NADH-Nitrate Reductase Inhibitor from Soybean Leaves

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ABSTRACT

A NADH-nitrate reductase inhibitor has been isolated from young soybean (Glycine max L. Merr. var. Amsoy) leaves that had been in the dark for 54 hours. The presence of the inhibitor was first suggested by the absence of nitrate reductase activity in the homogenate until the inhibitor was removed by diethylaminoethyl (DEAE)-cellulose chromatography. The inhibitor inactivated the enzyme in homogenates of leaves harvested in the light. Nitrate reductases in single whole cells isolated through a sucrose gradient were equally active from leaves grown in light or darkness, but were inhibited by addition of the active inhibitor.

The NADH-nitrate reductase inhibitor was purified 2,500-fold to an electrophoretic homogeneous protein by a procedure involving DEAE-cellulose chromatography, Sephadex G-100 filtration, and ammonium sulfate precipitation followed by dialysis. The assay was based on nitrate reductase inhibition. A rapid partial isolation procedure was also developed to separate nitrate reductase from the inhibitor by DEAE-cellulose chromatography and elution with KNO3. The inhibitor was a heat-labile protein of about 31,000 molecular weight with two identical subunits. After electrophoresis on polyacrylamide gel two adjacent bands of protein were present; an active form and an inactive form that developed on standing. The active factor inhibited leaf NADH-nitrate reductase but not NADPH-nitrate reductase, the bacterial nitrate reductase or other enzymes tested. The site of inhibition was probably at the reduced flavin adenine dinucleotide-NR reaction, since it did not block the partial reaction of NADH-cytochrome c reductase. The inhibitor did not appear to be a protease.

Some form of association of the active inhibitor with nitrate reductase was indicated by a change of inhibitor mobility through Sephadex G-75 in the presence of the enzyme. The inhibition of nitrate reductase was noncompetitive with nitrate but caused a decrease in Vmax.

The isolated inhibitor was inactivated in the light, but after 24 hours in the dark full inhibitory activity returned. Equal amounts of inhibitor were present in leaves harvested from light or darkness, except that the inhibitor was at first inactive when rapidly isolated from leaves in light. Photoinactivation of yellow impure inhibitor required no additional components, but inactivation of the purified colorless inhibitor required the addition of flavin.

Preliminary evidence and a procedure are given for partial isolation of a component by DEAE-cellulose chromatography that stimulated nitrate reductase. The data suggest that light-dark changes in nitrate reductase activity are regulated by specific protein inhibitors and stimulators.

MATERIALS AND METHODS

Plant Material. Soybeans (Glycine max L. Merr. var. Amsoy) were germinated in Vermiculite in a dark room for 3 days and grown for 5 additional days at 28 C in a chamber with about 360 μE m⁻² sec⁻¹ of continuous light from fluorescent lamps supplemented with tungsten lamps. Then part of the plants were exposed to darkness for an additional 54 hr, and others were kept continuously in the light. NR was induced by watering with 50 mM KNO3 in Hoagland nutrient solution every day after the 4th day of light. Young first leaves were used for enzyme and inhibitor preparations.

NR and Related Assays. NR (EC 1.6.6.2) with NADH, NADPH, FADH2, or reduced methylviologen was measured by a colorimetric determination of nitrite formation (8), as used previously by us (12). A unit of activity was 1 μmol of nitrite production/min. The unique feature of this assay was the use of varying amounts of KNO3 to distinguish between the NADH- and NADPH-NR. In the present work FAD was not in the reaction mixture for the routine NR assays and 1 mm cysteine was used throughout the isolation procedure for the inhibitor.

NADH-DCPIP reductase was measured by a decrease in A at 600 nm (26). NADH-Cyt c reductase activity was monitored at 550 nm in a recording spectrophotometer (7). These partial reactions of NR have been described in more detail (12). The in vivo NR activity was measured as the rate of nitrate excretion during a 30-min period by 10 leaf discs of 10-mm diameter (8).

