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Selective Inhibition of Auxin-Stimulated NADH Oxidase Activity and Elongation Growth of Soybean Hypocotyls by Thiol Reagents

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The NADH oxidase activity of isolated vesicles of soybean (*Glycine max* cv Williams 82) plasma membranes and elongation growth of 1-cm-long hypocotyl segments were stimulated by auxins (indole-3-acetic acid or 2,4-dichlorophenoxyacetic acid [2,4-D]). The auxin-induced stimulations of both NADH oxidase and growth were prevented by the thiol reagents *N*-ethylmaleimide, *p*-chloromercuribenzoate, 5,5′-dithiobis(2-nitrophenylbenzoic acid), dithiothreitol, and reduced glutathione. These same reagents largely were without effect on or stimulated slightly the basal levels of NADH oxidase and growth when assayed in the absence of auxins. In the presence of dithiothreitol or reduced glutathione, both 2,4-D and indole-3-acetic acid either failed to stimulate or inhibited the NADH oxidase activity. The rapidity of the response at a given concentration of thiol reagent and the degree of inhibition of the 2,4-D-induced NADH oxidase activity were dependent on order of reagent addition. If the thiol reagents were added first, auxin stimulations were prevented. If auxins were added first, the inhibitions by the thiol reagents were delayed or higher concentrations of thiol reagents were required to achieve inhibition. The results demonstrate a fundamental difference between the auxin-stimulated and the constitutive NADH oxidase activities of soybean plasma membranes that suggest an involvement of active-site thiols in the auxin-stimulated but not in the constitutive activity.

Morré et al. (1986) observed that the oxidation of NADH by isolated soybean (*Glycine max*) plasma membranes was stimulated by 2,4-D. This stimulation did not require addition of exogenous electron acceptors such as ascorbate radical or hexacyanoferrate III, and electron transfer presumably was either to oxygen or endogenous electron acceptors associated with the plasma membrane (Morré and Brightman, 1991). That this stimulation was distinct from that of the oxygen burst NAD(P)H oxidase of host defense was indicated by the observation that oxidation of NADPH was not stimulated by auxin (Morré and Brightman, 1991).

The auxin-stimulated NADH oxidase activity was purified from soybean plasma membrane by Brightman et al. (1988) as a complex of three proteins with molecular masses of 36, 55, and 72 kD. The NADH oxidase activity and the plasma membrane NADH:hexacyanoferrate III oxidoreductase did not co-purify and appeared to reside on different proteins (Morré and Brightman, 1991).

The present report demonstrates that the auxin-stimulated component of the NADH oxidase is inhibited by thiol reagents. The auxin-induced elongation growth of hypocotyl segments is inhibited specifically by these same thiol reagents. The findings provide evidence for a fundamental difference between the auxin-induced and the constitutive NADH oxidase activities of plasma membrane vesicles from soybean hypocotyls that also is observed for auxin-induced cell elongation. The difference appears to derive from an involvement of thiols at the active site in the auxin-stimulated but not in the constitutive activity.

MATERIALS AND METHODS

Isolation of Plasma Membrane

Soybean seeds (*Glycine max* L. cv Williams 82) were soaked for 4 h in water and grown in the dark (20–22°C) in moist vermiculite. After 4 to 6 d, 2-cm hypocotyl segments, cut just below the cotyledon, were harvested under diminished room light (0.15 μmol photons s^{-1} m^{-2}) and placed in cold water. Segments (40 g) were chopped with razor blades in 40 mL of homogenization medium (0.3 M Suc, 50 mM Tris-Mes [pH 7.5], 10 mM KCl, 1 mM $MgCl_2$, 1 mM PMSF). The homogenates were filtered through one layer of Miracloth (Chicopee Mills, New York, NY) and centrifuged for 10 min at 6,000*g* (HB-rotor, DuPont, Wilmington, DE). The supernatant was recentrifuged at 60,000*g* (SW 28 rotor; Beckman, Fullerton, CA) for 30 min and the pellets were resuspended in 0.25 M Suc with 5 mM potassium phosphate (pH 6.8). Plasma membrane vesicles were prepared using a 16-g aqueous two-phase partitioning system (Sandelius et al., 1987). Resuspended 60,000*g* pellets were mixed with 6.4% (w/w) PEG 3350 (Fisher), 6.4% (w/w) Dextran T500 (Pharmacia), 0.25 M Suc, and 5 mM potassium phosphate (pH 6.8). After mixing the tubes by 40 inversions, the phases were separated by centrifugation at 750*g* for 5 min. The lower phase was repartitioned with a fresh upper phase, and the two upper phases were repartitioned twice with fresh lower phases. The upper phases were diluted approximately 4-fold with buffer and collected by centrifugation at 100,000*g* for 30 min. The mem-

