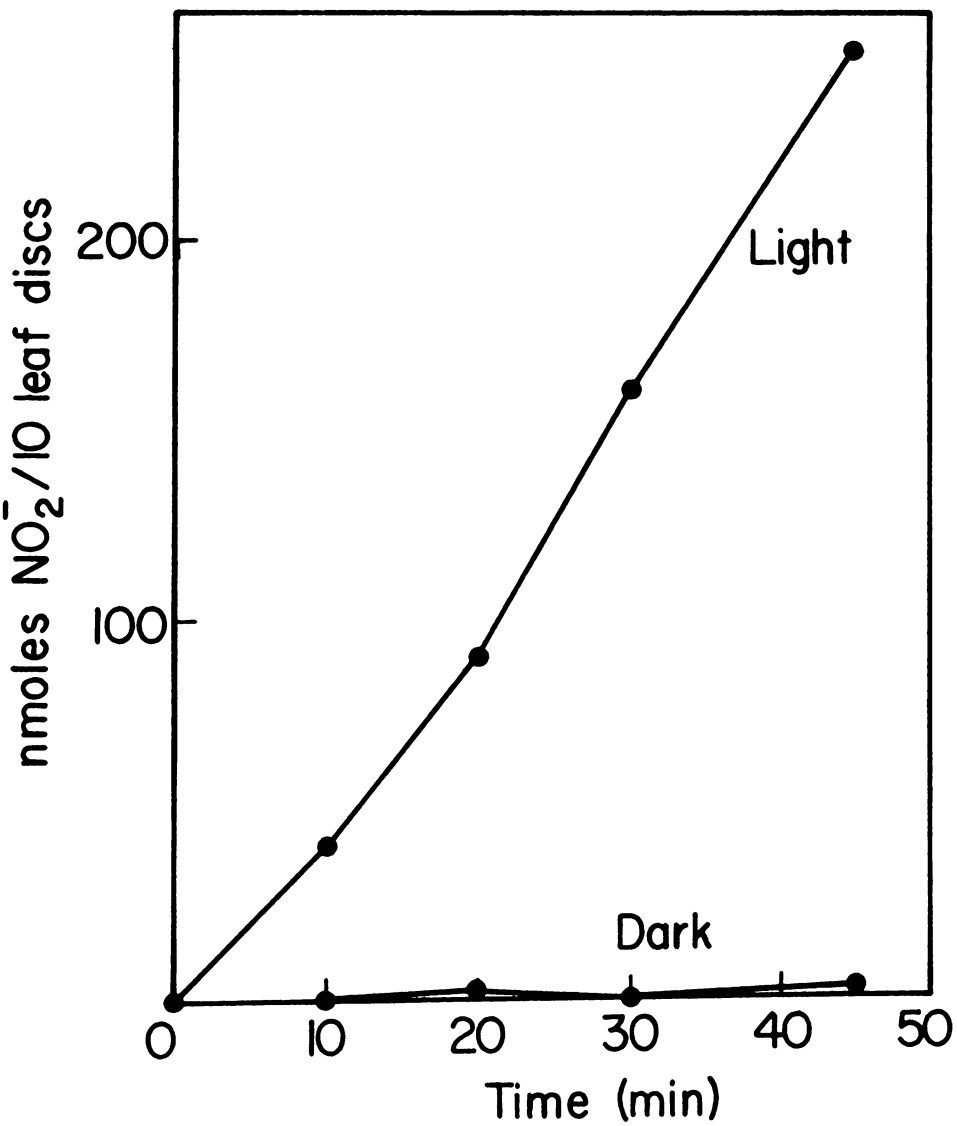

nmoles $NO_2^-$/10 leaf discs
200
100
Light
Dark
0
10
20
30
40
50
Time (min)

Figure 8

Figure 9. The distribution of nitrate reductase on isopycnic sucrose gradients. Homogenates of soybean leaves from plants exposed to darkness for the last 54 hours (-----) or grown under the continuous light (———) were applied. Enzyme activity was measured in terms of the rate of nitrite production by an aliquot of each fraction.

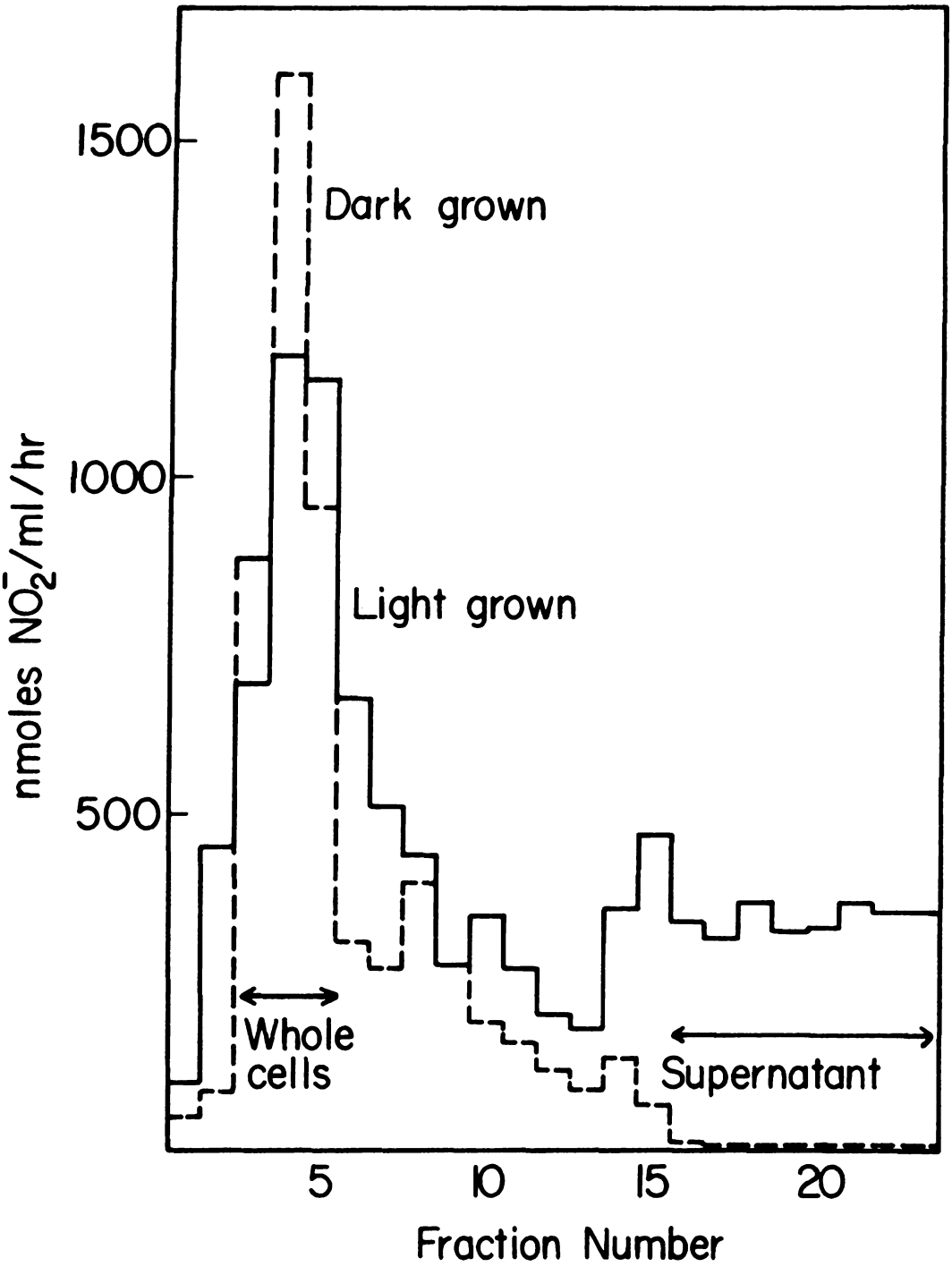

1500
1000
500
nmoles $NO_2^-$/ml/hr
Dark grown
Light grown
Whole cells
Supernatant
5
10
15
20
Fraction Number

Figure 9

top of the gradient from the dark grown leaves did not show nitrate reductase activity when compared to the supernatant from the light grown leaves. However, there were equal amounts of activity in the green heavy fraction in both gradients. The assays for nitrate reductase were done under the optimum condition for NADH-nitrate reductase and NADPH-nitrate reductase and almost the same distribution and amount of activities were obtained. By microscopic examination the green heavy fraction was whole cells. Because soybean leaves have a tough cell wall, many cells were not broken by my grinding procedure, but the leaf was nearly totally broken up into these cells. The supernatant fraction from dark grown plants inhibited the nitrate reductase activity present in both the supernatant of light grown plants and in the whole cell fraction (Table 5). Because nitrate reductase activity in whole homogenate from dark grown leaves was almost negligible, the high activity in the whole cell fraction may indicate a loss of an inhibitor from the cells during isolation in the high concentration of sucrose.

By passing the supernatant of dark grown plants through a small Sephadex G-25 column, about 20% of the inhibitory activity was lost. From this and data in the literature two types of inhibitor could be present in the supernatant from homogenates of dark grown soybean leaves: (1) low molecular weight inhibitor and (2) high molecular weight inhibitor. The presence of a low molecular weight, heat stable inhibitor in tobacco leaves was suggested by Dalling *et al.* (58). A series of organic acids were tested as inhibitors of impure nitrate reductase from spinach leaves (after the leaf homogenate had been fractionated by 25-45% ammonium sulfate and desalted by a

Table 5

The Inhibition of Nitrate Reductase by the Supernatant from an Isopycnic Sucrose Gradient of Homogenates of Soybean Leaves Which Had Been Kept in the Dark for 54 Hours

| Source of Nitrate Reductase Activity | Dark-Grown Leaf Supernatant | Inhibition |
|---|---|---|
| | ml | % |
| Whole cells (dark)* | 0 | 0 |
| | 0.1 | 43.4 |
| | 0.2 | 80.1 |
| Supernatant (light)** | 0 | 0 |
| | 0.1 | 46.5 |

*20 µl of the whole cell fraction from the sucrose gradient of the dark exposed leaves, fraction no. 4 in Figure 9, which produced 15.0 nmoles nitrite in 30 min.

**100 µl of supernatant fraction from the sucrose gradient of soybean leaves grown under continuous light, fraction no. 20 in Figure 9, which produced 17.0 nmoles nitrite in 30 min.

Sephadex G-25 column) (Table 6). All the organic acids at 5 mM inhibited nitrate reductase to some extent. Because we were interested in the effect of photorespiration upon nitrogen metabolism, the inhibition by glycolate and glyoxylate was further studied. Glycolate was somewhat a more powerful inhibitor than glyoxylate of spinach nitrate reductase preparation, which contained an active glycolate oxidase (Table 7). However, the inhibition by glycolate was lost after the nitrate reductase was further purified by DEAE-Sephadex A-25 column chromatography, but the inhibition by glyoxylate remained. The fraction containing nitrate reductase activity from the DEAE-Sephadex A-25 column did not contain glycolate oxidase activity. Glycolate inhibition of nitrate reductase activity in crude enzyme preparations is thus probably due to glyoxylate which was produced from glycolate by glycolate oxidase. The kinetics of glyoxylate inhibition was further examined with the partially purified spinach nitrate reductase (DEAE-Sephadex A-25 column chromatography after 25-45% ammonium sulfate fractionation of extracts). The $Km(NO_3^-)$ was $2.0 \times 10^{-4}$M and Ki value for glyoxylate was about $2.3 \times 10^{-3}$M. The Lineweaver-Burk plot indicated that the inhibition was noncompetitive. Further investigation might also examine the inhibitory effect by hydroxypyruvate which was noted after this phase of my investigations. It is unlikely that glyoxylate ever accumulates in the leaf cell in sufficient amount to inhibit nitrate reductase. The inhibition by hydroxypyruvate seemed more potent (Table 6).

Table 6

The Effect of Organic Acids on Nitrate Reductase Activity in a Crude Preparation from Spinach Leaves

The crude nitrate reductase was prepared by 25-45% ammonium sulfate fractionation of an extract from spinach leaves. Nitrate reductase was added to produce 15 nmoles $NO_3^-$ in 30 min at 30° in the control.

| Addition | Relative Activity of Nitrate Reductase | |
|---|---|---|
| | Addition 5 mM | Addition 10 mM |
| | % | |
| None | 100 | 100 |
| Glycolate | 62.9 | 32.0 |
| Glyoxylate | 70.5 | 42.2 |
| Oxalate | 68.7 | 50.3 |
| P-glycolate | 70.5 | 58.2 |
| Citrate | 78.4 | 44.0 |
| DL-Malate | 88.4 | 85.5 |
| L-Lactate | 83.6 | 72.7 |
| D-Lactate | 76.0 | 76.7 |
| α-Ketoglutarate | 83.0 | 60.9 |
| Serine | 93.3 | 82.3 |
| Glycine | 75.1 | 72.2 |
| Pyruvate | 85.4 | 73.8 |
| Glutamate | 73.5 | 91.6 |
| Oxalacetate | 73.5 | 56.5 |
| 3-P-glycerate | 63.9 | 52.0 |
| Glycerate | 14.5 | 6.2 |
| Hydroxypyruvate | 15.9 | 15.9 |

Table 7

The Effect of Glycolate and Glyoxylate on Crude and Partially Purified Nitrate Reductase from Spinach Leaves

| | Addition | | Relative Activity |
|---|---|---|---|
| | | | % |
| Crude nitrate reductase* | None | | 100 |
| | Glycolate | 2.5 mM | 60.7 |
| | Glycolate | 5.0 mM | 37.0 |
| | Glyoxylate | 2.5 mM | 91.8 |
| | Glyoxylate | 5.0 mM | 58.5 |
| Partially purified nitrate reductase** | None | | 100 |
| | Glycolate | 2.5 mM | 93.0 |
| | Glycolate | 5.0 mM | 91.0 |
| | Glyoxylate | 2.5 mM | 39.1 |
| | Glyoxylate | 5.0 mM | 21.2 |

*The extract of spinach leaf was fractionated by 25-45% ammonium sulfate and desalted and was then used in amounts which produced 11.1 nmoles $NO_2^-$ in 30 min.

**Crude nitrate reductase was further purified by DEAE-Sephadex A-25 column chromatography and was used in amounts which produced 13.6 nmoles $NO_2^-$ in 30 min.

## The Isolation and Purification of the High Molecular Weight Nitrate Reductase Inhibitor

Nitrate reductase activity and its inhibitory activity was separated by DEAE-cellulose column chromatography from soybean leaves which had had a five day light-period followed by a 54 hour dark-period. The typical purification procedures and results are summarized in Figure 10 and Table 8, respectively. The extraction procedure (with 5 mM cysteine) and the preparation of the DEAE-cellulose column (with 1 mM cysteine) for isolating the inhibitor were the same as for preparing the nitrate reductase. Because the inhibitor seemed to be unstable in crude extracts, as is the enzyme, the leaf homogenate was put directly on a DEAE-cellulose column without prior treatment except to remove most of the particulate materials by filtration and centrifugation. The inhibitor could be eluted with either $KNO_3$ or NaCl. Better resolution between nitrate reductases and the inhibitor was obtained with a $KNO_3$ gradient. The typical elution pattern of two nitrate reductases and the inhibitor by a linear gradient of $KNO_3$ is shown in Figure 11. The inhibitor was eluted at around 0.34M $KNO_3$.