Assay for NR Inhibitor. The rate of nitrite formation by a stock preparation of NR was measured with and without the inhibitor. An aliquot of the inhibitor was added to a reaction mixture which contained in 1 ml 25 μmol of K-phosphate at pH 6.5, 10 μmol of KNO3, and a specified amount of NADH-NR from soybean leaves. Without preincubation, the reaction was started by adding 0.2 μmol of NADH and was run for 30 min at 30 C. The amount of inhibitor was preselected by trial and error to give 30 to 55% inhibition, a range in which the inhibition was approximately linear with the amount of inhibitor. Data are expressed as a percent inhibition of the μmol of NR that were inhibited. The "μg of protein in the inhibitor fraction was measured by the Lowry

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2 Present address: The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, Mass. 02138.
3 Abbreviations: NR: nitrate reductase, DCPIP: dichlorophenolindophenol.
procedure (22).

Other Assays. Published assays were used for NR (21), glutamate dehydrogenase (31), glycolate oxidase (29), NAD-malate dehydrogenase (29), NAD-lactate dehydrogenase (15), α-glycerol phosphate dehydrogenase (4), acid phosphatase (14), and xanthine oxidase (23). β-Benzoyl-DL-arginine-p-nitroanilide from Sigma was hydrolyzed by Kruger's procedure (16) and l-leucine-p-nitroanilide by the method of Beever's (1). To test for protease activity (20) 1 ml of 0.4% casein solution of partially purified NADH-NR was incubated with a sample of the NR inhibitor at 30 C. Aliquots of 0.5 ml were removed after 0, 15, or 30 min, and the protein was precipitated with 0.5 ml of 10% trichloroacetic acid. After centrifugation, a 0.1-ml aliquot was added to a mixture that consisted of 0.5 ml of 1% ninhydrin in 0.5 M citrate at pH 5.5, 1.2 ml of glycerol, which had been passed through a column of Amberlite MB3, and 0.2 ml of 0.5 M citrate at pH 5.5. The mixture was heated in a boiling water bath for 12 min, cooled, and the color was read at 570 nm within 1 hr. Trypsophaan was used as a standard (20).

Cell and Organelle Separation by Sucrose Density Gradients. Soybean leaves (12.5 g) were ground with a cold mortar and pestle in 17.5 ml of buffer containing 25 mM K-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 C by layering into 50-ml Beckman cellulose nitrate tubes 8 ml of 2.3 M sucrose followed by 7.8 ml of 1.75 M, 7 ml of 1.7 M, and 5 ml of 1.6, 1.4, and 1.3 M sucrose solutions. The sucrose solutions were prepared in 25 mM K-phosphate at pH 7, 1 mM EDTA and 5 mM cysteine. Twenty ml of the leaf homogenate, that had been filtered through four layers of cheesecloth and then one layer of Miracloth, were layered on the top of the gradient. Centrifugation was at 3 C for 3 hr at 25,000 rpm (44,700g to 106,900g) in a Spincow SW 25.2 rotor (29). Fractions of 2.5 ml were collected from the bottom of the tube and aliquots were assayed for NR with NADH and NADPH.

Gel Filtration for Mol Weight Estimation. A Sephadex G-100 column (2.5 × 90 cm) was equilibrated with 25 mM K-phosphate at pH 7, 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/hr and 8-ml fractions were collected. The column was calibrated with blue dextran, BSA (68,000), ovalbumin (45,000), carboxy anhydrase (29,000), and Cyt c (12,400).

Polyacrylamide Gel Electrophoresis. The purity of the inhibitor was estimated by electrophoresis with gels 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 0.1 M K-phosphate at pH 7.5. For urea gel electrophoresis 8 M urea was added. Elution buffer was 0.1 M K-phosphate and 50 mM glycine at pH 7.5. The current was 8 mamp/5-mm diameter tube. For SDS-polyacrylamide gel electrophoresis, 0.1% SDS was added to 7.5% acrylamide gels and to the elution buffer at pH 7.1 (27). The protein was incubated at 100 C for 10 min in 10 mM Na-phosphate at pH 7.1, 1% SDS, 1% mercaptoethanol, and 10% glycerol (27). As references, 5 μg/tube of catalase (subunits of 60,000), ovalbumin (45,000), carboxy anhydrase (29,000), and lysozyme (14,300) were used. Protein mobility was calculated by the method of Weber and Osborn (35).