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Abbreviations: DTNB, 5,5′-dithiobis(2-nitrophenylbenzoic acid); 2-ME, 2-mercaptoethanol; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate.

branes were stored frozen at −70°C prior to assay. The yield was 1 to 2 mg of plasma membrane protein.

NADH Oxidase Activity

The assay for the plasma membrane NADH oxidase was in 50 mM Tris-Mes buffer (pH 7.0) and 150 μM NADH in the presence of 1 mM potassium cyanide, the latter to inhibit any mitochondrial NADH oxidases contaminating the plasma membranes. The assay was started by the addition of 0.1 mg of plasma membrane protein. The reaction was monitored by the decrease in the A_{340} with A_{430} as reference, using an SLM DW-2000 (SLM-Aminco, Urbana, IL) spectrophotometer in the dual-wavelength mode of operation or a Hitachi model U3210. The change of A was recorded as a function of time by a chart recorder. The specific activity of the plasma membrane was calculated using an absorption coefficient of 6.21 mM^{-1} cm^{-1} and expressed as nmol min^{-1} mg^{-1} protein.

Assays were initiated by addition of NADH and measured over 5 min after an equilibration period with NADH of 10 min to achieve an initial steady-state rate. Unless indicated otherwise, after each subsequent addition the assay was continued for 10 min with the steady-state rate between 5 and 10 min being reported. NADH was added first. With 2,4-D and thiol reagents, order of addition has been indicated for individual experiments. Control rates were linear for 50 min or longer. Unless indicated otherwise, results were averages from duplicate determinations from each of three different plasma membrane preparations ± SD values among the three different preparations.

Growth Studies

For growth studies, hypocotyl segments, 1 cm in length, were harvested from the zone of cell elongation under dim laboratory light (1.5 μmol photons s^{-1} m^{-2}) by cutting 5 mm below the cotyledons. The segments were floated on aqueous solutions, 2 mL/10 sections, containing the test substances. Incubations were in darkness at 24°C for 18 h. Growth was estimated from the increase in length of the segments after 3, 6, 12, and 18 h. Lengths were measured to the nearest 0.1 mm.

Protein

Protein content was determined by the bicinchoninic acid procedure (Smith et al., 1985). Standards were prepared with BSA.

RESULTS

Auxin-Induced Component of NADH Oxidase Activity Inhibited by Thiol-Reactive Reagents NEM, PCMB, and DTNB

NADH oxidase activity of plasma membranes isolated from soybean hypocotyls was stimulated by about 20% in the presence of 0.1 μM 2,4-D and by about 70% by 1 μM 2,4-D or IAA (Table I). After 10 min of pretreatment with 0.5 mM NEM, 1 μM PCMB, or 10 nM DTNB, no stimulation of NADH oxidase activity was observed with either 2,4-D or IAA. The concentrations of NEM, PCMB, and DTNB reported in Table I were determined from dose-response curves (e.g. Fig. 1) to be sufficient to inhibit the auxin-stimulated activity. In contrast to the auxin-stimulated component of the NADH oxidase activity, the basal NADH oxidase activity was not inhibited by any of the thiol reagents.

The dose response for NADH oxidase activity in the presence or absence of 1 μM 2,4-D is given in Figure 1A for NEM and Figure 1B for PCMB. The auxin-induced component of the activity was inhibited as concentrations of NEM approached or exceeded 10 μM. With PCMB (Fig. 1B), the auxin-induced component of the NADH oxidase was eliminated at about 1 μM. With both NEM and with PCMB there was a tendency for the compounds to stimulate the NADH oxidase activity in the absence of auxin, especially at high concentrations (Fig. 1) or with prolonged times of preincubation. Under no conditions was the constitutive NADH oxidase activity observed to be inhibited by thiol reagents. Similar results were obtained with DTNB except that inhibitions were observed in the nanomolar range (Table I).