After separation by DEAE-cellulose column chromatography, about 70% of the inhibitor was precipitated in a 40-60% ammonium sulfate fraction (Table 9). However, if the crude extract was fractionated by ammonium sulfate prior to DEAE-cellulose column chromatography, most of the inhibitor was found in the 20-40% ammonium sulfate fraction. Generally the 40-70% ammonium sulfate fraction of the inhibitor from the pooled fractions off the DEAE-cellulose column was collected by centrifugation and dissolved in a 25 mM potassium phosphate buffer

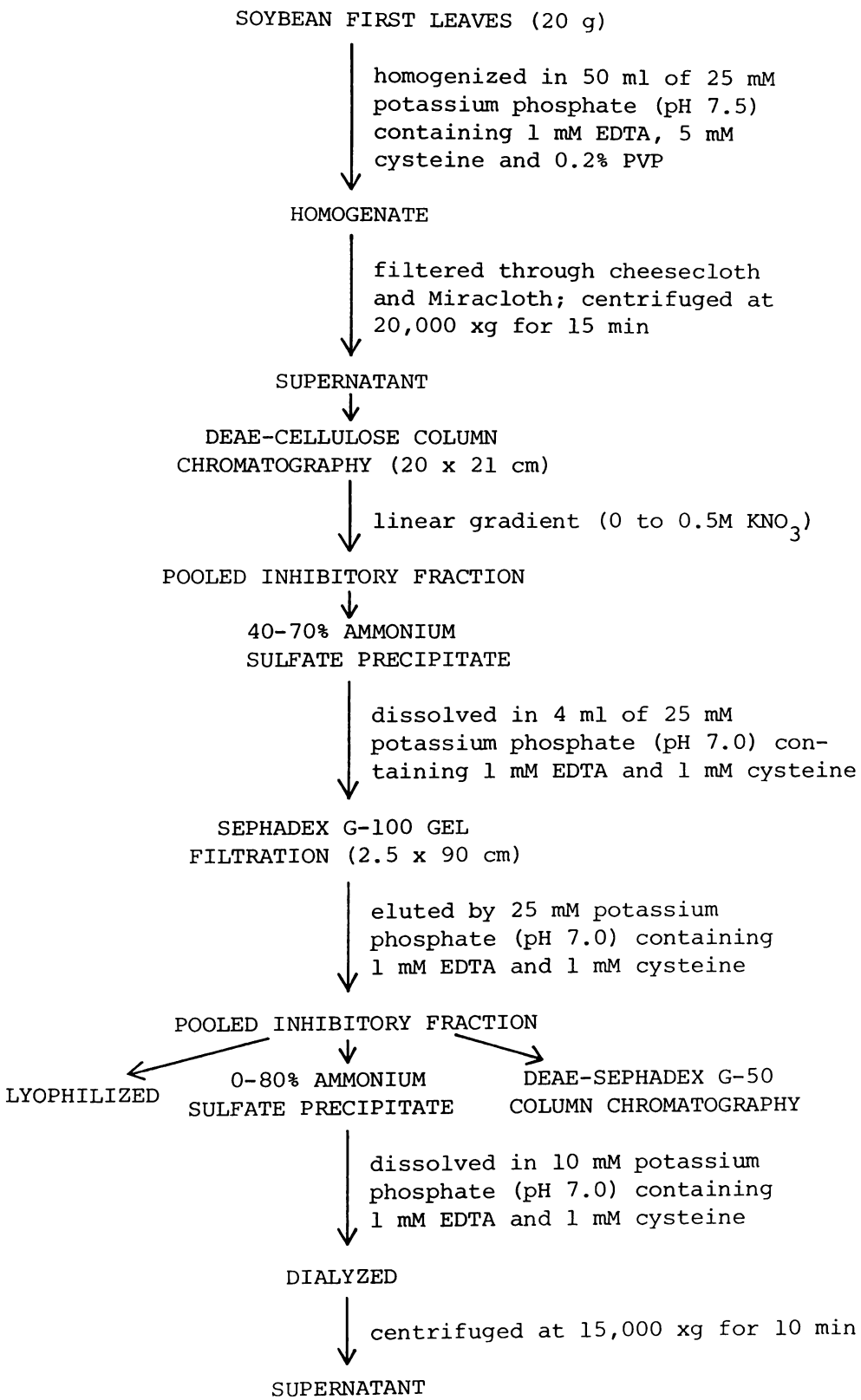



Figure 10. Flow diagram for the method of purification of the nitrate reductase inhibitor.

Table 8

Summary for Purification of the Nitrate Reductase Inhibitor

The inhibitor was purified from 24 g soybean first leaves by the procedure summarized in Figure 10. To assay of the inhibitory activity a partially purified NADH-nitrate reductase was used in amounts which produced 14.8 nmoles $NO_2^-$ in 30 min without the inhibitor.

| Stage of Procedure | Total Amounts of Inhibitor* | Total Protein | Specific Activity | Purification | Recovery |
|---|---|---|---|---|---|
| | nmoles/h | mg | nmoles/h/mg protein | fold | % |
| Centrifuged extract | 23,000 | 957.0 | 24 | 1 | 100 |
| Pooled inhibitory fraction from DEAE-cellulose column | 22,000 | 76.2 | 290 | 12 | 96 |
| 40-70% ammonium sulfate fraction | 34,000 | 26.4 | 1,300 | 54 | 148 |
| Pooled inhibitory fraction from Sephadex G-100 column | 20,000 | 1.87 | 11,000 | 460 | 87 |
| 0-80% ammonium sulfate fraction, after dialysis and centrifugation | 17,000 | 0.29 | 59,000 | 2,500 | 74 |

*Calculated from the size of an aliquot which gave approximately 50% inhibition and expressed in terms of the decrease in $NO_2^-$ production which the whole would cause.

Figure 11. Isolation of the nitrate reductase inhibitor by DEAE-cellulose column chromatography. The column (2 x 21 cm) was developed by a linear gradient of 0 to 0.5M $KNO_3$ in 10 mM potassium phosphate at pH 7.0 containing 1 mM EDTA and 1 mM cysteine. The NADH-nitrate reductase and NADPH-nitrate reductase activities of aliquots (50 µl) of each fraction were measured under their different optimum assay conditions. The reaction was for 20 min at 30°.

From alternate fraction, 100 µl aliquot was assayed for inhibition of nitrate reductase, using an amount of NADH-nitrate reductase which produced 16.65 nmoles nitrite in 30 min without the inhibitor. Inhibition is expressed as the decrease in the expected production of nitrite.

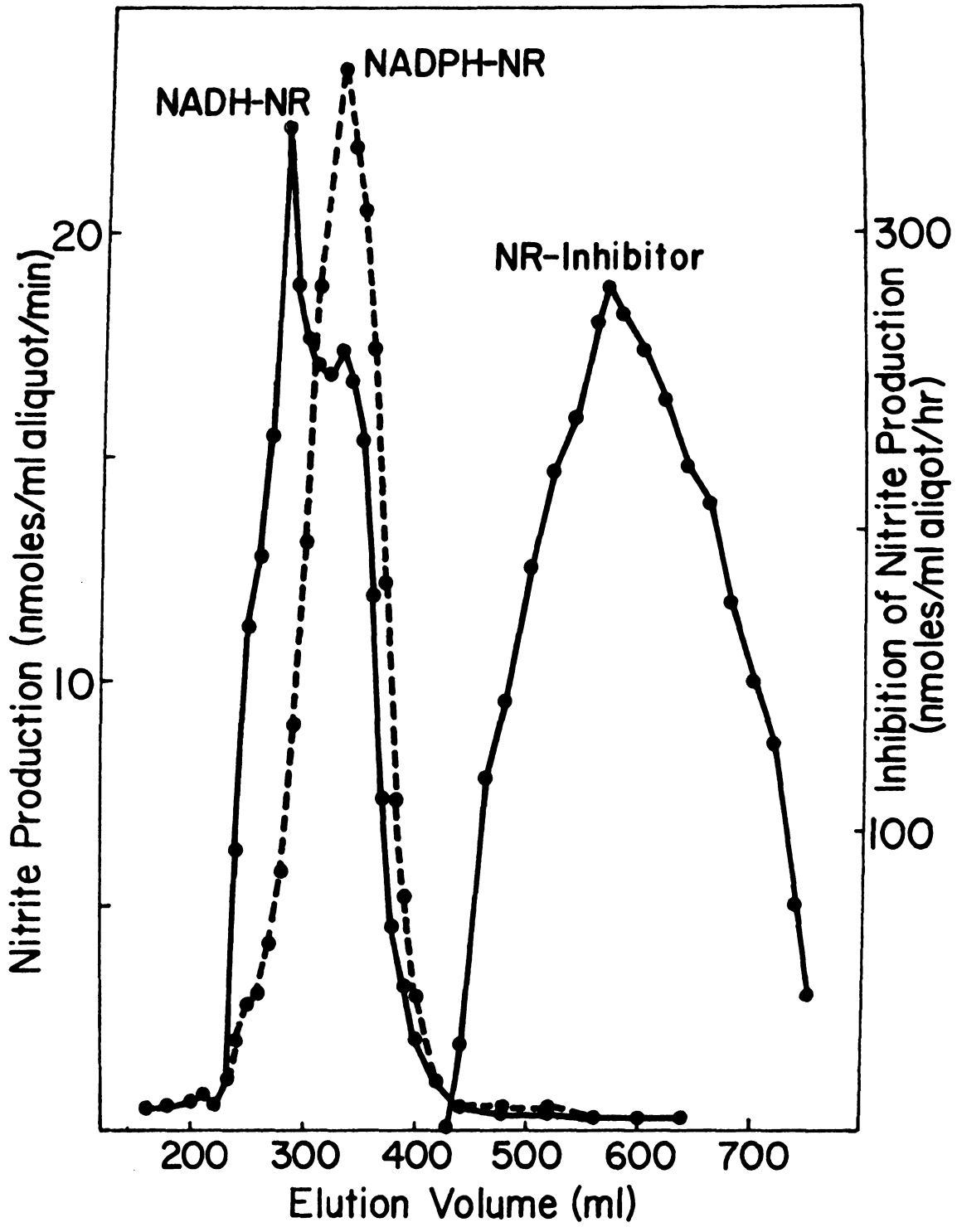

NADH-NR
NADPH-NR
NR-Inhibitor
Nitrite Production (nmoles/ml aliquot/min)
Inhibition of Nitrite Production (nmoles/ml aliqot/hr)
20
10
300
100
200
300
400
500
600
700
Elution Volume (ml)

Figure 11

Table 9

Ammonium Sulfate Fractionation of the
Nitrate Reductase Inhibitor

The inhibitor was isolated by DEAE-cellulose column chromatography with a stepwise gradient similar to that in Figure 10. The proteins which eluted between 0.3 and 0.5M $KNO_3$ were collected by ammonium sulfate as indicated.

| Ammonium Sulfate Fraction | Percent of Inhibitor Precipitating* |
|---|---|
| 0-20% | 0 |
| 20-40% | 16 |
| 40-60% | 70 |
| 60-80% | 14 |
| 80-90% | 0 |

*Based on proportion of precipitate required to give approximately 50% inhibition.

at pH 7.0 containing 1 mM EDTA and 1 mM cysteine. This solution of inhibitor was applied to a reverse flow Sephadex G-100 column (2.5 x 90 cm) for the further purification.

If there were enough protein in the inhibitor pool from the Sephadex G-100 column, it was concentrated by precipitation with 80% saturated ammonium sulfate followed by dialysis and centrifugation to remove insoluble protein. This greatly increased the specific activity without a great loss of total activity. When there was not enough protein to handle as an ammonium sulfate precipitate, the inhibitor was concentrated by lyophilization. By my purification procedure, the inhibitor was purified about 2,500-fold. Further purification by DEAE-Sephadex A-50 column chromatography or 2nd Sephadex G-100 gel filtration caused a tremendous loss of the activity and the specific activity did not significantly increase.

The increases in total inhibitory activity by the first ammonium sulfate fractionation (40-70%) is to be noted. This might be due to the removal of a nitrate reductase-stimulator which is eluted from DEAE-cellulose column between nitrate reductase and the inhibitor (to be further discussed with Figure 21), and which is mostly precipitated by a 70-90% ammonium sulfate solution as reported by Ku (152) and Ku and Meeks (153).

During the purification, it was necessary to separate the inhibitor completely from nitrate reductases by DEAE-cellulose column chromatography (Figure 11). When nitrate reductase was present, the inhibitor was eluted in the void volume from the Sephadex G-100 column with the enzyme, or the elution peak of the inhibitor was shifted to a region of higher molecular weight than its true molecular

weight. This was probably because of an association between the inhibitor and the enzyme which will be discussed in Figures 18 and 19. To avoid this, the DEAE-cellulose column could be washed with about four volumes of 0.25M $KNO_3$ in the elution buffer after loading the leaf extraction in order to remove the nitrate reductase, and then the inhibitor could be eluted by a 0.25M to 0.5M $KNO_3$ linear gradient. This technique for isolating the inhibitor was required when more leaves were used in larger preparations or when more nitrate reductase was found in the leaf extracts.

## Molecular Weight of the Inhibitor

To estimate the molecular weight of the inhibitor it was completely separated from nitrate reductase by repeating the DEAE-cellulose column chromatography. The pooled inhibitor from the DEAE-cellulose column was fractionated by 40-70% ammonium sulfate and dissolved in 3 ml of 25 mM potassium phosphate at pH 7.0, containing 1 mM EDTA and 1 mM cysteine. The solution of inhibitor was then applied to the bottom of Sephadex G-100 column (2.5 x 89 cm) and filtered by a reverse flow of the same buffer as above. The column was calibrated and the molecular weight of the inhibitor was estimated to be 31,000 (Figure 12).

## Gel Electrophoresis of the Inhibitor

The highly purified inhibitor (DEAE-cellulose column chromatography, ammonium sulfate fractionation and gel filtration) was concentrated by precipitation with 80% ammonium sulfate and dialysis overnight against 10 mM potassium phosphate at pH 7.0 containing 1 mM cysteine, after which the insoluble protein was removed by centrifugation. The

Figure 12. Molecular weight determination of the nitrate reductase inhibitor by Sephadex G-100 gel filtration. Ammonium sulfate precipitate (40-70%) of the inhibitor after the second DEAE-cellulose column chromatography was redissolved in 3 ml of 25 mM potassium phosphate at pH 7.0 containing 1 mM EDTA and 1 mM cysteine. The sample was applied to the bottom of the Sephadex G-100 column (2.5 x 89 cm) which had been equilibrated with the same buffer.