Photoinactivation and Recovery of Inhibitor. The reaction mixture for the NADH-NR assay minus the enzyme and NADH was mixed in 10-ml test tubes. After addition of the inhibitor they were illuminated at 0 C with about 360 μE m⁻2 sec⁻¹ from a Sunumbrella lamp. In dark treatments the tubes were covered with aluminum foil. After 2 hr, NR and NADH were added to assay for per cent inhibition of the reductase. To observe the recovery of the photoinactivated inhibitor, other samples were kept in the dark at 4 C for 24 hr after the 2 hr of illumination. Controls, minus inhibitor, were run.

RESULTS

APPARENT NR ACTIVITY IN LIGHT OR DARK-GROWN SOYBEAN LEAVES

After growing for 0 days in continuous light part of the soybean plants were transferred into a dark room. Fifty mM nitrate in a Hoagland nutrient solution was added every day after the 4th day of light. As observed by others, total NR by the in vitro assay disappeared in the dark but returned rapidly if placed back in the light (Fig. 1).

Soybean leaves from plants grown under continuous light (to be referred to as light-grown) or after 54 hr of darkness (dark-grown) were homogenized, the homogenate was filtered through Miracloth, and then subjected to isopycnic sucrose density gradient centrifugation (Fig. 2). The supernatant fraction at the top of the gradient from the dark-grown leaves had no NR activity whereas that from the light-grown leaves was active. Such results with homogenates in the past have been interpreted as dark inactivation of the enzyme. However, there were equal amounts

![FIG. 1. Effect of dark and light on total NR in soybean leaves. The in vitro assay was used for plants grown with Hoagland nutrient solution and 0.05 mM KNO3 in the light for 5 days and then for an additional indicated time in darkness followed by light.](image-url)
of activity in both gradients in the dense sucrose fraction, which, by microscopic examination contained whole cells from either light- or dark-grown plants. About equal activities of both NADH-NR and NADPH-NR were present. The supernatant fraction from the dark-grown plants severely inhibited the NR activity in the supernatant of light-grown plants as well as in the whole cell fractions (data not shown, see ref. 11). These data suggest that NR in the dark-grown leaves was present but inactive due to inhibitory substances that had been developed in the dark, and that these inhibitors were in the soluble supernatant fraction after homogenation and cell separation by a sucrose gradient.

Passage of the supernatant from dark-grown plants through a small Sephadex G-25 column removed only 20% of the inhibitory activity. The supernatant might have contained both low mol wt inhibitors and high mol wt inhibitors, but in the rest of this report we are concerned only with the latter component. The presence of a low mol wt, heat-stable inhibitor in tobacco leaves was suggested by Dalling et al. (5). Indeed some organic acids are inhibitors of NR from spinach leaves (11, 19).

**ISOLATION PROCEDURE FOR NR INHIBITOR**

In order to assay for the inhibitor of NR, it was first necessary to remove the inhibitor from the enzyme by DEAE-cellulose chromatography following the procedures used to separate NADH-NR from the NADPH enzyme (12). This step provided the NR for the assays and constituted an initial step in the purification of the inhibitor (Table I).

**Preparation of Homogenate.** For preparation of inhibitor, dark-grown soybean leaves were used, but light-grown leaves were better for NR preparations. Twenty to 24 g of soybean leaves were homogenized with a Waring Blender for 2 min in 50 ml of a buffer containing 25 mM K-phosphate at pH 7.5, 1 mM EDTA, 0.2% insoluble PVP, and 5 mM cysteine. The homogenate was filtered through four layers of cheesecloth and then one layer of Miracloth, and centrifuged for 10 min at 37,500g.

**DEAE-Cellulose Chromatography.** Both of the NR and the inhibitor were unstable in the crude homogenate. Therefore, the supernatant was immediately chromatographed on a DEAE-cellulose (Whatman DE52) column (2 x 21 cm) which had been equilibrated with a buffer containing 10 mM K-phosphate at pH 7.1, 1 mM EDTA, and 1 mM cysteine. After washing the column with 250 ml of equilibration buffer, it was developed with a linear NaCl or KNO₃ gradient obtained from mixing 375 ml of equilibration buffer and 375 ml of 0.5 M KNO₃ or NaCl in the equilibration buffer. Ten-ml fractions were collected. In our previous work a NaCl gradient was used to separate the two NR (12). The inhibitor could be eluted with either KNO₃ or NaCl but better resolution between the NR and the inhibitor was obtained with a KNO₃ gradient (Fig. 3). The inhibitor eluted at around 0.34 M KNO₃.