Auxin-Induced Growth Inhibited by Thiol Reactive Reagents

The rate of elongation growth of the soybean hypocotyl segments was approximately doubled by 10 μM 2,4-D. As with the auxin-induced component of NADH oxidase activity, growth induced by 10 μM 2,4-D (0.8 cm) was inhibited preferentially to the constitutive level (0.4 cm) by both NEM (Fig. 2A) and PCMB (Fig. 2B). With NEM (Fig. 2A) but not with PCMB (Fig. 2B), and to a lesser extent with DTNB (Fig. 2C), constitutive growth eventually was inhibited as well, but only as the NEM concentrations exceeded

Table I. *Inhibition by pretreatment with NEM, PCMB, or DTNB of the subsequent auxin (2,4-D or IAA) stimulation of NADH oxidase of plasma membranes of etiolated soybean hypocotyl segments*

The number of determinations is given in parentheses. Values are means ± SD among determinations.

Addition	Post-Addition	NADH Oxidase
		nmol min^{-1} mg^{-1} protein
None	None	1.06 ± 0.05 (5)[a]
	0.1 μM 2,4-D	1.24 ± 0.3 (5)[a]
	1 μM 2,4-D	1.8 ± 0.2 (3)[b]
	1 μM IAA	1.7 ± 0.2 (4)[b]
0.5 mM NEM	None	1.2 ± 0.1 (3)[a]
	0.1 μM 2,4-D	1.24 ± 0.14 (5)[a]
	1 μM 2,4-D	1.20 ± 0.15 (3)[a]
	1 μM IAA	1.0 ± 0.2 (3)[a]
1 μM PCMB	None	1.22 ± 0.20 (8)[a]
	1 μM 2,4-D	1.33 ± 0.15 (8)[a]
	1 μM IAA	1.2 ± 0.15 (3)[a]
10 nM DTNB	None	1.2 ± 0.1 (3)[a]
	1 μM 2,4-D	1.3 ± 0.1 (3)[a]
	1 μM IAA	1.1 ± 0.2 (3)[a]

[a,b] Values not followed by the same letter were statistically different ($P < 0.05$).

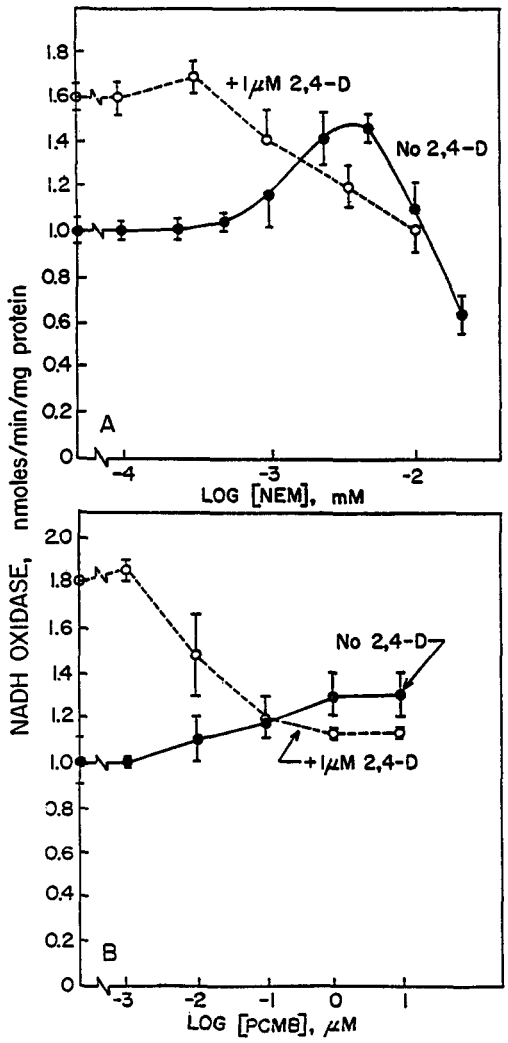

Figure 1. NADH oxidase activity of isolated soybean plasma membranes after 10 min of pretreatment as a function of the logarithm of NEM (A) and PCMB (B) concentration. The thiol reagents preferentially inhibit the 2,4-D-induced stimulation of the oxidase. 2,4-D was added first, followed by NEM or PCMB. Rates are steady-state rates measured 15 min after NEM or PCMB addition. Results are from three experiments with duplicate determinations in each experiment ± SD among the three experiments.