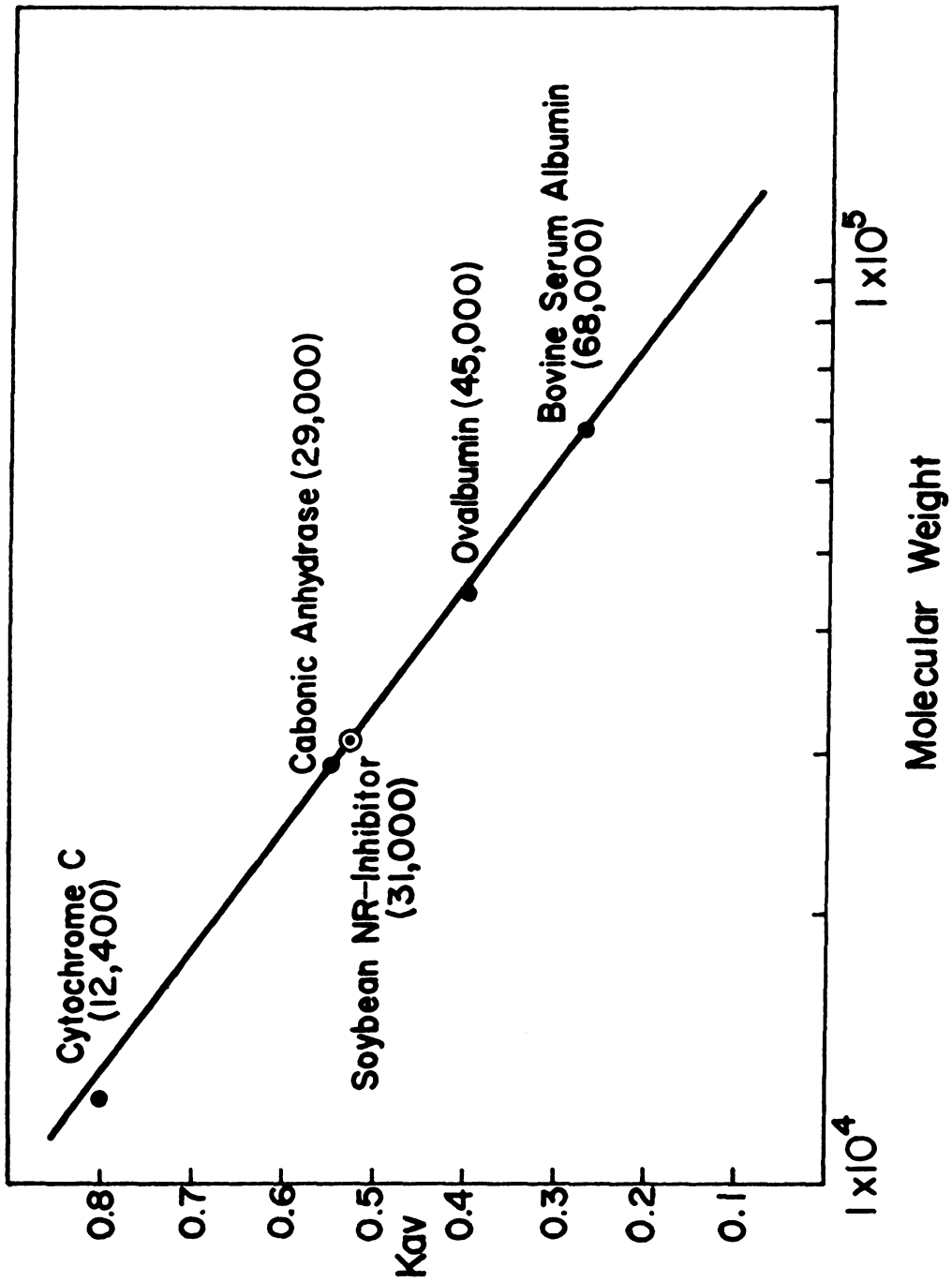

Cytochrome C
(12,400)
Cabonic Anhydrase (29,000)
Soybean NR-Inhibitor
(31,000)
Ovalbumin (45,000)
Bovine Serum Albumin
(68,000)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Kav
1x10^4
1x10^5
Molecular Weight

Figure 12

protein was electrophoresed on polyacrylamide gels, and gels were stained with coomassie brilliant blue. The protein(s) did not migrate in high pH gels as described by Davis (127), but at pH 7.5 the protein(s) migrated toward the anode. The inhibitor was extracted from 4 mm gel slices by immersing in the NADH-nitrate reductase reaction mixture minus the enzyme, FAD and NADH for two days at 4° in the dark. After the gel slices were removed, NADH-nitrate reductase and NADH were added and the inhibitory activity was assayed for 30 min at 30°. The gel scanning at 550 nm showed two protein bands (Figure 13). For convenience, the protein peak which migrated faster will be called protein I, and the protein peak which migrated slower will be called protein II. The relative amount of protein between the two bands seemed to be dependent on the freshness of the inhibitor or the purification procedure. When electrophoresis was carried out immediately after purification, the amount of protein I was much more than protein II as shown in Figure 13-A. However, after four days, protein II increased and protein I decreased as shown in Figure 13-B. The peak of inhibitory activity coincided with protein peak I (Figure 14).

The same sample of the inhibitor used for data in Figure 13 was subjected to SDS-gel electrophoresis. As shown in Figure 15, only one main protein band was observed. The molecular weight of this protein was estimated to be about 18,000 on SDS-gel. This molecular weight was slightly higher than a half of the molecular weight of the inhibitor (31,000) as estimated by Sephadex G-100 gel filtration (Figure 12). The highly purified inhibitor, which showed two main protein bands in acrylamide gel electrophoresis, was also treated

Figure 13. Polyacrylamide gel electrophoresis of the nitrate reductase inhibitor. About 20 μg protein from the most purified inhibitory preparation (about 2,500-fold purification) was applied to each polyacrylamide gel containing 4% acrylamide. Electrophoresis was performed at 8 mA per gel (5 mm diameter) for 5 hours at 4° in a buffer containing 0.1M potassium phosphate at pH 7.5 and 0.05M glycine. Following electrophoresis, the protein was stained with coomassie brilliant blue. Each gel was scanned at 550 nm with a Gilford linear transport scanner. Four days after the first gel electrophoresis (A), the inhibitory preparation which had been kept in the dark at 4° was again electrophoresed under the same condition (B).

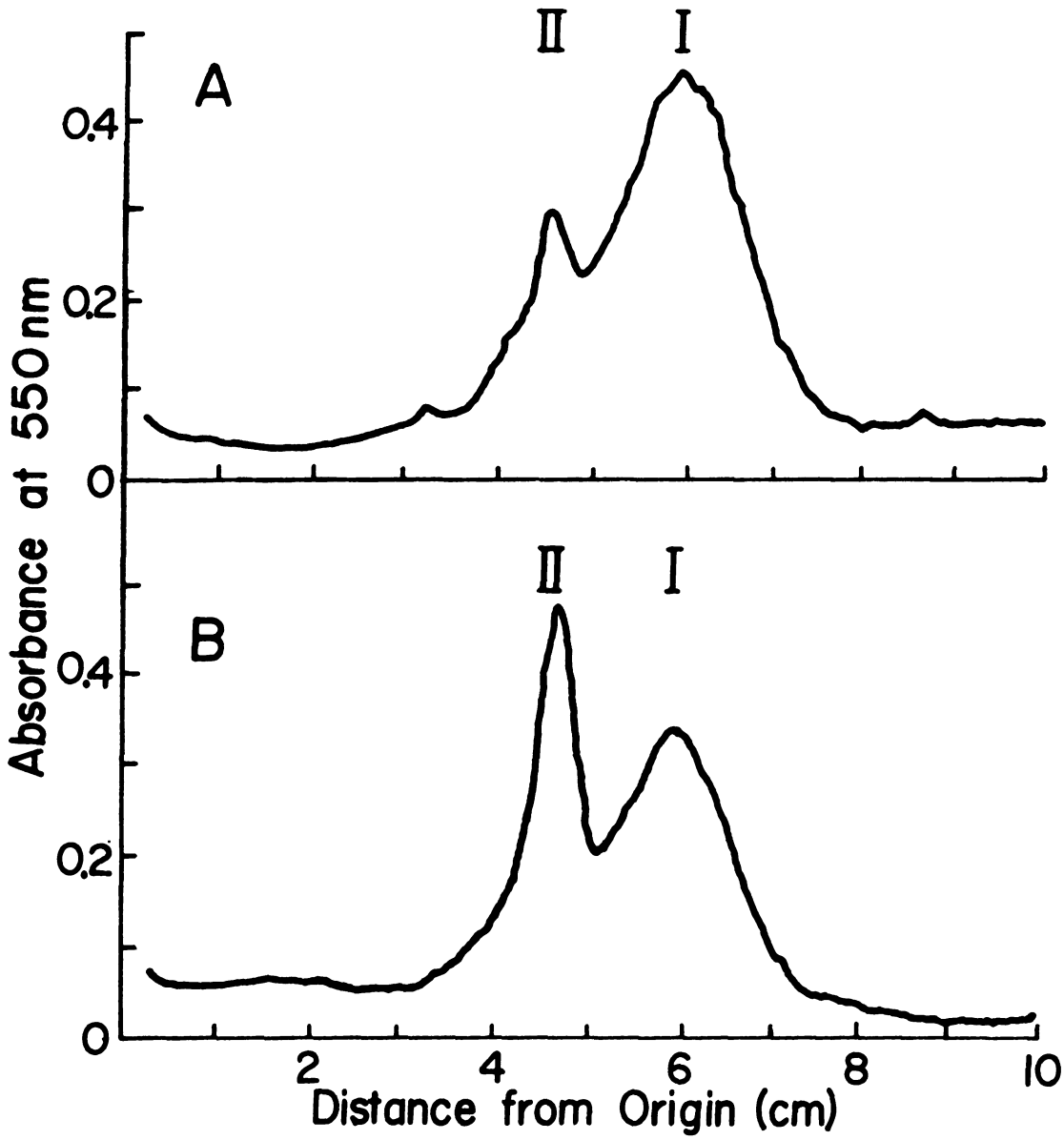

A
B
II
I
II
I
0.4
0.2
0
0.4
0.2
0
Absorbance at 550nm
2
4
6
8
10
Distance from Origin (cm)

Figure 13

Figure 14. Polyacrylamide gel electrophoresis of the nitrate reductase inhibitory preparation: total protein vs. inhibitory activity. After Sephadex G-100 gel filtration, the inhibitor was concentrated by lyophilization and dialyzed overnight. About 25 μg protein was applied to each gel. The procedure of the electrophoresis was the same as in Figure 13. Following electrophoresis, one gel was stained for protein and another gel was cut into 4 mm slices. Each slice was immersed in the NADH-nitrate reductase reaction mixture minus FAD, enzyme and NADH and kept in the dark at 4°. After two days, gel slices were removed from the reaction mixture and NADH-nitrate reductase and NADH were added to determine the inhibitory activity.

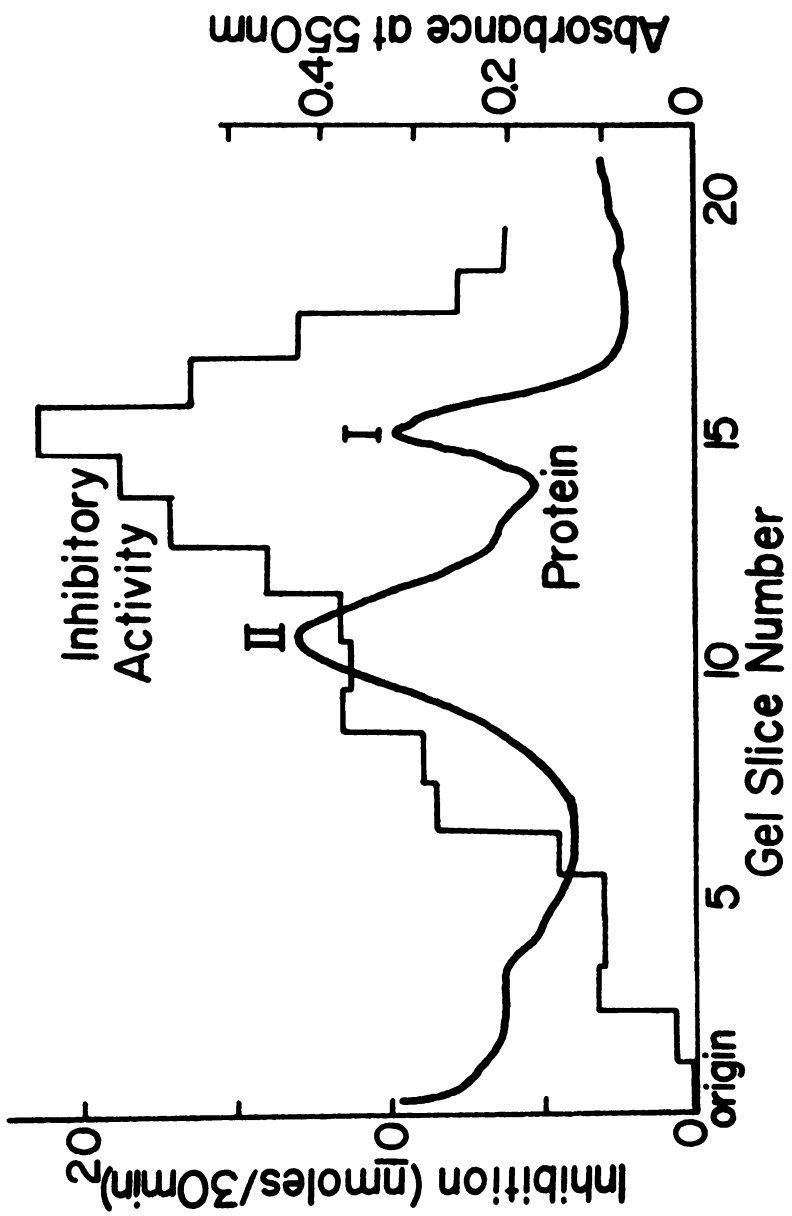

Absorbance at 550nm
0.4
0.2
0
Inhibition (nmoles/30min)
20
0
origin
5
10
15
20
Gel Slice Number
Inhibitory Activity
Protein
I
II

Figure 14

Figure 15. SDS-gel electrophoresis of the nitrate reductase inhibitor. The same sample of the inhibitor as used in Figure 13 was applied. The inhibitor was incubated at 100° for 10 min in 0.01M sodium phosphate at pH 7.1, 1% SDS, 1% mercaptoethanol and 10% glycerol (references 129,130). About 14 μg of protein was applied to each 7.5% acrylamide gel containing 0.1% SDS. The proteins used as standard were incubated together at 100° for 10 min with 1% SDS.

$$\text{Mobility} = \frac{\text{distance of protein migrated}}{\text{gel length after destaining}} \times \frac{\text{gel length before staining}}{\text{distance of dye migration}}$$

(reference 131)