**Table I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total NR Inhibited nmol/hr</th>
<th>Total Protein mg</th>
<th>NR Inhibited by precipitating inhibitor</th>
<th>Purification fold</th>
<th>Recovery %</th>
</tr>
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<tr>
<td>Homogenate</td>
<td>23,000</td>
<td>957</td>
<td>24</td>
<td>1</td>
<td>100</td>
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<tr>
<td>Pooled inhibitory fraction from DEAE-cellulose column</td>
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<td>76</td>
<td>290</td>
<td>12</td>
<td>96</td>
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<tr>
<td>40-70% ammonium sulfate fraction</td>
<td>34,000</td>
<td>26</td>
<td>1,300</td>
<td>54</td>
<td>148</td>
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<tr>
<td>Pooled inhibitory fraction from Sephadex G-100 column</td>
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<td>1,87</td>
<td>11,000</td>
<td>460</td>
<td>87</td>
</tr>
<tr>
<td>0-50% ammonium sulfate fraction after dialysis and centrifugation</td>
<td>17,000</td>
<td>0.29</td>
<td>59,000</td>
<td>2,500</td>
<td>74</td>
</tr>
</tbody>
</table>

**Fig. 3.** Separation of NR and inhibitor by DEAE-cellulose column chromatography. The column (2 x 21 cm) was developed by a linear gradient of 0 to 0.5 M KNO₃ in a buffer of 10 mM K-phosphate at pH 7.1, 1 mM EDTA, and 1 mM cysteine. The NADH-NR and NADPH-NR activities of aliquots (50 μl) of each fraction were measured under their different optimum assay conditions (12). The reaction was for 20 min at 30 C. Aliquots of 100 μl were also assayed for inhibition of another NADH-NR preparation which produced 16.65 nmol of nitrite in 30 min without the inhibitor. Inhibition is expressed as decrease in production of nitrite.

**Ammonium Sulfate Fractionation.** The peaks of NADH-NR, NADPH-NR, and inhibitor (Fig. 3) were pooled. The two enzyme fractions were precipitated by 50% saturated ammonium sulfate, collected by centrifugation, and dissolved in a small volume of buffer containing 10 mM K-phosphate at pH 7.1, 1 mM EDTA, 5 mM cysteine, and 0.5 M sorbitol. These enzyme preparations were passed through a Sephadex G-25 column (2 x 18 cm) which had been equilibrated with the same buffer. The NR were collected in the void volume, frozen in liquid N₂ and stored at −18 C.

After DEAE-cellulose column chromatography, the inhibitor was precipitated between 40 to 70% saturated ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 25 mM K-phosphate at pH 7 containing 1 mM EDTA and 1 mM cysteine. If the crude extract had been fractionated by ammonium sulfate prior to DEAE-cellulose column chromatography, most of the inhibitor would have been in the 20 to 40% ammonium sulfate fraction along with the NR.

The increases in total inhibitory activity by the first ammonium sulfate fractionation is to be noted (Table I). Two factors may contribute to this phenomenon. The inhibitor was activated in the dark, which continued during its isolation. Also, a NR stimulator was eluted from a DEAE-cellulose column between NR and the inhibitor, and its separation from the inhibitor would result in an apparently more active inhibitor. The NR stimulator was precipitated by 70 to 90% ammonium sulfate (17, 18).