1 mM. Even at 1 mM NEM, where constitutive growth was still unaffected, the auxin-stimulated component of growth was inhibited by about 50%.

In contrast to NEM, PCMB was virtually without effect on the constitutive growth of sections in the absence of auxin (Fig. 2B). However, auxin-induced growth was strongly inhibited by PCMB.

With DTNB (Fig. 2C), inhibition of growth in the absence of auxin was slight and not significant statistically. However, auxin-induced growth was eliminated almost completely. The inhibition of growth over 18 h by 2,4-D was further increased only slightly by first abrading the hypocotyl segments to facilitate cuticular penetration of the DTNB (Fig. 2C, △).

The Auxin-Induced Component of NADH Oxidase Activity Inhibited by GSH and DTT

Differences between the auxin-induced and constitutive NADH oxidase activities became even clearer when responses to GSH and DTT were compared. The NADH oxidase of plasma membrane vesicles in the absence of auxin was largely unaffected by these compounds (Table II). However, the 2,4-D-stimulated increment of NADH oxidase was inhibited completely.

At concentrations of GSH (Fig. 3A) or DTT (Fig. 3B) of 1 and 10 μM, not only was the auxin stimulation of NADH

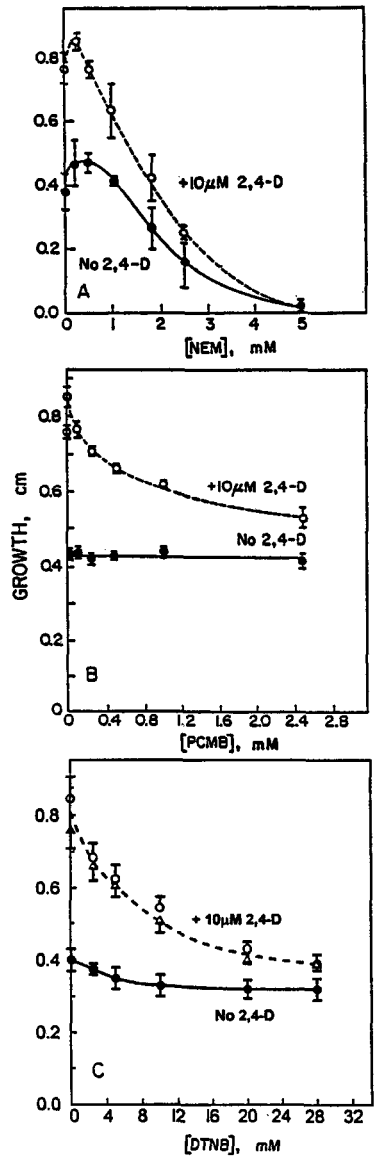

Figure 2. NEM (A), PCMB (B), and DTNB (C) preferentially inhibit auxin-induced (open symbols, dotted lines) elongation of 1-cm segments of etiolated hypocotyls of soybean (cut 0.5 cm below the hook) compared to controls (filled symbols, solid lines). With DTNB (C), comparisons were with abraded hypocotyl segments plus 10 μM 2,4-D (open triangles). Results are means ± SD among experiments of three experiments, each involving measurements of 10 tissue segments incubated for 18 h.

oxidase activity eliminated but the activity in the presence of 1 μM 2,4-D was actually less ($P < 0.01$) than in its absence, i.e. 2,4-D appeared to inhibit rather than to stimulate NADH oxidation in the presence of GSH or DTT. In contrast, the NADH oxidase activity in the absence of 2,4-D was largely unaffected by all concentrations of either GSH or DTT tested.

2-ME did not elicit the same effects on the NADH oxidase as did GSH or DTT. 2-ME was without effect on NADH oxidase activity in the absence of 2,4-D and did not abrogate the 2,4-D or IAA responsiveness of the isolated plasma membrane vesicles at concentrations up to and including 100 μM (Table II).