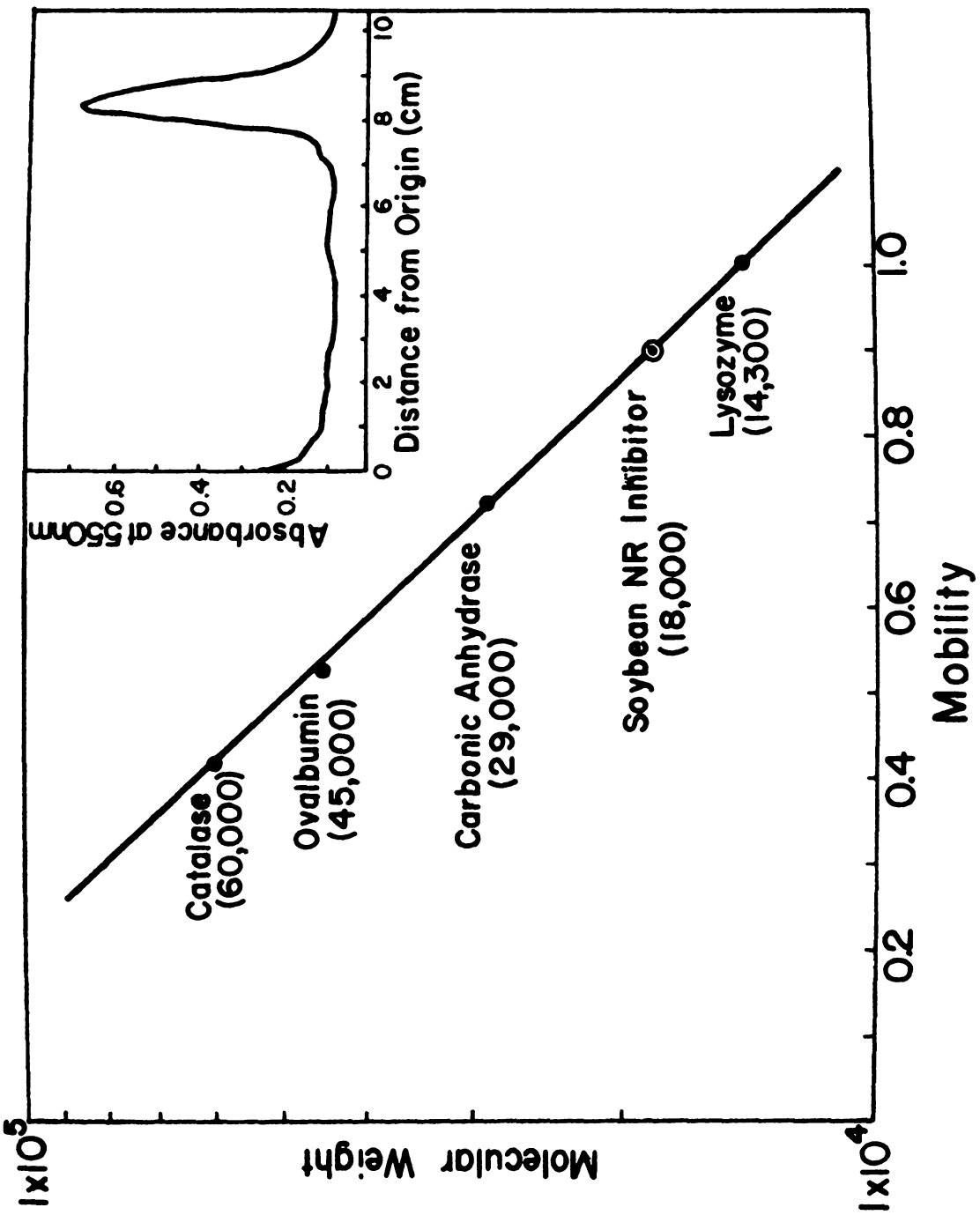

Molecular Weight
1x10^5
1x10^4
0.2
0.4
0.6
0.8
1.0
Mobility
Catalase (60,000)
Ovalbumin (45,000)
Carbonic Anhydrase (29,000)
Soybean NR Inhibitor (18,000)
Lysozyme (14,300)
Absorbance at 550nm
0.2
0.4
0.6
0
2
4
6
8
10
Distance from Origin (cm)

Figure 15

with 8M-urea and subjected to gel electrophoresis in 8M urea. Under these conditions, also only one main protein band was observed. The data indicate that proteins I and II could be composed of a common subunit and that the two proteins could be interconverted between an active form and a less active form.

## Stability of the Inhibitor

The nitrate reductase inhibitor was much more stable than soybean nitrate reductase. However, about 50% activity was lost after incubation at 50° and 100% at 100° for 10 min (Table 10). The inhibitor was kept in solution at pH 7.0 and 0° for at least one week without significant loss of the activity. However, it was unstable at acid or alkaline pH. As shown in Table 11, the inhibitor lost activity completely in 30 min at 0° in solution at pH 2 and pH 12. The inhibitor seemed relatively more stable in an acid pH range in comparison to the alkaline pH (Table 11).

## Specificity of the Inhibitor

The inhibitor seemed to be specific for NADH-nitrate reductases from three plants and it did not have an effect on a number of other enzymes (Table 12). Partially purified NADH-nitrate reductases from spinach and pigweed leaves were inhibited by the inhibitor as much as soybean NADH-nitrate reductase. However, the respiratory nitrate reductase from *Escherichia coli*, which cannot use NADH or NADPH as electron donor but uses $FMNH_2$, $FADH_2$ and reduced viologens (84), was not inhibited (Table 12). The activities of nitrite reductase and glutamate dehydrogenase, subsequent enzymes in nitrate assimilation by plants, were not influenced by the inhibitor. Xanthine oxidase,

Table 10

Heat-Lability of the Nitrate Reductase Inhibitor

The inhibitor used was from the inhibitor peak in DEAE-cellulose column chromatography of soybean leaves kept in the dark for 54 hours.

| Inhibitor Heat Treatment | Nitrate Reductase Activity |
|---|---|
| | nmoles/30 min |
| Untreated | 4.4 |
| 50° for 10 min | 6.2 |
| Boiled for 10 min | 8.3 |
| Nitrate reductase minus inhibitor | 8.3 |

Table 11

Lability of the Inhibitor in Acid and Alkaline Solution

The inhibitor was a preparation after Sephadex G-100 gel filtration (Figure 10). The NADH-nitrate reductase produced 7.7 nmoles nitrite in 30 min without the inhibitor, and the inhibitor stored at pH 7 prevented the production by 4.1 nmoles nitrite.

| Treatment at 0° | | Inhibition Remaining |
|---|---|---|
| | | % |
| pH 7 | 30 min | 100 |
| | 60 min | 100 |
| pH 2 | 30 min | 8 |
| | 60 min | 0 |
| pH 4 | 60 min | 94 |
| pH 10 | 60 min | 53 |
| pH 12 | 30 min | 0 |
| | 60 min | 0 |

Table 12

The Specificity of the Inhibitor

The assay methods for each enzyme are described in Methods.

| Enzymes | Source | Inhibition |
|---|---|---|
| NADH-nitrate reductase | Soybean leaves | + |
| | Spinach leaves | + |
| | Pigweed leaves | + |
| NADPH-nitrate reductase | Soybean leaves | - |
| $FMNH_2$-nitrate reductase | *Escherichia coli** | - |
| Nitrite reductase | Soybean leaves | - |
| | Spinach leaves | - |
| Glutamate dehydrogenase | Spinach leaves | - |
| | Bovine liver | - |
| Glycolate oxidase | Spinach leaves | - |
| Malate dehydrogenase | Spinach leaves | - |
| Lactate dehydrogenase | Rabbit muscle* | - |
| Xanthine oxidase | Buttermilk* | - |
| Glycerol-P-dehydrogenase | Rat liver | - |
| Acid phosphatase | Wheat germ* | - |

*Commercial enzymes.

a flavo-protein with Fe and Mo components, which has been reported to have similar protein subunits to $FMNH_2$-nitrate reductase in *Neurospora crassa* (87), was also not affected. Xanthine oxidase can also reduce nitrate to nitrite in anaerobic conditions with xanthine. However, the inhibitor did not affect this nitrate reduction either. None of the other dehydrogenases and acid phosphatase tested here were affected by the inhibitor.

Partially purified soybean NADPH-nitrate reductase was inhibited by the factor only 9% as much as NADH-nitrate reductase. When NADPH-nitrate reductase was prepared without cysteine, a procedure to eliminate most of the NADH-nitrate reductase activity, it was inhibited by the factor only 3.6% as much as NADH-nitrate reductase (Table 13).

The site of the inhibition in NADH-nitrate reductase complex was studied (Table 13). When the electron donor was changed to reduced methylviologen or $FADH_2$, the inhibitory effect on nitrate reductase decreased 50% or 29%, respectively. However, when the electron acceptor was changed to cytochrome c or DCPIP instead of nitrate, the inhibitor had no effect on the reaction. The data suggest that the $FADH_2$-nitrate reductase component in NADH-nitrate reductase complex is the main site of the action of the inhibitor, but that the diaphorase (NADH-cytochrome c reductase) component in the NADH-nitrate reductase complex is not affected.

Because the inhibitor did not affect the respiratory nitrate reductase from *Escherichia coli*, which still had $FMNH_2$-nitrate reductase activity, it might be possible that the $FMNH_2$-nitrate reductase component in nitrate reductase complex from leaves may be different from the respiratory nitrate reductase from the bacteria.

Table 13

The Effect of the Inhibitor on the Components of Nitrate Reductase Complex

NADH-nitrate reductase was prepared with 20 mM cysteine in the grinding and with 10 mM in the elution buffer used in the DEAE-cellulose column chromatography. NADPH-nitrate reductase was prepared without cysteine. The inhibitor was purified to the Sephadex G-100 gel filtration step (Figure 10). Without inhibitor, NADH- and NADPH-nitrate reductase each produced 22 nmoles nitrite per 30 min assay. The inhibitor prevented the production of 18.1 nmoles nitrite in the NADH-$NO_3^-$ system, and this amount of inhibition is assigned the value of 100. In each system controls were run without the inhibitor.

| Enzyme | Electron Donor | Electron Acceptor | Relative Inhibition |
|---|---|---|---|
| NADH-nitrate reductase | NADH | $NO_3^-$ | 100 |
| | Reduced methylviologen | $NO_3^-$ | 50 |
| | $FADH_2$ | $NO_3^-$ | 71 |
| | NADH | Cytochrome c | 0 |
| | NADH | DCPIP | 0 |
| NADPH-nitrate reductase | NADPH | $NO_3^-$ | 3.6 |

Kinetic Studies with the Inhibitor

The Lineweaver-Burk plot in Figure 16 indicated that the type of inhibition is noncompetitive with nitrate. Therefore, it might bind to nitrate reductase at different site from the nitrate site. The Km value for $NO_3^-$ was not changed significantly by the inhibitor, but the Vmax of nitrate reductase was decreased by increasing the inhibitor concentration. The activity of the inhibitor was dependent upon the amount of nitrate reductase and the concentration of the inhibitor. When the amount of nitrate reductase was fixed, the inhibition curve was sigmoidal (Figure 17). These data may indicate that the inhibitor has an allosteric effect on nitrate reductase.

Evidence for binding of the inhibitor to nitrate reductase was obtained with Sephadex G-75 columns (0.85 x 19 cm) (Figure 18). The partially purified NADH-nitrate reductase from soybean leaves was layered on a small column and eluted with 25 mM potassium phosphate at pH 6.5 and 1 mM cysteine (Column A). Under these unfavorable conditions for keeping NADH-nitrate reductase active (low pH, low cysteine concentration and very low concentration of protein), only about 14% of the nitrate reductase activity was recovered in the void volume during the one hour experiment. When nitrate reductase was mixed with the inhibitor and quickly passed through the same column (Column B), little nitrate reductase activity was detected in the void volume. After elution the protein fractions from Column A and Column B were kept at 4° in the dark for one day, during which time the nitrate reductase from both columns lost activity completely. At this time, the inhibitory activity was measured with another active NADH-nitrate reductase preparation. The peak of the inhibitor

Figure 16. Effect of nitrate concentration on the activity of the nitrate reductase inhibitor. The fraction from Sephadex G-100 column having the greatest inhibitory effect was used. Variable amounts of the inhibitory protein were used as indicated. NADH-nitrate reductase was passed through Sephadex G-100 column (1 x 60 cm) which had been equilibrated with 25 mM potassium phosphate at pH 7.0 containing 1 mM EDTA, 5 mM cysteine and 0.5M sorbitol, and collected in the void volume.

v: production of $NO_3^-$ (nmoles/hour)

[S]: concentration of $KNO_3$ (mM)

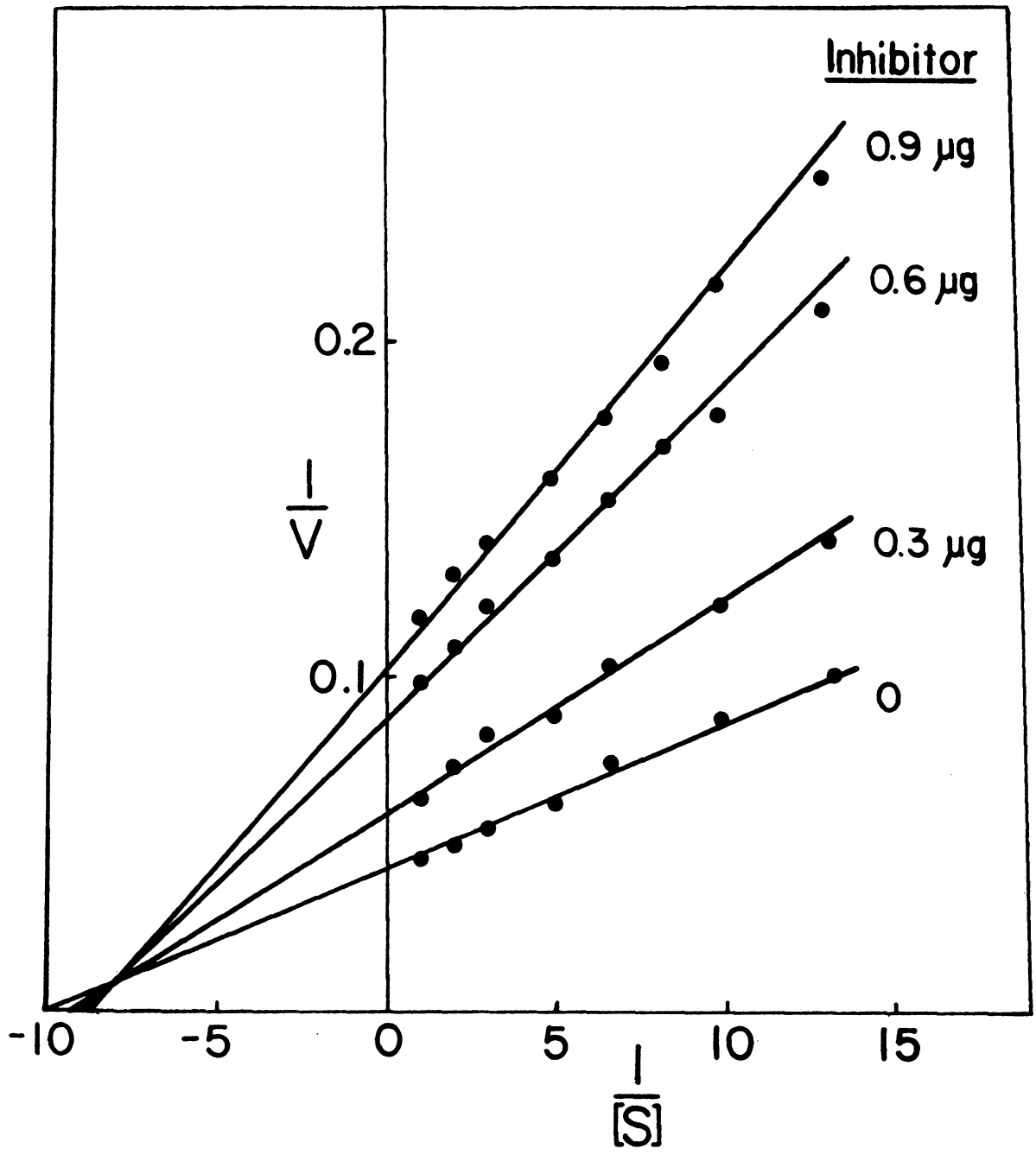



Figure 16

Figure 17. Effect of inhibitor concentration on NADH-nitrate reductase. The fraction from a Sephadex G-100 column having the greatest inhibitory effect and the NADH-nitrate reductase which had been purified to the Sephadex G-200 gel filtration step were used for this study. Without the inhibitor, 5 nmoles nitrite were formed per 30 min assay.

