Effect of Ethylene Treatment on Polar IAA Transport, Net IAA Uptake and Specific Binding of N-1-Naphthylphthalamic Acid in Tissues and Microsomes Isolated from Etiolated Pea Epicotyls

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ABSTRACT

The effect of ethylene treatment on polar indole-3-acetic acid (IAA) transport, net IAA uptake in the presence and absence of N-1-naphthylphthalamic acid (NPA) and [3H]NPA binding characteristics was investigated in tissue segments or microsomes isolated from etiolated pea (Pisum sativum L. cv Alaska) epicotyls. Basipetal IAA transport in 5 millimeter segments isolated from ethylene-treated seedlings was inhibited by ethylene in a dose-dependent manner. Threshold, half-maximal and saturating concentrations of ethylene were 0.01, 0.55, 10.0 microliters per liter, respectively. This inhibition became apparent after 6 to 8 hours of ethylene treatment. Transport velocity in both control and ethylene-treated tissues was estimated to be 5 millimeters per hour. Net IAA uptake was stimulated in ethylene-treated tissues and the relative affinity of the phytoptropin NPA to enhance net IAA uptake was reduced in treated tissues. Specific binding of [3H]NPA to microsomes prepared from both control and ethylene-treated tissues was saturable and consistent with the existence of a single class of binding sites with an apparent affinity (Kd) toward NPA of 8 to 9 nanomolar. The density of these binding sites (per milligram protein) was lower (36% of control) in ethylene-treated tissues. Direct application of ethylene to microsomal preparations isolated from untreated seedlings had no effect on the level of specific [3H]NPA binding.

The physiological interactions between endogenous plant growth substances are important determinants in the regulation of plant development. In particular, the reciprocal interactions between endogenous auxins (notably IAA) and ethylene have been extensively documented. Physiologically, interactions between these two classes of growth substances can have a profound effect on plant development. In the case of leaf abscission, the physiological balance between auxins and ethylene serves to regulate both the timing and extent of leaf drop (8, 15). It is generally accepted that the initiation of leaf abscission is preceded by a decline in leaf-derived auxins in the abscission zone (1, 8, 15). This decline can occur as a result of the disruption of basipetal auxin transport in the petioles (3, 30). Conditions known to stimulate leaf abscission such as ethylene fumigation, advanced leaf senescence, water stress and defoliating treatment have been shown to result in impaired basipetal auxin transport (3, 5, 25, 30).

The ability of ethylene to modify seedling growth and alter the normal distribution of endogenous auxins was noted by Van der Laan (28). Subsequent studies using [14C]IAA demonstrated that ethylene treatment of intact cotton plants and etiolated pea seedlings resulted in the inhibition of basipetal IAA transport (4, 16). In cotton tissues, this inhibition could not be explained on the basis of enhanced metabolism of the applied auxin (2). To date, the biochemical bases for this ethylene effect remain unknown. The net basipetal movement of auxins through tissue segments is thought to occur as a result of the asymmetric localization of active auxin efflux porters at the basal end of cells in the transporting tissues (7, 9, 20). Synthetic compounds such as NPA,2 morphactins, and TIBA disrupt polar auxin transport by binding to and inhibiting the action of the efflux porters (6, 24, 27). The availability of high specific activity [3H]NPA allows for the determination of molecular characteristics of this auxin efflux system. In this report, the effects of ethylene treatment on IAA transport and NPA binding characteristics in etiolated pea epicotyls are described. This tissue was selected for study because it exhibits ethylene-induced inhibition of auxin transport and it is a relatively rich source of NPA binding sites (4, 9). Portions of this research have been presented in abstract form (26).

MATERIALS AND METHODS

Plant Material and Experimental Procedure. Pea (Pisum sativum L. cv Alaska; Sunseeds, Twin Falls, ID, lot No. 40-019-020) seeds were surface-sterilized using a 1:4 (v/v) dilution of commercial bleach (5.3% Na hypochlorite by weight) and were sown in flats containing vermiculite and watered with tap water. After 5 d of growth in the dark (27 ± 1°C), seedlings were transplanted to treatment chambers. All manipulations, prior to harvest, were conducted under dim green light. Unless otherwise noted, intact seedlings were exposed to ethylene using plexiglass chambers and were maintained in the dark. Tissue segments or microsomes were then isolated from the treated seedlings. All experiments described in this paper were conducted a minimum of three times. Where possible, individual experiments were replicated. Data from typical experiments are presented.

Auxin Transport Studies. The basipetal movement of [14C]IAA was determined using previously described methods (25). Internodal segments (5 mm) were excised 5 to 7 mm below the

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1 Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that also may be suitable.