Auxin-Induced Growth Inhibited by GSH and DTT

Neither GSH nor DTT inhibited growth of excised 1-cm segments of soybean hypocotyls in the absence of 2,4-D (Fig. 4). However, in the presence of 2,4-D, growth was preferentially inhibited by GSH to constitutive levels or below (Fig. 4A). With DTT (Fig. 4B), 2,4-D-stimulated growth also was preferentially inhibited, but not to the same extent as with GSH (Fig. 4A).

2-ME stimulated growth slightly over the range 0.01 to 10 mM and inhibited growth in the presence of 10 μM 2,4-D by about 10% over the same range of concentrations as for GSH (data not shown). GSSG did inhibit auxin-induced growth but at concentrations higher than were required for inhibition of auxin-induced growth by GSH.

Growth Responses Were Steady State

Experiments with each of the growth conditions shown in Figures 2 and 4 were repeated with measurements at 3, 6, 12, and 18 h to verify that growth kinetics were steady state and that steady-state rates were attained within 3 h both in the presence and absence of 2,4-D (Fig. 5).

Table II. *Inhibition by thiol protectants (0.1 mM) of the auxin (1 μM 2,4-D or 1 μM IAA) stimulation of NADH oxidase*

The number of determinations is given in parentheses. Values are means ± SD among determinations.

Addition	Post-Addition	NADH Oxidase
		nmol min^{-1} mg^{-1} protein
None	None	1.6 ± 0.15 (7)[a]
	2,4-D	2.3 ± 0.2 (4)[b]
	IAA	2.4 ± 0.2 (4)[b]
GSH	None	1.7 ± 0.3 (5)[a]
	2,4-D	1.4 ± 0.2 (4)[a]
	IAA	1.5 ± 0.1 (3)[a]
DTT	None	1.8 ± 0.3 (5)[a]
	2,4-D	1.4 ± 0.15 (3)[a]
	IAA	1.3 ± 0.1 (3)[a]
2-ME	None	1.6 ± 0.3 (7)[a]
	2,4-D	2.1 ± 0.4 (6)[a]
	IAA	2.0 ± 0.4 (3)[a]

[a,b] Values not followed by the same letter were statistically significant ($P < 0.05$).

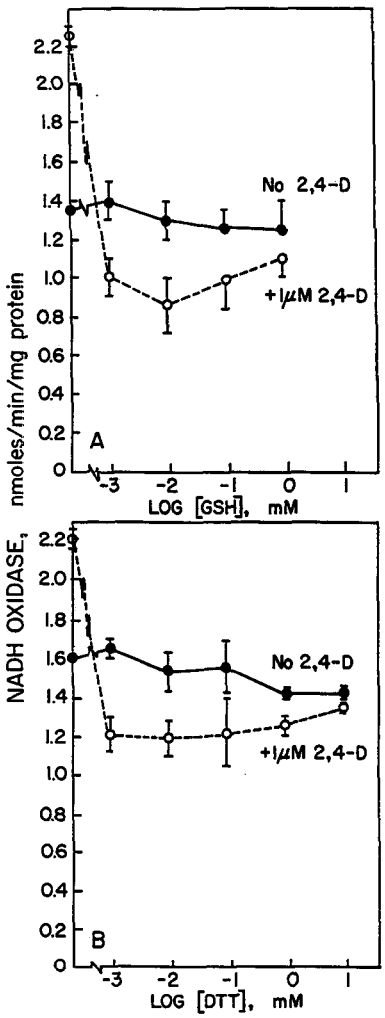

Figure 3. NADH oxidase activity of isolated vesicles of plasma membrane from soybean in response to GSH (A) and DTT (B). The rates shown are steady-state rates following a 5-min pretreatment with the concentrations of GSH and DTT shown with subsequent assay at the same concentrations of GSH and DTT as the pretreatment. Results are means ± SD among experiments of three experiments, with duplicate determinations within each experiment. With membranes pretreated for 5 min with the GSH concentrations shown, the subsequent response to 2,4-D was inhibited.