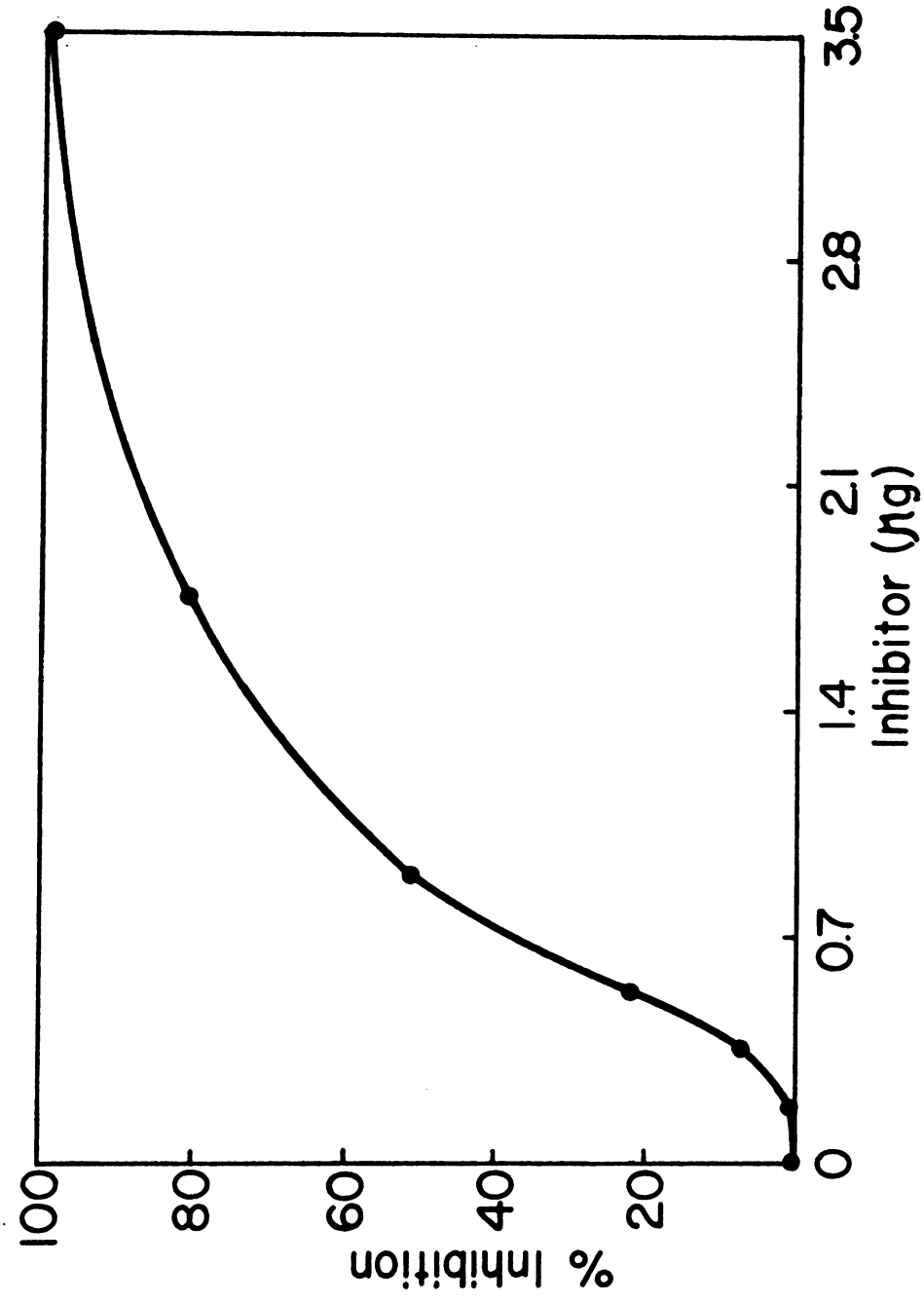



Figure 17

Figure 18. Studies on binding of inhibitor to NADH-nitrate reductase. NADH-nitrate reductase was partially purified by DEAE-cellulose column chromatography followed by ammonium sulfate fractionation and desalting by Sephadex G-25 column. The inhibitor was purified through the Sephadex G-100 gel filtration step.

(A) Nitrate reductase alone: 0.5 ml solution containing NADH-nitrate reductase (1.42 units) and 0.4M sorbitol was layered on a small Sephadex G-75 column (0.85 x 19 cm), which had been equilibrated by 25 mM potassium phosphate at pH 6.5 and 1 mM cysteine, and then the protein was eluted by the same buffer. One milliliter fractions were collected and the void volume was 6 ml.

(B) Nitrate reductase plus inhibitor: 0.5 ml solution containing NADH-nitrate reductase (1.42 units), the inhibitor (inhibited 1.03 units of the NADH-nitrate reductase) and 0.4M sorbitol was filtered by Sephadex G-75 in the same manner as in (A).

(C) Inhibitor only: 0.5 ml solution containing the inhibitor (inhibited 1.03 units of NADH-nitrate reductase) and 0.4M sorbitol was passed through the Sephadex G-75 column under exactly the same conditions as in (A) and (B).

Nitrate reductase (———) and the inhibitory activity (-----) were assayed in 0.5 ml reaction mixtures for 30 min at 30°. To measure the nitrate reductase activity, $KNO_3$ and NADH were added to 0.45 ml of each fraction. To measure the inhibitory activity, $KNO_3$, NADH-nitrate reductase (5 nmoles nitrite/assay) and NADH were added to 0.45 ml of each fraction. All assays were run immediately after the gel filtration except the assay for the inhibitory activity in part B, which was measured after 24 hours of storage in the dark at 4°, during which time the accompanying nitrate reductase lost all of its activity.

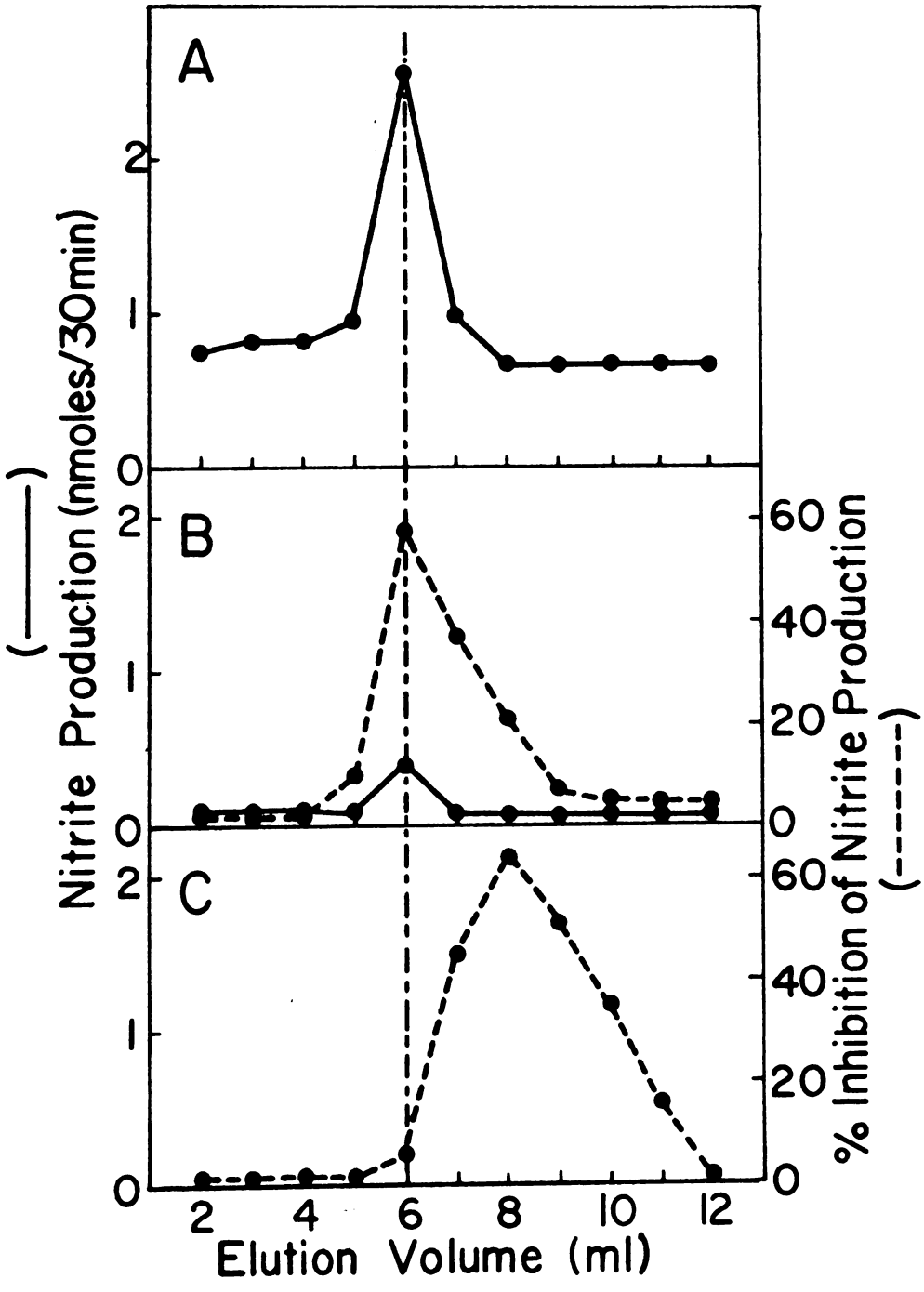

A
B
C
Nitrite Production (nmoles/30min)
(——)
% Inhibition of Nitrite Production
(- - - -)
Elution Volume (ml)
0
1
2
20
40
60
2
4
6
8
10
12

Figure 18

from Column B was in the same fraction (void volume) as nitrate reductase. When the inhibitor alone was passed through the same column (Column C), the activity peak of the inhibitor was found in two fractions (2 ml) after the void volume.

Because the nitrate reductase was not pure, it is possible that a contaminating protein in the preparation (presumably RuDP carboxylase) bound to the inhibitor and changed the elution position of the inhibitor from Sephadex column. However, purified spinach RuDP carboxylase or bovine serum albumin did not affect the elution position of the inhibitor when it was passed through the Sephadex G-75 column with these proteins (Figure 19).

## Absence of Protease Properties of the Inhibitor on Nitrate Reductase

Proteases, which are active in the neutral pH range, can usually be classified as sulfhydryl, serine or metal dependent (154). A range of specific inhibitors of proteases was tested on the nitrate reductase inhibitor (Table 14). The sulfhydryl group inhibitor, *p*-chloromercuribenzonic acid, at a concentration of 0.1 mM had no effect on the activity of the inhibitor, but inhibited the soybean NADH-nitrate reductase activity about 30%. Pnenylmethylsulfonyl fluoride is an inhibitor of serine-dependent enzymes (e.g., trypsin [155]). At 0.25 mM it did not have any influence on either NADH-nitrate reductase or the inhibitor. Commercial trypsin inhibitors from soybean and ovomucoid did not affect the inhibitory activity. The metal-chelating agents, EDTA and *o*-phenanthroline did not decrease the inhibition even at high concentrations.

Figure 19. Inhibitor is not bound to RuDP carboxylase or albumin. NADH-nitrate reductase and inhibitor were purified to the same stage of purification in Figure 18. RuDP carboxylase was purified from spinach leaves. The procedure of Sephadex G-75 gel filtration was the same as in Figure 18. The amounts of protein in samples applied on the column were as follows:

(A) Inhibitor (0.014 mg)
(B) Inhibitor (0.014 mg) plus NADH-nitrate reductase (0.360 mg)
(C) Inhibitor (0.014 mg) plus RuDP carboxylase (1.183 mg)
(D) Inhibitor (0.014 mg) plus bovine serum albumin (0.750 mg).

All the fractions from the column were stored in the dark at 4° for about 24 hours and then the inhibitory activity was measured by assaying 0.9 ml aliquots. The amount of NADH-nitrate reductase used in the assay produced 9.5 nmoles nitrite in the absence of the inhibitor in 30 min reaction. Elution volumes of nitrate reductase, RuDP carboxylase and bovine serum albumin were 6 ml.