2 Abbreviations: NPA, N-1-Naphthylphthalamic acid; FCCP, carbonyl-cyanide-p-trifluoromethoxyphenylhydrazide; TIBA, 2,3,5-triiodobenzoic acid.
plumular hook and, with the exception of the velocity study, transport was allowed to proceed in the dark (27 ± 1°C) for 2.5 h. Dose-response studies were conducted using seedlings that had been enclosed for 18 to 20 h. For the time-course studies, both control and treated seedlings were enclosed for the indicated period. Velocity studies were conducted after 17 h of enclosure ± ethylene. In all cases, a mercuric perchlorate trap was included in control chambers to absorb endogenously produced ethylene. All ethylene treatment levels were verified by GC at the end of the enclosure period.

**IAA Uptake Studies.** Seedlings were enclosed for 23 ± 45 μL/L ethylene. Thin (2-3 mm) cross-sectional segments were isolated (freehand) from the upper 2 cm of subapical tissue (avoiding nodal tissues). Epicytol segments (35-40) were placed in a 25 mL flask containing 3 mL of uptake solution (50 mM Mes/KOH [pH 5.7], 5 μM IAA containing 0.3 μCi [14C]IAA, plus NPA at the indicated level). Uptake was allowed to proceed for 60 min in an oscillating water-bath shaker (27 ± 1°C). Following the uptake period, segments were removed, washed successively with 10 mL of glass-distilled water, and twice with 10 mL of 100 μM unlabeled IAA in Mes buffer. The washed segments were blotted dry, frozen and the radioactivity taken up was determined following sample combustion using a Packard tissue oxidizer.

**Binding Studies.** For the initial studies, seedlings were enclosed for 18 h and exposed to <0.01 (control), 0.31 (ethylene-low) or 27 (ethylene-high) μL/L of ethylene. For the equilibrium saturation studies, seedlings were enclosed for 18 h ± 45 μL/L ethylene. Studies examining the in vitro effectiveness of various agents were conducted using microsomes prepared from untreated tissues. In this case, the assay buffer was amended such that it contained 10 μM of the displacing compound (flurenon, TIBA) or the entire assay tube was sealed with a serum cap and ethylene gas added to the indicated level. In all cases, the upper 2 to 3 cm of epicotyl tissue (-plumule) were excised. Microsomes were prepared using the media and procedures of Ray et al. (19) with the following modifications. The initial homogenization buffer to tissue ratio was 5:1 (v/w) and both ultracentrifugation steps were extended to 90 min (100,000g). Following the second spin, the resultant pellet was suspended in assay buffer (10 mM Na citrate, 5 mM MgCl2 [pH 5.0], 0.25 mM sucrose) such that 1 mL contained the membranes from 300 to 600 mg fresh weight of tissue. Binding studies were conducted on ice and were allowed to proceed for 60 min. One mL of the microsomal preparation (in assay buffer) was added to 2 mL of assay buffer containing 0.06 to 0.08 μCi [3H]NPA, 2 to 200 pmol unlabeled NPA (added in 10 μL DMSO), and 1 μM FCCP. The protein content of the microsomal preparations was determined using the biocinchonic acid reagent (23). Nonspecific binding was defined using 10 μM unlabeled NPA. Separation of free from bound ligand was accomplished by filtration through Whatman GF/B glass-fiber filters using a multiport filtration apparatus (Millipore). After the 60 min assay period, the membranes were diluted by adding 5 mL of cold resuspension buffer, poured onto the filtration apparatus and washed with two additional 5 mL aliquots of cold resuspension buffer. The entire procedure (from dilution to dried filter) required less than 20 s; any preparations requiring additional filtration times were not included in the subsequent computer analysis. Analysis of the binding data was performed according to the method of Scatchard (21) using an unweighted, nonlinear curve fitting technique originally developed by Munson and Rodbard (18) as modified for the IBM-PC by McPherson (14).

**Chemicals.** 3-Indoyl[1-14C]acetic acid (59 mCi/mmol) was obtained from Amersham (Arlington Heights, IL) [2,3,4,5-3H]Naphthylphthalamic acid (55 Ci/mmol) was obtained from Research Products International Corp. (Mount Prospect, IL) and had a radiochemical purity in excess of 96%. NPA was purchased from Pfaltz and Bauer Inc. (Waterbury, CT) and was purified by recrystallization prior to use. All other chemicals used were of the highest grade commercially available.

**RESULTS**

The inhibition of polar auxin transport by ethylene fumigation has been examined previously using several plant species and excised tissues (2–4, 16, 17, 25). The use of various species precludes the development of a unified picture of this aspect of ethylene action in a given tissue. For this reason and because our plant material and growing conditions differed from earlier studies, a detailed examination of this ethylene effect was conducted using 5-d-old etiolated peas. Direct application of ethylene to the isolated epicotyl segments during the transport period (2.5 h) had no effect on IAA movement. Therefore, unless otherwise noted, all ethylene treatments were applied to intact seedlings while auxin transport was measured in 5 mm segments isolated 5 to 7 mm below the seedling hook.