Preincubation with Auxin Delays Inhibition by Thiol Reagents

Inhibition of NADH oxidase activity by thiol reagents was influenced by the order of reagent addition (Table III). If membranes were first preincubated for 10 min with or without 2,4-D prior to addition of the thiol reagent, 2,4-D stimulation was retained for at least 10 min as if active site thiols were being protected by the 2,4-D. These experiments were conducted together with controls that paralleled the treatments listed in Tables I and II to demonstrate that the thiol reagents did block the stimulation by 2,4-D when the order of addition was reversed. Over 15 min after NEM addition, the apparent protective effects were lost gradually in the presence of 2,4-D (Fig. 6) and were no

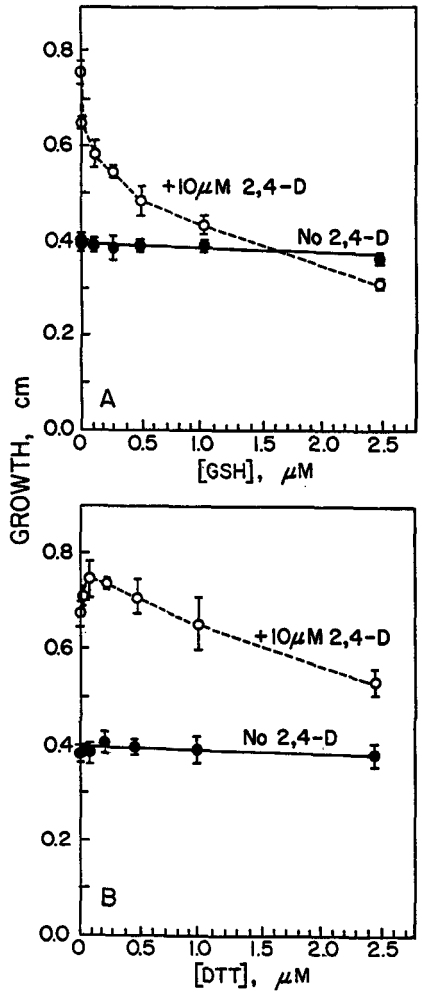

Figure 4. GSH (A) and DTT (B) inhibit auxin (10 μM 2,4-D)-induced but not control (no 2,4-D) elongation of 1-cm segments of etiolated hypocotyls of soybean (cut 0.5 cm below the hook). Results are means ± SD among experiments of three experiments, each involving measurements of 10 tissue segments incubated for 18 h.

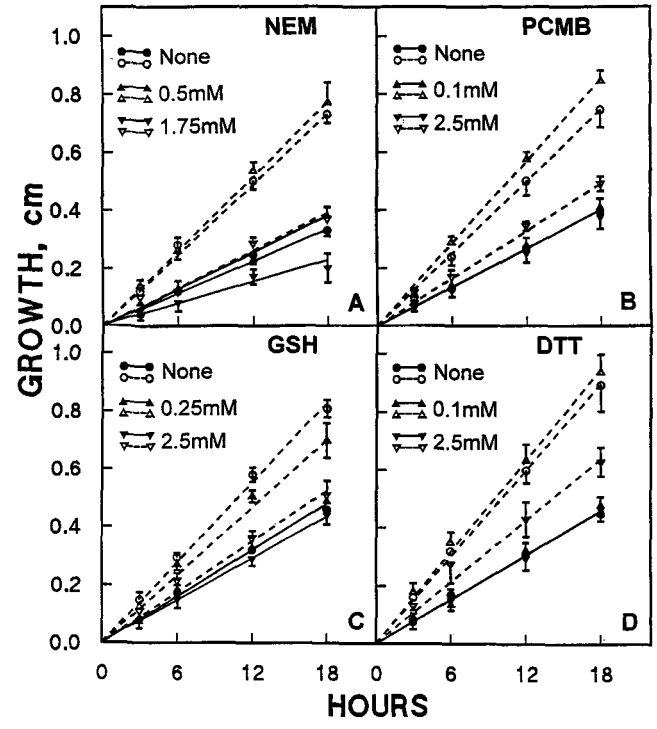

Figure 5. Growth kinetics of 1-cm segments of etiolated hypocotyl of soybean at selected concentrations of NEM (A), PCMB (B), GSH (C), and DTT (D) in the presence (open symbols, dotted lines) or absence (filled symbols, solid lines) of 10 μM 2,4-D. Within the error of the determinations, growth was steady state over the observation period. Results are means ± SD among experiments of three experiments, each involving measurements of 10 tissue segments.

longer evident after 10 min of incubation with 10 μM NEM (Fig. 6B). Protection by 2,4-D decreased with increasing concentrations of NEM (Fig. 6B). The NADH oxidase activity in the absence of 2,4-D was largely unaffected by even prolonged treatment with NEM at concentrations of 10 μM or less (Fig. 6A).