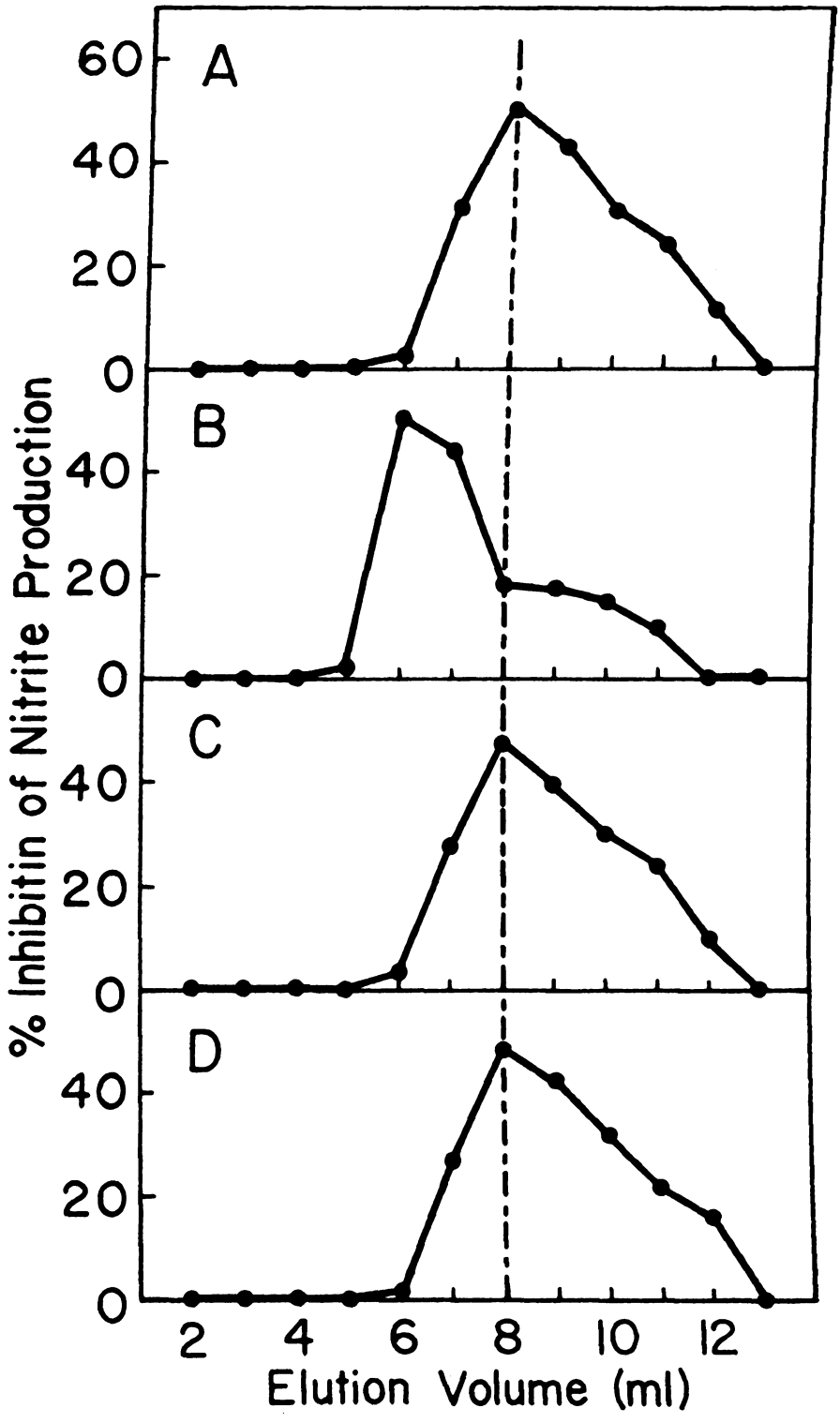

A
B
C
D
60
40
20
0
% Inhibitin of Nitrite Production
2
4
6
8
10
12
Elution Volume (ml)

Figure 19

Table 14

Influence of Protease Inhibitors on the Activity of the Nitrate Reductase Inhibitor

The NADH-nitrate reductase formed 8.9 nmoles nitrite in 30 min without the inhibitor. In the absence of any protease inhibitor, the nitrate reductase inhibitor lowered the production of nitrite 3.9 nmoles. This amount of inhibition (5.0 nmoles not produced) is arbitrarily assigned value of 100%.

| Protease Inhibitor | Concentration | Relative Inhibition |
|---|---|---|
| | | % |
| None | - | 100 |
| *p*-Chloromercuribenzonic acid | 0.1 mM | 94 |
| Phenylmethylsulfonyl fluoride | 0.25 mM | 93 |
| Trypsin inhibitor (soybean) | 20 µg | 100 |
| | 100 µg | 95 |
| Trypsin inhibitor (ovomucoid) | 40 µg | 98 |
| | 200 µg | 92 |
| *o*-Phenanthroline | 1.0 mM | 97 |
| EDTA ($Na_2$) | 10.0 mM | 90 |

The nitrate reductase inhibitor, which was purified through the Sephadex G-100 gel filtration step, did not hydrolyze the synthetic peptides, α-benzoyl-L-arginine-*p*-nitroanilide or L-leucine-*p*-nitroanilide. The inhibitor also did not release amino acids from a casein solution nor a partially purified NADH-nitrate reductase (data are not shown).

If the inhibitor were to act as a protease for nitrate reductase, the level of inhibition should be dependent on preincubation time of nitrate reductase with the inhibitor. Nitrate reductase was incubated with and without inhibitor in the reaction mixture minus NADH at 30° for various times, after which the reaction was started by adding NADH. As shown in Figure 20, the difference of $NO_2^-$ production with and without inhibitor did not change (a horizontal line in Figure 20) with an increasing preincubation time from 0 to 30 min. When the inhibitor was purified only by DEAE-cellulose column chromatography, the inhibition was increased by increasing the preincubation time as if the impure inhibitor preparation contained some protease activity.

When nitrate reductase activity was recorded at 340 nm by measuring NADH oxidation, the inhibition was observed immediately after adding the inhibitor or NADH-nitrate reductase, and the inhibition rate did not change for at least 15 min at 30°.

Effect of Light and Darkness on the Inhibitor

In most of my work the same extract from a batch of soybean leaves was used to separate NADH-nitrate reductase, NADPH-nitrate reductase and the nitrate reductase inhibitor by one DEAE-cellulose column chromatogram. Because the nitrate reductases were unstable,

Figure 20. Lack of effect of preincubation of the inhibitor with nitrate reductase on the inhibitory activity. NADH-nitrate reductase was partially purified by DEAE-cellulose column chromatography followed by ammonium sulfate fractionation and passed through Sephadex G-100 column. The inhibitor was purified through the Sephadex G-100 gel filtration step. NADH-nitrate reductase (7 units) was suspended in 65 mM potassium phosphate at pH 6.5 in 3 ml with or without an amount of the inhibitor which inhibited 3 units of nitrate reductase activity, and incubated at 30°. At each time interval, 0.4 ml of the sample was taken out and added to the NADH-nitrate reductase reaction mixture. The NADH-nitrate reductase activity was measured in 10 min assay. The inhibitory activity (-----) was calculated by subtraction of nitrite produced with the inhibitor from nitrite produced without inhibitor.

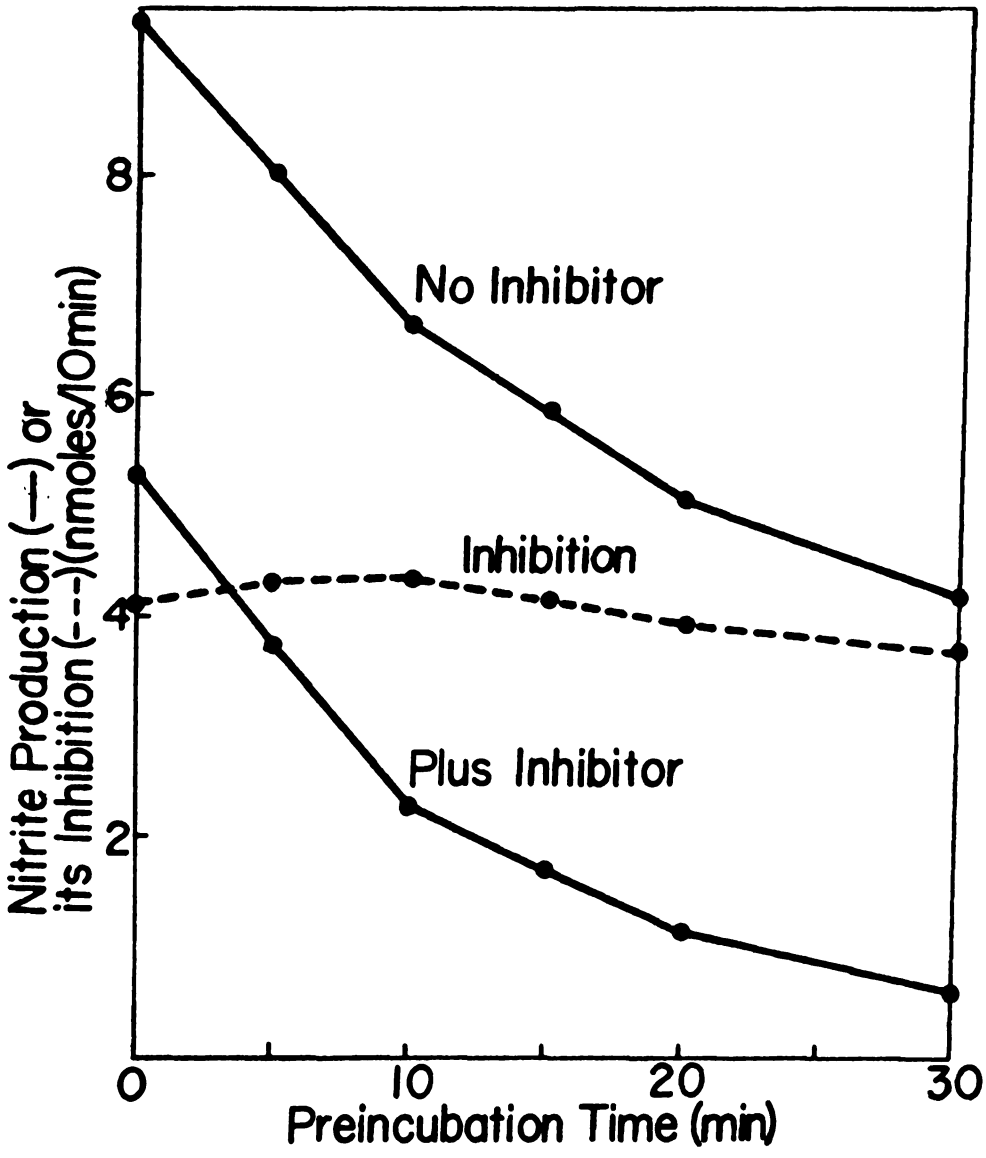

Nitrite Production (—) or
its Inhibition (---) (nmoles/10min)
8
6
4
2
No Inhibitor
Inhibition
Plus Inhibitor
0
10
20
30
Preincubation Time (min)

Figure 20

they were assayed as fast as possible and concentrated and passed through a Sephadex G-25 column to desalt and change buffers. The more stable inhibitor was assayed later which was more than 24 hours after the leaves were harvested. In this procedure the DEAE-cellulose column chromatography gave the same pattern of nitrate reductase activities and inhibitory activity from soybean leaves grown under continuous light as from leaves exposed to a 54 hour dark-period after the light-period as shown in Figure 11. However, if the inhibitor were isolated and assayed in three hours, more inhibitor was observed from soybean leaves exposed to the dark than from leaves kept under continuous light. For quick separation, only 5 g of leaves were chromatographed on a small DEAE-cellulose column (2.5 x 5 cm). The inhibitory activity from the soybean leaves grown under the continuous light varied from none to 30% of that from leaves exposed to darkness (Figure 21-A). The variability seemed to depend on leaf material and the time used for the preparation and isolation of the inhibitor. After loading leaf extracts onto the DEAE-cellulose column, the column was washed with six volumes of 10 mM potassium phosphate at pH 7.0 containing 1 mM EDTA, 1 mM cysteine and 0.25M $KNO_3$ to remove the nitrate reductases. The columns were then eluted with a 0.25M to 0.5M $KNO_3$ linear gradient and assayed against another preparation of nitrate reductase. The fractions (0 to 16) which were first eluted from the column stimulated the nitrate reductase activity, but these fractions by themselves did not have nitrate reductase activity. This stimulator of nitrate reductase is probably similar to the component reported by Ku (152). The inhibitor(s) eluted in subsequent fractions (20 to 32) from the columns

Figure 21. Rapid isolation of the inhibitor. Five grams of soybean first leaves were harvested from plants exposed to a 54 hour dark-period after a 5 day light-period (dark grown soybeans, ———) or from plants grown under the continuous light (light grown soybeans, -----). Leaves were homogenized with a Waring blendor for 1.5 min in 50 ml of buffer containing 25 mM potassium phosphate at pH 7.5, 1 mM EDTA, 5 mM cysteine and 0.2% insoluble PVP. The homogenate was filtered and centrifuged at 39,000 xg for 10 min. The supernatant was layered on a DEAE-cellulose column (2.5 x 5 cm) which had been equilibrated with 0.25M $KNO_3$ in 10 mM potassium phosphate at pH 7.0, 1 mM EDTA and 1 mM cysteine. The column was washed with 150 ml of the equilibrating buffer, and then developed with a linear gradient of 0.25 to 0.5M $KNO_3$ (120 ml). Two and five-tengths milliliter fractions were collected. The inhibitory activity in each fraction was measured at 3 hours (A) and at 24 hours (B) after harvesting the leaves.

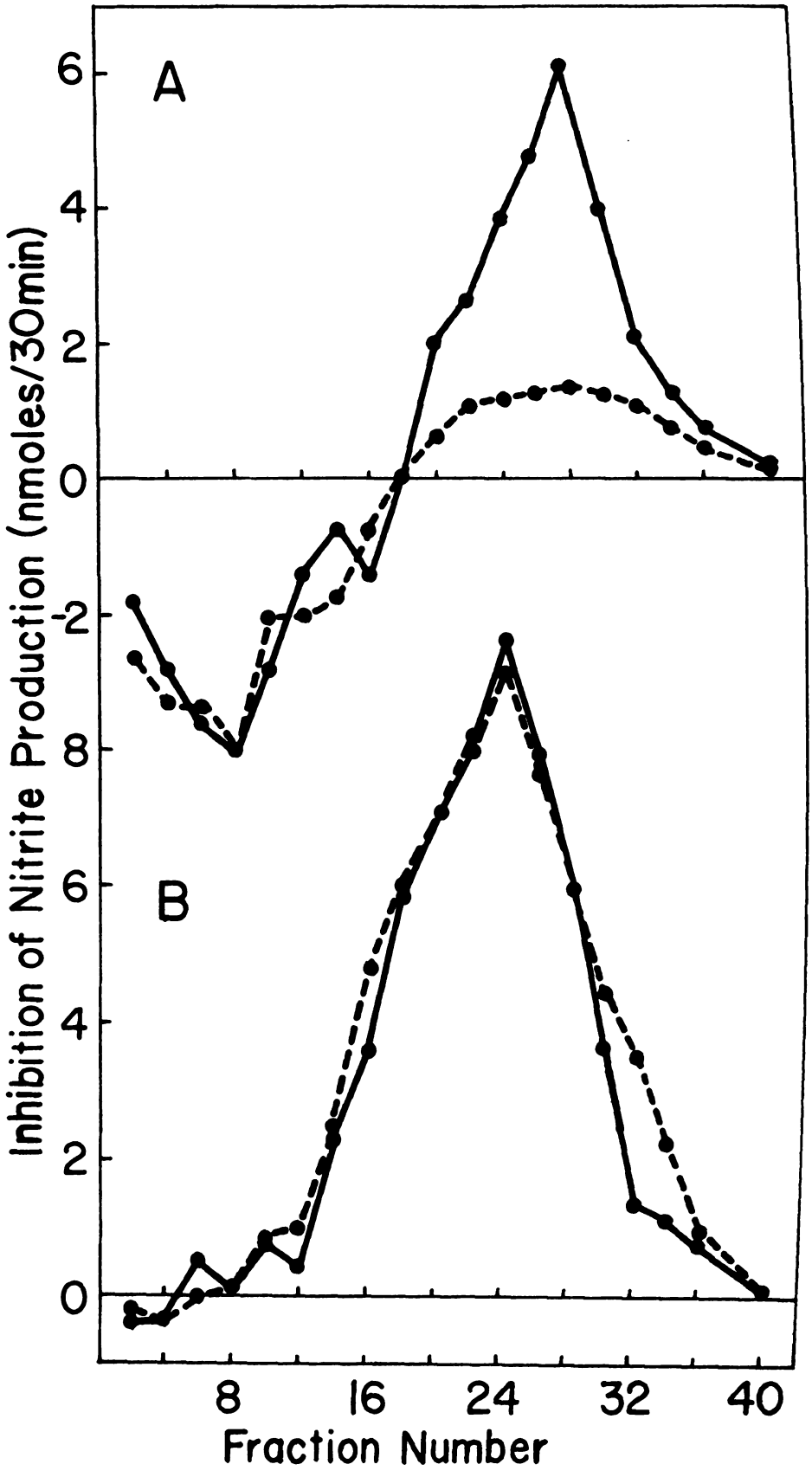

A
B
6
4
2
0
2
8
6
4
2
0
Inhibition of Nitrite Production (nmoles/30min)
8
16
24
32
40
Fraction Number

Figure 21

inhibited nitrate reductase. After assaying they were stored at 4° in the dark, and after about 24 hours, the activities were assayed again. As shown in Figure 21-B, the stimulatory activity had disappeared and the inhibitory activity had greatly increased in the fractions from leaves grown under continuous light. Also the total inhibitory activity in the fraction from leaves exposed to darkness was increased about 50% and its peak was shifted a few fractions to a lower salt concentration. The elution patterns for inhibitory material from leaves grown under the light and exposed to darkness became almost identical after storage in the dark for 24 hours. Because the peak of inhibitor was shifted to lower salt concentration after 24 hour storage, at least two factors or components may be involved in increasing the inhibitory activity. Besides dark activation of the inhibitor, inactivation of the stimulator could account for increased inhibitory activity and the shift in the elution profile.