Exposure of pea seedlings to ethylene for 18 to 20 h resulted in a dose-dependent inhibition of IAA transport (Fig. 1). This inhibition was first observed at an ethylene concentration of 10 mL/L, reached half-maximal at 55 mL/L, and was saturated at 1 to 10 μL/L. Using a saturating concentration of ethylene (25 μL/L), the inhibition of IAA transport was detected after 8 h of fumigation (JC Suttle, unpublished data). After 24 h of fumigation, IAA transport in segments isolated from treated seedlings was inhibited by over 95%.

The amount of IAA transport during an assay is a function of both the capacity and velocity of the transport system. Using the intercept method of Van der Weij (29), the velocity of IAA movement was determined in both control and ethylene-treated tissues. Using this method, transport in both tissues was linear for at least 4 h and exhibited an apparent velocity of 5 mm/h (Fig. 2). Thus, the reduction of IAA movement in ethylene-treated tissues is the result of reduced transport capacity rather than reduced velocity.

**FIG. 1.** Effect of increasing concentrations of ethylene on basipetal transport of [14C]IAA through 5 mm segments isolated from etiolated pea epicotyls. Intact seedlings were treated for 18 to 20 h in the dark. Note: ppm = μL/L. Bars indicate SE (n = 6).
As described in the introduction, the net basipetal movement of auxins in tissues is thought to occur as a result of the action of auxin efflux proteins preferentially located (or active) in the plasmalemma at the basal ends of cells in the transporting tissues (for a complete discussion, see 7, 10, 20). Thus, the reduction in auxin transport capacity in ethylene treated tissues could be the result of a decrease in the concentration or activity of these efflux proteins. NPA and other synthetic compounds have been shown to bind to and thereby inhibit the action of this efflux protein resulting in a stimulation of net IAA uptake in treated tissues (24). Recently, we introduced a method for assaying the in vivo activity of these porters using the ability of the phyto tropin NPA to stimulate net IAA uptake. Furthermore, a dose-response study can be used to determine if any treatment (in this case ethylene) resulted in an altered affinity of the efflux porters toward NPA. Exposure of untreated segments to NPA resulted in a dose-dependent stimulation of net IAA uptake (Fig. 3). The threshold concentration of NPA was between 10 and 100 nM and the half-maximal stimulatory concentration was just greater than 100 nM. In segments isolated from ethylene fumigated seedlings, NPA-stimulated net IAA uptake was less pronounced but still observable. Net IAA uptake in these segments in the absence of NPA was nearly twice that of untreated segments, suggesting that a functional loss of auxin efflux porters had resulted from ethylene treatment. Although the NPA effect was less pronounced in ethylene-treated segments, the threshold and half-maximal NPA concentrations were unaltered.

NPA binding sites can readily be detected in microsomes prepared from etiolated pea seedlings (9). The effect of ethylene treatment on NPA binding to pea microsomes was examined next. Specific [3H]NPA binding to microsomal preparations from both control and ethylene-treated epicotyls was linear with respect to protein content in the range of 100 to 600 μg membrane protein per assay (JC Suttle, unpublished data). Therefore in the binding assays described herein, membrane protein content was adjusted so as to fall within this range.

Exposure of pea seedlings to subsaturating concentrations of ethylene (0.3 μL/L) resulted in an 83% inhibition of polar auxin transport in isolated segments and also in a 37% inhibition of specific [3H]NPA binding to isolated microsomes (Table I). Using a saturating concentration of ethylene (27 μL/L) the inhibition of auxin transport was even greater (6% control) and a further decrease (41% of control) in specific [3H]NPA binding was observed. Even at this high treatment level, specific [3H]NPA binding was still apparent and, during the course of over 20 independent determinations, did not decline to zero (JC Suttle, unpublished data).

The observed reduction in specific binding could have resulted from either a decrease in affinity toward NPA (i.e. an increased Kd value) or from a decline in total binding sites (Bmax) or both. In order to differentiate between these possibilities, equilibrium binding studies were conducted using a range of NPA concentrations (2–200 pmoles/assay). The resulting data were fitted using an iterative nonlinear least squares procedure (18). Data from a

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**Table I. Effect of Ethylene Treatment on Basipetal Auxin Transport and Specific Binding of [3H]NPA in Etiolated Pea Tissues**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>IAA Transport</th>
<th>Specific [3H]NPA Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1091 ± 146*</td>
<td>439 ± 3</td>
<td>*</td>
</tr>
<tr>
<td>ETH-LOW</td>
<td>190 ± 19</td>
<td>275 ± 8</td>
<td></td>
</tr>
<tr>
<td>ETH-HIGH</td>
<td>66 ± 11</td>
<td>179 ± 9</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± se (n = 6 or 3; transport or binding, respectively).
typical effect of ethylene-treated (26 to NPA text).