**Sephadex G-100 Filtration.** A solution of the inhibitor was applied to a reverse flow Sephadex G-100 column (2.5 x 90 cm) equilibrated and eluted with 25 mM K-phosphate at pH 7 containing 1 mM EDTA and 1 mM cysteine. If there was enough protein in the inhibitor fraction from the Sephadex G-100 column, it was concentrated by precipitation with 80% saturated ammonium sulfate, redissolved in the same buffer, dialyzed, and centrifuged to remove insoluble protein. This greatly increased the specific activity without a great loss of total activity. The inhibitor was purified about 2,500-fold (Table I) and was used for subsequent investigations in this report. When there was less protein in the final preparation it could be concentrated by lyophilization. At-
tempts at further purification by DEAE-Sephadex A-50 column chromatography or a second Sephadex G-100 gel filtration resulted in tremendous losses of activity and the specific activity did not significantly increase.

During the purification, it was necessary to separate the inhibitor completely from NR. When NR was present, as occurred with a NaCl gradient during DEAE-cellulose column chromatography, the inhibitor would elute 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 mol wt than its true mol wt. This was assumed to indicate an association between the inhibitor and the enzyme, which will be discussed in a subsequent section. To avoid this, the DEAE-cellulose column could be washed with about 4 volumes of 0.25 M KNO₃ in the elution buffer after loading to remove all of the NR, and then the inhibitor could be eluted by a 0.25 M to 0.5 M KNO₃ linear gradient (data not shown). This procedure was required with larger preparations or when more NR was present in the leaf extract.

GEL ELECTROPHORESIS

The inhibitor was electrophoresed on polyacrylamide gels, and then stained with Coomassie brilliant blue. The proteins did not migrate in high pH gels (6), but at pH 7.5 they migrated toward the anode. The inhibitor could be extracted from 4-mm gel slices by immersing them in a reaction mixture for the NADH-NR assay minus the enzyme and NADH for 2 days at 4°C in the dark. After the slices were removed, NADH-NR and NADH were added and the reductase assay was assayed for 30 min at 30°C.

The gel scanning at 550 nm showed two protein bands; the faster migrating one was designated as I (Fig. 4). The relative amount of protein between the two bands was dependent on the age of the inhibitor preparation. When electrophoresis was carried out immediately after purification, there was much more protein I than II (Fig. 4A). After 4 days, protein II had increased and I decreased proportionally (Fig. 4B). The peak of inhibitory activity coincided with protein I (Fig. 4C). Apparently the protein was changing into an inactive form on standing.

MOL. WEIGHT AND STABILITY OF NR INHIBITOR

All of the inhibitory activity was destroyed at 100°C and 50% at 50°C for 10 min. At pH 7 and 0°C the inhibitory activity was stable for at least 1 week, but it was unstable at acid or alkaline pH. All inhibitory activity was lost after 30 min at 0°C at pH 2 or 12.

The NR inhibitor had an approximate mol wt of 31,000 by Sephadex G-100 column chromatography. From SDS-gel electrophoresis, only one protein band was observed with an estimated mol wt of 18,000. This one subunit size was obtained from freshly prepared or aged preparations which showed different ratios of protein I and II by electrophoresis. Treatment with 8 M urea and electrophoresis in 8 M urea also indicated that there was only one type of subunit. These data suggest that protein bands I and II of the inhibitor were composed of two common subunits.

SPECIFICITY OF INHIBITOR

The isolated protein inhibited NADH-NR preparations from leaves of soybeans, spinach, and pigweed plants, but it did not inhibit the NADPH-NR from soybean leaves (12). It also did not inhibit the respiratory NR from Escherichia coli, which cannot use NADH or NADPH but uses FMNH₂, FADH₂, and reduced violagens as electron donors. The activities of NR and glutamate dehydrogenase, subsequent enzymes in nitrate assimilation by plants, were not influenced by the inhibitor. Xanthine oxidase, a flavoprotein with Fe and Mo components, which can also reduce nitrate to nitrite anaerobically with xanthine, was not affected. Other enzymes that were not affected by the inhibitor were glycolate oxidase and malate dehydrogenase from spinach leaves, lactate dehydrogenase from muscle, glyceral-P dehydrogenase from liver, and acid phosphatase from wheat germ. Thus, the inhibitor seemed to be specific for NADH-NR from plants.