DISCUSSION

Studies of the effects of thiol reagents on the auxin-responsive NADH oxidase of soybean plasma membranes have revealed several characteristics of the activity and its regulation by auxin. The results showed that the auxin-responsive component of the oxidase was distinct in its response to thiol reagents from the basal or constitutive activity. The constitutive NADH oxidase activity was resistant to thiol reagents and was not inhibited by NEM, PCMB, DTNB, DTT, or GSH. The 2,4-D-stimulated component of the NADH oxidase, on the other hand, was strongly inhibited by these reagents and reduced or eliminated by concentrations that had little or no effect on the constitutive activity. Thus, it appeared that interference with thiol interchange, prior to or during 2,4-D treatment, either by reaction of free thiol groups of the plasma membrane with NEM, PCMB, or DTNB or by the presence of a redox buffer strongly favoring thiol formation such as DTT or GSH,

Table III. *Protection by pretreatment with 2,4-D from the action of NEM, PCMB, DTNB, GSH, and DTT in blocking of the auxin (2,4-D or IAA) stimulation of NADH oxidase of plasma membranes of etiolated soybean hypocotyl segments*

2-ME also was tested. Each entry is the average of three determinations. Values are means ± SD among determinations.

Addition	Post-Addition	NADH Oxidase
		nmol min^{-1} mg^{-1} protein
None	None	1.15 ± 0.2[a]
0.1 μM 2,4-D	None	1.35 ± 0.15[a]
0.1 μM 2,4-D	0.1 mM NEM	1.5 ± 0.2[a]
1 μM 2,4-D	None	1.95 ± 0.2[b]
1 μM 2,4-D	0.1 mM NEM	2.0 ± 0.15[b]
1 μM 2,4-D	1 μM PCMB	1.9 ± 0.23[b]
1 μM 2,4-D	10 nM DTNB	2.0 ± 0.2[b]
1 μM 2,4-D	0.1 mM GSH	2.1 ± 0.2[b]
1 μM 2,4-D	0.1 mM DTT	2.1 ± 0.3[b]
1 μM 2,4-D	0.1 mM 2-ME	2.2 ± 0.1[b]

[a,b] Values not followed by the same letter were statistically significant ($P < 0.05$).