From the above experiments, the inhibitor seems to be in an inactive form in the light and turns into an active form in the dark. However, there are other possibilities to explain these data. One is the inactivation of a stimulator in the first part of the fractions. Another possibility is that the inhibitor, which appeared after 24 hour storage in the dark may be a completely different substance. To check this latter possibility the inhibitory fraction from extract of leaves exposed to darkness was prepared by the stepwise gradient and eluted proteins (inhibitor[s]) between 0.25 to 0.5M $KNO_3$ were then chromatographed in a DEAE-Sephadex A-50 column. The nitrate reductase inhibitor eluted in only one peak (data are not shown).

The following experiments indicate photoinactivation of the isolated inhibitor. The active inhibitor, freshly isolated from leaves grown under continuous light by DEAE-cellulose column chromatography, was added to the NADH-nitrate reductase reaction mixture minus the enzyme and NADH. The solution was illuminated with about 2,000 ft. candle at 0° for the light pretreatment, and an identical portion was covered by aluminum foil for the dark pretreatment. After two hours of pretreatment, NADH-nitrate reductase and NADH were added, and the inhibitory activity was measured. By illumination, all of the inhibitor was inactivated (Table 15). When higher concentrations of the inhibitor were treated by light under the same condition, less inactivation was observed. If the yellowish inhibitor, just after the DEAE-cellulose column chromatography was used, exogenous flavin was not required for the photoinactivation. However, the colorless inhibitor after Sephadex G-100 gel filtration required the addition of flavin for photoinactivation (Table 15).

The loss of the inhibitory effect on nitrite production by the light pretreatment could be caused by (A) the generation of an electron donor ($FADH_2$) (156), with which nitrate reductase may be able to reduce nitrate even in the presence of the inhibitor, (B) generation of the superoxide radical upon reoxidation in air of photochemically reduced flavin (157) and the $O_2^-$ destroys the inhibitor, or (C) a conformational change in the inhibitor by light mediated through the action of a flavin (158,159).

If the inhibitor blocks electron transport between NADH and FAD, the $FADH_2$ generated during illumination or added in excess could reduce nitrate even in the presence of the inhibitor. However, when

Table 15

Photoinactivation of the Nitrate Reductase Inhibitor

For pretreatment the inhibitor was added to a NADH-nitrate reductase reaction mixture minus NADH and enzyme. In the system of "minus FAD", the FAD was omitted during pretreatment but was added just before the assay of nitrate reductase. The system was illuminated by 2,000 ft. candle for 2 hours at 0° (Light Pretreatment) or covered by aluminum foil (Dark Pretreatment). After pretreatment, enough NADH-nitrate reductase to produce 9.15 nmoles nitrite in 30 min assay was added with NADH and then nitrite production was measured. In each case, a control without inhibitor was run. The yellowish inhibitory fraction from DEAE-cellulose column reduced nitrite production by 5.1 nmoles for 30 min after the dark pretreatment, and the colorless inhibitor after Sephadex G-100 column reduced the activity by 4.2 nmoles.

| | | % Inhibition of Nitrate Reductase | |
|---|---|---|---|
| | FAD | Pretreatment of the Inhibitor for 2 hrs in Dark | Pretreatment of the Inhibitor for 2 hrs in Light |
| Yellowish inhibitor after DEAE-cellulose | ± | 56 | 0 |
| Colorless inhibitor after Sephadex G-100 | + | 46 | 13 |
| | - | 46 | 41 |

NADH was not added after illumination with or without inhibitor, no nitrate was reduced to nitrite. Moreover, as shown before in Table 13, the inhibitor was also effective when chemically reduced FAD was used as the electron donor. Therefore it is unlikely that the loss of the inhibitory activity by light is solely due to reduced FAD.

To determine if $O_2^-$ from $FADH_2$ inactivates the inhibitor, the following experiments were done. The superoxide radical was generated by xanthine and xanthine oxidase in a small beaker at 25° (160,161, 162). The inhibitor in a small dialysis tube was dialyzed against this xanthine-xanthine oxidase system in the dark for one hour. Water and the suspension buffer of the inhibitor were also dialyzed. The increase in absorption at 290 nm was recorded to measure the formation of uric acid from the oxidation of xanthine (163). After one hour dialysis, the inhibitory activity was measured (Table 16). The inhibitor when exposed to the $O_2^-$ lost 70% of its activity in one hour, while the water exposed to the $O_2^-$ caused a 36% decrease in activity of the undialyzed inhibitor (Table 16). In another experiment, the addition of superoxide dismutase to the solution of the inhibitor prevented about 24% of the inactivation during illumination at 25° for 45 min (Table 17). These exploratory experiments suggest that the $O_2^-$ may attack the inhibitor and may be one factor for its photoinactivation.

If the photoinactivation of the inhibitor was only due to destruction by $O_2^-$ or by other high energy radicals, the inactivation should be irreversible. However, as shown in Table 18, the photoinactivated inhibitor regained nearly 100% of its inhibitory activity after standing in the dark for 24 hours at 4°. The old preparation

Table 16

Effect of a Superoxide Generating System (SOGS)
on the Nitrate Reductase Inhibitor

The superoxide generating system (SOGS) consisted of 9.5 mg xanthine and about 3 units of xanthine oxidase from buttermilk (commercial) in 50 ml of 25 mM potassium phosphate at pH 7.5. The inhibitor from the DEAE-cellulose column was either placed in a small dialysis bag and dialyzed against the SOGS for one hour at 25° or was kept at the same temperature in a beaker for the same time. The suspension buffer of the inhibitor and water were also dialyzed against the SOGS. The nitrate reductase produced 12.3 nmoles nitrite per assay in the presence of a 0.1 ml aliquot of dialyzed buffer without inhibitor, and a 0.1 ml sample of the undialyzed inhibitor reduced nitrite production by 4.2 nmoles in 30 min reaction.

| Addition (0.1 ml) | % Inhibition of Nitrate Reductase |
|---|---|
| Inhibitor *not* dialyzed against the SOGS | 66 |
| Inhibitor *not* dialyzed against the SOGS + water dialyzed against the SOGS | 42 |
| Inhibitor dialyzed against the SOGS | 20 |
| Buffer dialyzed against the SOGS | 0 |

Table 17

Protection by Superoxide Dismutase Against Photoinactivation of the Nitrate Reductase Inhibitor

The inhibitor was placed in the reaction mixture of NADH-nitrate reductase minus NADH and enzyme with and without superoxide dismutase (about 20 units). The systems were pretreated by light or dark at 25° for 45 min. In each case a minus inhibitor control was run. After pretreatment of the inhibitor NADH and nitrate reductase were added to produce 11.8 nmoles nitrite in 30 min reaction in control.

| | % Inhibition of Nitrate Reductase | |
|---|---|---|
| Superoxide Dismutase | Pretreatment of the Inhibitor for 45 min in Dark | Pretreatment of the Inhibitor for 45 min in Light |
| - | 67.4 | 8.1 |
| + | 65.7 | 20.8 |

Table 18

Recovery of the Photoinactivated Nitrate Reductase Inhibitory Activity in the Dark

The inhibitor was pretreated in the light or dark for 2 hours as described in Table 15, and the inhibitory activity was measured immediately and after 24 hours in the dark at 4°. The NADH-nitrate reductase produced 13.2 nmoles nitrite in 30 min reaction without the inhibitor. The inhibitor was added to inhibit the production of nitrite by 10.7 nmoles for 30 min after the dark pretreatment (81% inhibition of nitrite production).

| | % Inhibition of Nitrate Reductase | |
|---|---|---|
| Dark Period after Pretreatment | Pretreatment of the Inhibitor for 2 hrs in Dark | Pretreatment of the Inhibitor for 2 hrs in Light |
| 0 hour | 81 | 18 |
| 24 hours | 81 | 78 |

of the inhibitor lost these characteristics of the photoinactivation and its dark recovery. It was also observed that if the inhibitor was exposed to a high concentration of sodium chloride or potassium phosphate, but not potassium nitrate during its isolation, it was less sensitive to photoinactivation and recovery in the dark, although it had a normal amount of activity.

The completely photoinactivated inhibitor did not compete or prevent the active inhibitor from inhibiting nitrate reductase (Table 19).

Table 19

The Effect of the Photoinactivated Inhibitor on the Action of the Active Inhibitor

NADH-nitrate reductase without inhibitor produced 11.15 nmoles nitrite in 30 min reaction.

| Active Inhibitor* | Photoinactivated Inhibitor** | % Inhibition of Nitrate Reductase |
|---|---|---|
| 50 µl | None | 48 |
| None | 50 µl | 0 |
| 50 µl | 50 µl | 48 |

*A 50 µl aliquot containing about 20 µg protein of the inhibitory fraction from DEAE-cellulose column chromatography.

**The active inhibitor was illuminated for 2 hours as described in Table 15.

# DISCUSSION

1) Nitrate reductase from higher plants has been isolated and studied most thoroughly from spinach, corn and soybean leaves (4). The enzyme from soybean leaves appears to differ from that from other plant species (2) (A) by a difference of pH optimum with NADH as co-factor, (B) by the fact that FAD appears to be bound less tightly, and (C) by the ability of the soybean enzyme(s) to utilize NADPH as an electron donor almost as well as NADH. For this last reason and because of the economic importance of soybeans, special effort in recent years has been made to isolate NADH-nitrate reductase and NADPH-nitrate reductase from soybean leaves.

Evans and Nason (1) isolated soybean nitrate reductase without using sulfhydryl reagents in their buffers and found the Km($NO_3^-$) with NADPH to be 7.5 mM, Km(NADPH) to be 0.023 mM, and Km(NADH) to be 0.032 mM. They also observed that added FAD was required for maximum activity and that FMN could not substitute for FAD. Comparison of the properties of nitrate reductase isolated by Evans and Nason (1) with the NADPH-nitrate reductase isolated by me indicates that the two preparations are similar. Thus when soybean leaves are extracted without cysteine, the surviving nitrate reductase activity which can be isolated by DEAE-cellulose column chromatography is indistinguishable from the NADPH-nitrate reductase fraction isolated with 5 mM or 10 mM cysteine. It is to be emphasized, however, that in most plants only

NADH-nitrate reductase is present and that even in young soybean leaves the NADH specific enzyme is the major component. Soybean leaves differ in that they also contain a NADPH specific enzyme.

Beevers *et al.* (2) found that NADH-nitrate reductase activity of soybean extracts could be markedly increased or preserved by the addition of 10 mM cysteine to the extraction medium. Under this condition the ratio of the NADPH-nitrate reductase activity to the NADH-nitrate reductase activity was not altered during several purification steps. However, they obtained only one peak of nitrate reductase activity by DEAE-cellulose column chromatography, when the fractions were assayed with 10 mM $KNO_3$. However, I find that this procedure will resolve the activity into two fractions, an NADH-nitrate reductase and an NADPH-nitrate reductase and that this resolution is much more apparent if the reductase is assayed in 10 mM $KNO_3$ with NADH and in 80 mM $KNO_3$ with NADPH. Beevers *et al.* (2) also investigated the properties of the soybean nitrate reductase when it was prepared with and without 10 mM cysteine. They observed Km values for nitrate reductase prepared without cysteine which were similar to those found by Evans and Nason (1) and to those reported here for the NADPH-nitrate reductase. All these data strongly indicate that NADPH-nitrate reductase from young soybean leaves can be prepared free of the NADH-nitrate reductase activity by the simple omission of sulfhydryl reducing agent from the extraction medium. When Beevers *et al.* (2) extracted soybean leaves with 10 mM cysteine, they probably obtained a mixture of the two nitrate reductases as I have done.

Since Beevers *et al.* (2) could not separate the nitrate reductase of soybean leaves into NADH-nitrate reductase and NADPH-nitrate reductase, they looked for a transhydrogenase reaction or a NADPH specific phosphatase as alternative explanations for the NADPH-nitrate reductase activity. Recently, Wells (7) and Wells and Hageman (8) found that desalted crude extracts of soybean leaves contained an active NADPH phosphatase. On DEAE-cellulose column, the majority of NADPH phosphatase activity separated from the nitrate reductase peak. However, their nitrate reductase peak, which had activity with both NADH and NADPH, still contained sufficient NADPH phosphatase activity to account for the NADPH-nitrate reductase activity (7,8).

As presented in this dissertation, NADH-nitrate reductase and NADPH-nitrate reductase activity from soybean leaves can be separated by DEAE-cellulose column chromatography. Three major factors were involved: rapid isolation before the activities decay, the use of cysteine to protect the NADH enzyme activity, and the use of adequate amounts of $KNO_3$ for full activity of the NADPH enzyme. When it was found that NADPH-nitrate reductase had a higher Km constant for $NO_3^-$ (4.5 mM) than NADH-nitrate reductase (0.11 mM), assays were run with 80 mM $KNO_3$ for NADPH-nitrate reductase and 10 mM $KNO_3$ for NADH-nitrate reductase (Figure 1). When the fractions of the two nitrate reductases, which had been separated by DEAE-cellulose column chromatography, were assayed for NADPH phosphatase activity (111), only insignificant amounts of the phosphatase were found to be present.

In addition, several properties of the NADPH-nitrate reductase indicated that a NADPH phosphatase could not account for the NADPH

nitrate reductase activity. Potassium fluoride, an inhibitor of phosphatase (7,8,149,150), did not inhibit NADPH-nitrate reductase even at 10 mM. Phosphate is also reported to inhibit NADPH phosphatase (7,8,147,148), but the putative NADPH-nitrate reductase was as active in phosphate buffer as in MES buffer. Forti *et al.* (75) reported that the Km(NADPH) for NADPH phosphatase from peas was 150 μM, whereas the Km(NADPH) of NADPH-nitrate reductase is about two orders of magnitude lower (Table 3). Therefore, it seems unlikely that NADPH-nitrate reductase is an artifactual association of an NADPH phosphatase with an NADH-nitrate reductase. In addition, the fact that there are two nitrate reductase peaks by DEAE-cellulose column chromatography even with $FADH_2$ as an electron donor (Figure 1), and the fact that the proteins in these peaks have different electrophoretic mobilities, are strong evidence for two nitrate reductases in soybean leaves. The reasons why others have not separated the two nitrate reductases might be because they used 10 mM $KNO_3$ in their assays, under which condition activity would be mainly due to the NADH-nitrate reductase.