When the electron donor for NR was changed from NADH to reduced methylviologen or FADH₂, the inhibitory effect of the protein was still present though decreased 50 or 29%, respectively. When the electron acceptor was changed from nitrate to NAD⁺ or DCPIP, the inhibitor had no effect on the reaction. The data suggest that the FADH₂-NR component in the NADH-NR complex might be the main site of action of the inhibitor, and that the diaphorase (NADH-Cyt c reductase) component in the NADH-NR complex is not affected. Because the inhibitor did not affect the NR from E. coli, which has an FMNH₂-NR activity, it is postulated that the flavin-NR component in the NR complex from leaves may be different from NR from the bacteria.

KINETIC STUDIES WITH INHIBITOR

The Lineweaver-Burk plot in Figure 5 was characteristic of noncompetitive inhibition of NR although the intercepts suggested that it was not strictly noncompetitive. The Kᵢ(KNO₃) was not significantly changed by the inhibitor, but Vₘₐₓ decreased with increasing amount of inhibitor. The activity of the inhibitor was dependent upon the amount of NR and of the inhibitor. Thus, with a fixed amount of NR the inhibition curve was sigmoidal (Fig. 6). For this reason, assays during the isolation procedure were run with dilutions of the inhibitor to provide 30 to 55% inhibition.

Evidence for binding of the inhibitor to NADH-NR was obtained with Sephadex G-75 columns (0.85 × 19 cm), through which the reductase passed in the void volume (Fig. 7A), but...
which retained the inhibitor (Fig. 7C). In combination with NR, the inhibitor eluted in the void volume (Fig. 7B). Because the NR was not pure, it was possible that a contaminating protein in the preparation might have bound the inhibitor. However, the known contaminant in the NR preparation, ribulose-5'-carboxylase, or BSA did not affect the elution position of the inhibitor when a pure preparation of these proteins was co-chromatographed with the inhibitor on the Sephadex G-75 column.

**ABSENCE OF PROTEASE ACTIVITY IN INHIBITOR**

A NR inhibitor from corn roots has been identified as a protease which degraded casein and which was phenylmethylsulfonyl-sensitive (33). Therefore, a series of protease inhibitors (9) and substrates were tested on our protein inhibitor from soybean leaves and found to be without effect. p-Chloromercuribenzoic acid, 0.1 mM, had no effect on the activity of the inhibitor, but inhibited the soybean NADH-NR activity about 30%. Phenylmethylsulfonyl fluoride, an inhibitor of serine-dependent enzymes, at 0.25 mM, did not have any effect on either NADH-NR or the inhibitor. Commercial trypsin inhibitors from soybean and ovo-
mucoid did not affect the inhibitory activity. EDTA and o-
phenthanthrolene even at high concentrations did not decrease the inhibitory activity.

The purified NR inhibitor did not hydrolyze the synthetic peptides, a-benzoyl-tyr-arginine-p-nitroanilide or t-leucine-p-nitroanilide, nor did it release amino acids from a casein solution or a partially purified NADH-NR. The inhibition was independent of preincubation time with NR (data not shown, see ref. 11), as if there were rapid binding of the inhibitor to the reductase but no inhibition by a time-dependent protease activity. Also, when the NR activity was recorded at 340 nm by measuring NADH oxida-

**EFFECT OF LIGHT AND DARKNESS ON INHIBITOR ACTIVITY**

Because of the literature on dark inactivation of NR (3), and because of changes in amount of inhibitor observed during studies on its isolation (11), it appeared necessary to develop a rapid procedure for isolating the inhibitor in order to evaluate light-
dark changes in the amount of it in leaves. For this the homogenate from 5 g of leaves was chromatographed on a small DEAE-

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**Fig. 5.** Effect of nitrate and inhibitor concentration on activity of NR. V: nmol of nitrite produced/hr; [S]: mM KNO₃.

**Fig. 6.** Effect of inhibitor concentration of NADH-NR activity. Without the inhibitor, 5 nmol of nitrite were formed in a 30-min assay.