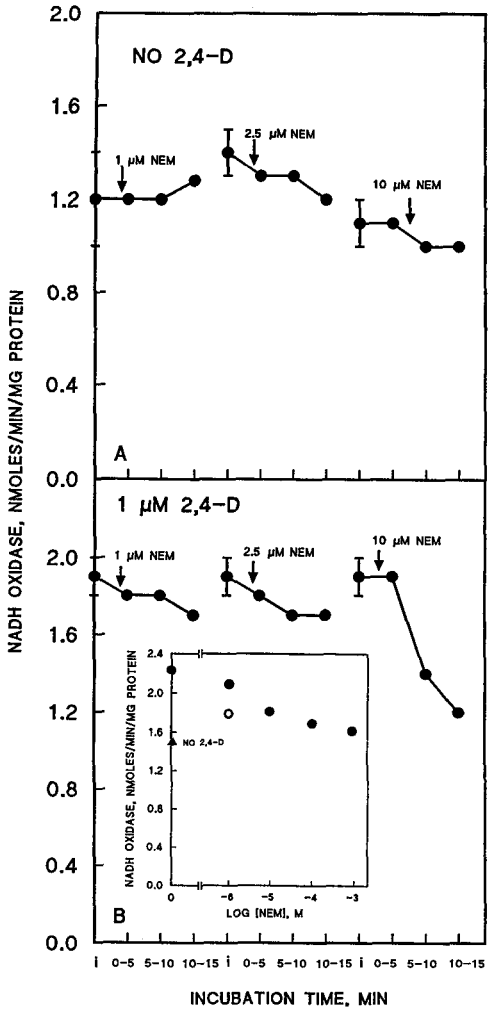

Figure 6. Inhibition by NEM of NADH oxidase activity of soybean plasma membranes as a function of time of incubation and concentration. A, NADH oxidase activity in the absence of 2,4-D. i, Initial steady-state rate after NADH addition. NEM was added as indicated by the arrows and the rate was measured over three successive 5-min intervals. B, As in A except with 1 μM 2,4-D present during the initial incubation with NADH (i). NEM was then added as indicated by the arrows and the rate was again determined over three successive 5-min intervals. Inset, Rate of NADH oxidation in the presence of 1 μM 2,4-D between 5 and 10 min of NEM addition (filled symbols) as a function of NEM concentration. The open symbol is the rate for 1 μM NEM between 10 and 15 min of NEM addition. The 1-μM concentration of NEM had already inhibited 2,4-D-induced NADH oxidase activity after 10 min. The rate without 2,4-D is indicated by the solid triangle.

reduced or prevented the ability of 2,4-D to stimulate the NADH oxidase.

A similar response was seen in the growth of excised stem segments. The 2,4-D-induced growth of soybean hypocotyl segments was much more sensitive to inhibition by DTNB, DTT, PCMB, and GSH than was the basal elongation. With PCMB, for example, basal elongation was poorly inhibited at all PCMB concentrations, whereas auxin-induced elongation was nearly completely inhibited by millimolar concentrations of PCMB. Also inhibitory to 2,4-D-induced elongation of soybean hypocotyls were both GSH and DTT, whereas neither of these compounds elicited a significant response with basal elongation. Only with NEM was the constitutive or basal growth strongly inhibited and only then at very high concentrations. Thus, not only do thiol groups appear to be involved in the response of the plasma membrane NADH oxidase to 2,4-D, but thiol modifications also appear to influence the response of the membranes to 2,4-D. The latter is suggested by the lack of a 2,4-D response after treatment of the membranes with DTT or GSH.

One possible interpretation of the results is that a disulfide bond is required for 2,4-D stimulation of the NADH oxidase activity and that any treatment that prevents disulfide formation interferes with the auxin stimulation. Such a mechanism would be consistent both with the results of Spring et al. (1988) as well as with a mechanism of auxin stimulation of the NADH oxidase based on thiol-disulfide interchange. With the latter, the thiol intermediate of the exchange would be trapped by reaction with NEM or PCMB and the overall activity would be inhibited. At least with NEM there appeared to be an apparent competition between NEM and 2,4-D. This was indicated from the experiments where the membranes were incubated first with 2,4-D and then the sulfhydryl reagents were added. With 2,4-D preincubation, the 2,4-D stimulations were retained for 5 min or longer depending on the sulfhydryl reagent and concentration. However, when the order was reversed and the sulfhydryl reagents were administered first, the 2,4-D stimulations were prevented.

A relationship between auxins and thiols was noted early by Key and Wold (1961) for intact soybean seedlings. They reported that auxins increased the tissue concentration of ascorbic acid, soluble thiols, and protein thiols. Subsequently, Pilet and Dubois (1968) reported a relationship between thiol content and growth rate in vitro. A direct correlation between auxin-induced growth and thiols was demonstrated subsequently for excised hypocotyl segments of soybean using thiol titration methods with silver salts (Morré, 1970). Since these early studies, the role(s) of thiol groups in the plant growth process have received little additional attention, except in the report by Spring et al. (1988), where NEM was suggested to inhibit growth by reacting with thiol groups unmasked by auxin treatment. Reported possibilities for involvements of thiols in the action of mammalian hormones at the plasma membrane include interactions with insulin (Maturo et al., 1983), muscarinic (Aronstam et al., 1978), dopamine (Suen et al., 1980; Sidhu et al., 1986), opiate (Larsen et al., 1981), β-adrenergic (Bottari et al., 1979), vasopressin (Favo and Farenholz, 1990), leukotirene B4 (Falcone and Aharony, 1990), and peptide (El Battari et al., 1988) receptors.

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