NADH-nitrate reductase in most plants is rather specific for NADH, yet in my fractions from the DEAE-cellulose column this enzyme from soybeans used NADPH about 30% as well as NADH. It is possible that the NADPH-nitrate reductase activity associated with the NADH-nitrate reductase fraction could have been due to phosphatase activity. The pH optima of the NADH-nitrate reductase when using NADPH as electron donor was shifted to pH 6 (Figure 3), which would be expected if an acid-phosphatase contributes to the overall reaction. Moreover, the Km constant for NADPH was in the range reported

for pea NADPH phosphatase (75). However, no direct evidence was found for the presence of NADPH phosphatase in the NADH-nitrate reductase preparation.

Most properties of the two nitrate reductases from soybean leaves were similar to those for the nitrate reductase from other higher plants (4). A number of different reactions are catalyzed by higher plant nitrate reductases (4), and all these reactions occurred with both of the soybean nitrate reductases. The pyridine nucleotide-cytochrome c reductase activity was present as an NADH-cytochrome c reductase in NADH-nitrate reductase and an NADPH-cytochrome c reductase in NADPH-nitrate reductase.

The Km values for $FADH_2$ with both nitrate reductase enzymes are similar to those reported for other plant nitrate reductases (3,51). However, endogenous FAD appears to be bound less tightly to the nitrate reductase of soybean leaves (2). The optimum activity of the soybean nitrate reductase isolated without cysteine (NADPH-linked enzyme) was not obtained unless FAD was added to the reaction mixture (2). Likewise my NADPH-nitrate reductase appeared to have FAD loosely bound, as evidenced by the enhancement of activity by added FAD after ammonium sulfate fractionation and Sephadex G-25 gel filtration, which could have removed part of the FAD. However, this enhancement was not found for NADH-nitrate reductase similarly treated. Nitrate reductase from spinach is normally not stimulated by added FAD or FMN; however, after extensive purification, it was converted to a form which was stimulated by addition of FAD (181,182). The NADH-nitrate reductase of soybean leaves was not tested for stimulation by FAD after such a purification procedure.

When reduced methylviologen and $FADH_2$ were used as electron donors, higher activities for both nitrate reductases were obtained than with the pyridine nucleotides. With these electron donors only part of the enzyme complex is probably involved in the reduction of nitrate (3,4). Furthermore, when $FADH_2$ was used as the electron donor, Km constant for $NO_3^-$ was increased for both nitrate reductases.

Molecular weights of 330,000 for NADH-nitrate reductase and 220,000 for NADPH-nitrate reductase from soybean leaves are lower than reported by some authors for nitrate reductases from other plants (4,182,192). However, Notton *et al.* (133) reported that nitrate reductase from spinach leaves has a molecular weight of 230,000, as determined by gel filtration. The molecular weights of nitrate reductases from the diatom, *Thalassiosira pseudonana* (134) and from barley leaves (196) have been reported to be 330,000 and 230,000, respectively.

NADPH-nitrate reductase has been reported in *Lemna minor* (72) and rice (66), but its properties have not been described. The nitrate reductase of *Neurospora crassa* is of the NADPH type (81,184), and it has certain properties similar to soybean NADPH-nitrate reductase. The molecular weight of the enzyme from *Neurospora* is 228,000, and it contains cytochrome b and loosely bound FAD (81,184). The form of our soybean NADPH-nitrate reductase showed no visible spectrum for a cytochrome characteristic even when treated with sodium dithionite.

Notton *et al.* (133) also reported finding two isoenzymes of spinach nitrate reductase when the enzyme was purified by isoelectric

focusing. One of the isoenzymes had a more acidic isoelectric point and appeared to be formed from the other when the nitrate reductase preparation was exposed to more acidic conditions. The only reported difference between these two isoenzymes of spinach leaves was the isoelectric point. Both isoenzymes could use NADH and reduced benzylviologen and could not use NADPH as electron donor. Since 10 mM $KNO_3$ was used for all the nitrate reductase assays, it is possible that one of the two isoenzymes might have been NADPH enzyme. However, when I attempted the separation of the two nitrate reductases from spinach leaves by the same procedure as from soybean leaves, only NADH-nitrate reductase was observed. Since the nitrate reductases from the studies reported here were not exposed to any extremes of pH, it seems unlikely that the NADPH linked enzyme resulted from the effects of acid or base on the NADH-nitrate reductase.

The molecular weight of NADPH-nitrate reductase was smaller by 110,000 than NADH-nitrate reductase. Thus, it is possible that one subunit, which has an active sulfhydryl site, because of the need for cysteine protection, could be associated with the utilization of NADH. This subunit might have been separated from NADH-nitrate reductase to produce the NADPH-nitrate reductase with the resultant higher Km for nitrate. If this were the case, when a low concentration of cysteine was used for purification, the NADPH-nitrate reductase activity should have increased as much as there was a decrease in NADH-nitrate reductase activity. This did not occur but, rather, the NADPH-nitrate reductase remained at the same level in lower or higher concentration of cysteine (Figure 1).

2) The rapid fluctuations of nitrate reductase activity that occur daily (11,13,15,19), or seasonally (42), or when plants are placed in the dark (9-12,14,18), or when they are exposed to heat or drought (183) indicate an effective *in vivo* regulatory mechanism. Fluctuations in activity could be brought about by changes in relative rates of synthesis or activation and breakdown or deactivation of the enzyme. All the experiments referred to above measured nitrate reductase activity by an *in vivo* assay with leaf discs or by using crude leaf extracts as source of nitrate reductase. No one has measured the amount of nitrate reductase protein which might be changing but only the activity. In all these tests, if there were inhibitors of nitrate reductase formed in the leaves after exposed to darkness, nitrate reductase activity would have been observed to decrease.

The studies with inhibitors of protein synthesis (14,16,64,65,100) constitute the best evidence that increase in activity during induction is due to *de novo* synthesis of nitrate reductase rather than activation. While these studies do show that a prerequisite for induction of nitrate reductase is RNA and protein synthesis, they do not demonstrate conclusively *de novo* synthesis of the enzyme. In fact, protein synthesis inhibitors could prevent the formation of a protein inhibitor as well as formation of the nitrate reductase itself. Thus far all attempts to demonstrate *de novo* synthesis of nitrate reductase have been inconclusive. Ingel (32) observed that when nitrate reductase was induced in the presence of [$^{3}$H]uridine plus [$^{14}$C]leucine, the incorporation of the radioactivity into nitrate reductase was negligible in comparison to the total amount of labeled

protein formed, while nitrate reductase activity was greatly increased. He concluded that the requirement of protein synthesis for nitrate reductase induction was not necessarily directly *de novo* synthesis, but may involve the synthesis of an effector necessary for nitrate reductase activity. Travis *et al.* (16) found that a loss of nitrate reductase activity in the dark in barley leaves was prevented by cycloheximide, actinomycin D and low temperature, indicating that protein synthesis was necessary for inactivation.

The thrust of my work is that, in soybean leaves, a nitrate reductase inhibitor protein is formed in the dark. I have isolated and partially characterized this inhibitor.

When the soybean plants were exposed to a 54 hour dark-period following a five day light-period, nitrate reductase, as detected by an *in vivo* assay with leaf discs, was a very low level. The enzyme was also inactive in the supernatant of leaf homogenates after sucrose gradient centrifugation. However, in the whole cell fraction after sucrose gradient centrifugation nitrate reductase activity was observed to be equally high level in cells from leaves kept in darkness or under continuous light (Figure 9). Also when the supernatant of the leaf extract from soybeans exposed to a dark period was chromatographed on a DEAE-cellulose column, nitrate reductase was observed at a level comparable to that from the leaf extracts of plants grown under continuous light. From the same DEAE-cellulose column, a nitrate reductase inhibitor was eluted after the nitrate reductases (Figure 11). These observations clearly indicate that the loss of nitrate reductase activity in the leaves in darkness is not due to a disruption of the enzyme but to the presence of an inhibitor.

Recently high molecular weight nitrate reductase inhibitors were isolated from roots of corn (36,37,38) and rice (39), but not from green leaves. This dissertation may be the first report of purification and characterization of a nitrate reductase inhibitor from green leaves. The heat-unstable, nitrate reductase inhibitor protein was purified about 2,500-fold (Table 8). The molecular weight of the inhibitor was estimated to be 31,000 by Sephadex G-100 gel filtration (Figure 12). When the inhibitor was treated with 1% SDS, the subunit molecular weight was about 18,000 (Figure 15). It is possible that the nitrate reductase inhibitor may be a dimer with 18,000 D subunits.

This inhibitor was very specific for NADH-nitrate reductases from soybean, spinach and pigweed leaves, but it did not inhibit NADPH-nitrate reductase from soybean leaves and respiratory $FMNH_2$-nitrate reductase from *Escherichia coli* (Table 12). The inhibitor did not affect any other enzyme activities tested, including nitrite reductase, glutamate dehydrogenase, xanthine oxidase, acid phosphatase and several other dehydrogenases (Table 12).

The nitrate reductase inhibitors from roots of corn and rice seedlings, as reported by Wallace (36,37,38) and Kadama (39), seemed to be specific proteases for nitrate reductase. My nitrate reductase inhibitor from soybean leaves does not seem to be a protease. This view was supported by the following observations:

(a) Its activity was not inhibited by a range of specific inhibitors of proteases (Table 14).

(b) The inhibition was observed instantly after mixing the inhibitor with nitrate reductase when the activity was assayed by measuring NADH utilization.

(c) Increasing the preincubation time of the inhibitor with nitrate reductase did not change the inhibition (Figure 20).

(d) It did not hydrolyze partially purified NADH-nitrate reductase or casein or synthetic peptides to amino acid and it did not hydrolyze any of the other enzymes tested to the extent of inhibiting their activities.

The soybean leaf inhibitor blocked the $FADH_2$-nitrate reductase reaction component in the nitrate reductase enzyme complex, but it did not block the NADH-cytochrome c reductase reaction (Table 13). On the other hand the NAD(P)H-cytochrome c reductase component of nitrate reductase complex was the main site of action of the inhibitors from roots of corn and rice seedlings (36-39).

Preliminary data indicate that the soybean nitrate reductase inhibitor may specifically bind to the nitrate reductase protein. If the inhibitor were not separated from the reductase by chromatography on DEAE-cellulose column, it was precipitated with nitrate reductase by 20-40% ammonium sulfate but, when it was separated from the reductase, 40-60% ammonium sulfate was required for precipitation. An incomplete separation of the inhibitor from the reductase by DEAE-cellulose column chromatography caused the inhibitor to be eluted in a higher molecular weight range and in the void volume from Sephadex G-100 column, but when the reductase was removed it moved differently on these columns. When the inhibitor was prepared

from nitrate depleted leaves which had low nitrate reductase activity, this phenomenon did not occur.

ESR spectroscopic studies have indicated that the nitrate is directly bound to the Mo(V) center of nitrate reductase from *Micrococcus definitrificans* before its reduction (91,92). When extra Mo(V) was added to soybean NADH-nitrate reductase before the addition of the inhibitor, it did not lessen the effect of the inhibitor on nitrate reductase. This implies that the inhibitor probably does not function by reacting with molybdenum of the reductase. Kinetic studies with the inhibitor indicated that the inhibition might be noncompetitive (Figure 16). Therefore, the inhibitor may bind to the enzyme at a different site from nitrate.

When the leaf extracts from soybeans grown under continuous light were chromatographed quickly on a DEAE-cellulose column, so that separation and assay were finished within three hours after harvesting the leaves, low activity of the inhibitor was found in comparison to the amount from plants exposed to a prior 54 hour dark-period (Figure 21A). After 24 hour storage of the inhibitory fraction from the DEAE-cellulose column at 4° in the dark, the amount of inhibition from both leaf extracts was equal (Figure 21B). The isolated inhibitor could be photoinactivated in the presence of flavin (Table 15). It is possible that $O_2^-$ or other high energy state radicals might destroy the inhibitor in this condition. If this were the case, the inactivation should be irreversible; however, the photoinactivated inhibitor was reactivated nearly completely by storage at 4° in the dark (Table 18). The photoinactivation of the inhibitor *in vitro* might involve some damage by the $O_2^-$ as well as some reversible protein conformational

change. How much superoxide is actually produced in a leaf cell and whether it would act *in vivo* in this manner are unanswered questions (185). *In vivo* it is more likely that the nitrate reductase inhibitor is in an active form in the dark and is inactivated by light through some conformational change. It is proposed that in the dark the inhibitor is in an active form which depresses nitrate reductase activity, and that it is changed by light to an inactive form.

Erlager and colleagues (175,176,177) have studied how systems normally insensitive to light can be photoregulated by means of photochromic effector molecules. Their molecules shared a common *p*-phenylazophenyl group which, under the influence of light undergoes a reversible configurational change to yield a *cis* or *trans* isomer. Their model can be used to explain the nitrate reductase activity cycle in the light and dark through the photoinactivation-reactivation of the inhibitor described in this dissertation (158). Their effector, N-*p*-phenylazophenyl carbamyl choline iodide, was converted from the *trans* to the *cis* isomer at pH 7.0 by sunlight and it was reversed to the *trans* isomer in the dark. The *trans* isomer was more stable and stronger inhibitor of acetylcholinesterase.

In a living system, regulation by means of an irreversible light-sensitive effector would require that the organism synthesize new enzyme in order to return to its former state. With reversible inhibitors, no new synthesis would be required; the conversion of the form would restore enzyme activity.

At this time it is not known whether the nitrate reductase inhibitor is induced by nitrate. Because soybeans have large cotyledons, it is almost impossible to obtain young nitrate-depleted

plants at the stage used in my work. In exploratory experiments cotyledons were removed when the first leaves had started to develop, and the plants were grown in vermiculite with only water. From these leaves, much lower amounts of nitrate reductase were present than from normal plants, but the amount of the inhibitor remained almost the same.

The evidence presented in this dissertation appears to establish that NADH-nitrate reductase, NADPH-nitrate reductase and the nitrate reductase inhibitor exist in the young soybean leaves. The metabolic role(s) and subcellular locations of the two nitrate reductases remain to be established. In other exploratory experiments it was found that isolating organelles by sucrose gradient centrifugation from soybean leaves is difficult. It has been thought that NADPH-specific enzymes in leaves are related to utilization of NADPH from photosynthesis. If this were the case for the NADPH-nitrate reductase in soybean leaves, this reductase might only function in the light to use excess NADPH and it would not need to be regulated by a light-sensitive effector as is the NADH-nitrate reductase. In contrast, the NADH-nitrate reductase may have to be inhibited in the dark, lest the nitrite it produces prove toxic in the absence of chloroplast-generated reductant.

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