**Fig. 7.** Binding of inhibitor to NADH-NR. NADH-NR had been partially purified by DEAE-cellulose column chromatography followed by ammonium sulfate fractionation and desalting with Sephadex G-25 column. A: NR (1.42 units) in 0.5-ml solution of 0.4 mM sorbitol was chromatographed on a Sephadex G-75 column (0.85 x 19 cm), which had been equilibrated with 25 mM K-phosphate at pH 6.5 and 1 mM cysteine. The void volume was 6 ml. B: NR (—) plus inhibitor (---) were chromatographed in the same manner. The amount of inhibitor inactivated 1.03 units of the reductase. C: Inhibitor only. To measure NR activity, KNO₃ and NADH were added to 0.45 ml of each fraction. To measure inhibitory activity KNO₃, NADH-NR (5 nmol of nitrate/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 hr of storage in the dark at 4 C, during which time the accompanying NR lost all of its activity in the presence of only 1 mM cysteine.
active inhibitor. An additional complexity was the presence of a NR stimulator, which eluted from the DEAE-cellulose column in the first 14 fractions free of NR. In Figure 8A this is shown by the negative values for inhibition of NR. The stimulator is probably the component reported by Ku (17) and Ku et al. (18). After storage for 24 hr in the dark this stimulator was inactive. The in vitro shift in the peak fraction of inhibitor, after storage, toward the location of the stimulator fraction probably reflects the total effect on NR activity from the two components acting in an opposite manner.

A qualitative photoinactivation and dark reactivation of the isolated inhibitor could be demonstrated. Inhibitor from light-grown leaves was isolated on a DEAE-cellulose column and placed in the assay medium without NR and NADH, and stored in the dark or illuminated with 360 £E m⁻¹ sec⁻¹ at 0°C for 2 hr. Afterwards, the dark sample exhibited good inhibition of NADH-NR, whereas the illuminated sample was inactive. If the inactive illuminated sample was now placed in the dark for 24 hr at 4°C, it regained nearly all of the inhibitory activity of the fresh sample that had been stored in the dark for 2 hr. The inhibitor fraction from the DEAE-cellulose column was yellow in color, and required no flavin for photoinactivation; but the purified inhibitor after Sephadex G-100 gel filtration was colorless and required the addition of a flavin for this photoinactivation. The completely photoinactivated inhibitor did not compete or prevent the active inhibitor from inhibiting NR. It was also observed that if the inhibitor was exposed to a high concentration of NaCl or K-phosphate, but not KNO₃ during its isolation, it was less sensitive to photoinactivation and recovery in the dark, although it had a normal amount of activity.

Some of the mechanisms for light inactivation of the NR inhibitor were explored by preliminary experiments. (a) Light might have generated an electron donor (FADH₂) (24), for NR by the reductase even in the presence of the inhibitor. This seemed unlikely because NADH was required by any NR with or without inhibitor. (b) In the light in the presence of a flavin the generation of O₂⁻ might inactivate the inhibitor. Indeed, the inhibitor lost 70% of its activity in 1 hr in a small dialysis tube placed in a xanthine-xanthine oxidase solution in the dark for O₂⁻ generation. Addition of superoxide dismutase to a solution of the inhibitor prevented some (up to 24%) of the inactivation during illumination at 25°C. These experiments suggest that the O₂⁻ can attack the inhibitor and may be one factor for its photoinactivation. However, destruction of NR inhibitor by O₂⁻ or other high energy radicals should be irreversible, but most of the photoinactivation of the inhibitor observed in a solution of the inhibitor was reversed after standing in the dark for 24 hr at 4°C. (c) A third hypothesis, which we favor, is that there is a reversible, conformational change in the inhibitor which is light-mediated through some pigment component. The reversible light inactivation and dark activation of the inhibitor in vivo and vitro have already been cited to support this hypothesis.

**DISCUSSION**

Rapid fluctuations of NR indicate an effective in vivo regulatory mechanism (2), which could be brought about by changes in relative rates of synthesis and breakdown or activation and deactivation of the enzyme. In the past all such experiments measured NR activity by an in vivo assay with leaf discs or by using crude leaf extracts as the source of the enzyme. Only activity was measured and no evaluation of the amount of NR protein present could be done. In those tests, if inhibitors of NR were present in the leaves and were activated in the dark, NR activity would have been observed to decrease.

Previous studies with protein synthesis inhibitors (3, 28, 30) constituted evidence that increases in NR activity during induction were due to de novo synthesis of the enzyme rather than activation. While these studies did show that a prerequisite for induction of NR activity was RNA and protein synthesis, they did 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 NR itself. Attempts to demonstrate de novo synthesis of NR by others have been inconclusive. Ingel (10) observed that when NR was induced in the presence of [³H]uridine plus [¹⁴C]leucine the incorporation of the radioactivity into NR was negligible in comparison to the total amount of labeled protein formed, in spite of the fact that NR activity greatly increased. He concluded that the requirement of protein synthesis for NR induction was not necessary for direct de novo synthesis, but might involve the synthesis of an effector necessary for NR activity. Travis et al. (30) found that a loss of NR activity in the dark in barley leaves was prevented by cycloheximide, actinomycin D, and low temperature, indicating that protein synthesis was necessary for inactivation, but this too could have been an effect upon the inhibitor.

The thrust of our work is that in soybean leaves, a NR inhibitor protein is active in the dark. The presence of a stimulator protein for NR was also indicated, but until it is also isolated and characterized, a full understanding of the regulation of NR is not feasible. Our initial experiment indicated complete loss of NR activity after 54 hr of darkness when assayed in vivo with leaf discs, or in vitro in the homogenate. However, in whole cells after sucrose gradient centrifugation NR activity was as high as in whole cells from leaves kept in continuous light. Also, when the supernatant of the leaf extract from soybeans exposed to a dark period was chromatographed on DEAE-cellulose column, NR
was observed at a level comparable to that from the leaf extracts of plants grown under a continuous light. From the same DEAE-cellulose column, a NR inhibitor was eluted after the NR. These observations indicate that the loss of NR activity in the leaves in darkness was not due to a destruction of the enzyme but to the presence of an inhibitor. The isolated inhibitor was a heat-labile protein of about 31,000 with two identical subunits. The inhibitory activity of the isolated protein was reversibly light-inactivated and dark-activated.

The single cells from dark-grown soybean leaves without NR activity by the in vivo leaf disc assay had much enzyme activity when isolated in a sucrose gradient and assayed with added NADH and nitrate. Addition of the inhibitor protein of 31,000 mol wt stopped the reductase activity in these cell suspensions. The results suggested that both NADH and the inhibitor crossed the membranes of these whole cells, unless the 50% sucrose isolation medium had somehow destroyed the semipermeability of this membrane. It is also possible then in the presence of sucrose the inhibitor in the whole cells on the gradient was inactivated so that the NR activity was expressed.

NR inhibitors previously isolated from roots of corn and rice (13, 32-34) were proteases. That our NR inhibitor from soybean leaves was probably not a protease is indicated by the following observations. (a) The inhibitor activity was not prevented by specific inhibitors of proteases. (b) The inhibition of NR occurred immediately after mixing the inhibitor with the enzyme. A preincubation time of the inhibitor with NR did not change the degree of inhibition. (c) The isolated inhibitor did not hydrolyze partially purified NADH-NR, casein, or synthetic peptides to ninhydrin-positive material, and it did not hydrolyze or inactivate any of the other enzymes tested. These tests would not exclude a specific limited protease. (d) However, inhibition of NR was reversed by removal of the inhibitor, which should not occur from limited proteolysis.

Preliminary data indicate that the soybean NR inhibitor may be specifically bound to the NR protein. If the inhibitor were not separated from the reductase by chromatography on DEAE-cellulose column, it was precipitated with NR by 20 to 40% saturated ammonium sulfate but, when it was separated from the reductase, 40 to 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 mol wt range and in the void volume from a Sephadex G-100 column. When the reductase was completely separated from the inhibitor, then its chromatographic properties were that of a protein with a mol wt of about 31,000.

It is well known that NR is induced by growth of plants on nitrate. In our experience this phenomenon is related to the synthesis of the enzyme. It is not known whether some nitrate is required to induce the synthesis of the inhibitor, because soybeans have large cotyledons, and nitrate-depleted young plants were not obtained. In exploratory experiments cotyledons were removed, when the first leaves had started to develop, and plants were grown in Vermiculite with only water. From these leaves, much lower amounts of NR were present than from normal plants, but the amount of the inhibitor remained almost the same as from control plants.

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