The result is an integrated and internally consistent picture of the molecular events that underlie ethylene action in this system. In general, the characteristics of ethylene-induced inhibition of polar auxin transport described herein resemble those previously reported by Morgan and colleagues using light-grown cotton seedlings (2, 3, 16, 17) and Burg and Burg (4) who used etiolated peas of slightly greater age than those used here. In light-grown cotton seedlings, concentrations of ethylene required for threshold, half-maximal and saturating effects were 0.1, 1.0, and 10 μL/L, respectively (3). The etiolated peas used in this study were somewhat more sensitive to ethylene, requiring 0.01, 0.06, and 1 to 10 μL/L for threshold, half-maximal, and saturation of the inhibitory response (Fig. 1). Both cotton (2) and peas (4) exhibited a reduction in auxin transport after 3 h of ethylene fumigation. Under the conditions used here, a clear-cut inhibition was observed only after 8 h of ethylene fumigation (JC Suttle, unpublished data). In either case, the response to ethylene is relatively slow. As reported before (2–4), the inhibition of auxin transport by ethylene is a reflection of a decline in transport capacity not velocity (Fig. 2). Using the chemiosmotic hypothesis for IAA uptake and transport as a working paradigm, the reduction in transport capacity can be interpreted as reflecting a decrease in the number of active IAA efflux porters. Others (24, 27) have shown that phytotropins such as NPA bind to and thereby inhibit the action of the IAA efflux system. Using NPA as a probe, the effect of ethylene treatment on this IAA efflux system has been assessed. Following ethylene treatment, there is a decline in the relative ability of NPA to stimulate net IAA uptake without a measurable change in the sensitivity of the treated tissue toward exogenous NPA (Fig. 3). This observation is consistent with the above-mentioned hypothesis concerning ethylene action.

Direct measurement of [3H]NPA binding characteristics in both control and ethylene-treated tissues confirms this interpre-

typical run are presented in Figure 4. Specific binding of [3H] NPA to microsomes prepared from control epicotyls was saturable and, when transformed according to the method of Scatchard (Fig. 4, upper panel), was consistent with the existence of a single class of binding sites (i.e., a linear Scatchard plot). Using the method of Scatchard, a $K_d$ of 8.7 nM and a binding site density ($B_{max}$) of 1.4 nmol/mg membrane protein were calculated. Specific binding of [3H]NPA to microsomes prepared from ethylene-treated epicotyls was also found to be saturable and consistent with a single class of binding sites (Fig. 4, lower panel). Again using Scatchard analysis, a $K_d$ of 8.1 nM and a binding site density of 0.6 nmol/mg membrane protein were determined. These results were highly reproducible. When the results of five independent experiments were combined (Table II), ethylene treatment was found to have no effect on the $K_d$ value but resulted in a 66% decline in the apparent density of NPA binding sites. Thus, the reduction in specific NPA binding observed in ethylene-treated tissues was entirely due to a decline in binding site density.

When microsomes prepared from untreated seedlings were exposed in vitro to various concentrations of ethylene during the binding assay, no effect of ethylene was observed (Table III). Inclusion of unlabeled NPA, TIBA, or the morphactin Flurenol resulted in a dramatic inhibition of [3H]NPA binding.

**DISCUSSION**

The failure to define the physiological relevance of in vitro binding studies is a recurrent criticism of this type of investigation. For this reason, the studies described here have addressed the mechanism of ethylene-induced inhibition of IAA transport using a number of complementary experimental approaches.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$K_d$</th>
<th>$B_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.0 ± 0.4$^a$</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Ethylene</td>
<td>8.2 ± 0.5</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

$^a$ Mean ± se, (n = 5).

Table II. Effect of Ethylene Treatment on NPA Binding Characteristics in Etiolated Pea Epicotyl Microsomes: Summary of Data

A compilation of data from five separate experiments examining the effect of ethylene fumigation on NPA binding to microsomes isolated from 5-d-old etiolated pea epicotyls. Experimental details are given in the text.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>[3H]NPA Bound (fmol/assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>681 ± 10$^a$</td>
</tr>
<tr>
<td>CH$_4$</td>
<td>2 μL/L</td>
<td>635 ± 1</td>
</tr>
<tr>
<td></td>
<td>15 μL/L</td>
<td>651 ± 9</td>
</tr>
<tr>
<td></td>
<td>100 μL/L</td>
<td>653 ± 8</td>
</tr>
<tr>
<td>TIBA</td>
<td>10 μM</td>
<td>354 ± 3</td>
</tr>
<tr>
<td>Flurenol</td>
<td>10 μM</td>
<td>162 ± 3</td>
</tr>
<tr>
<td>NPA</td>
<td>10 μM</td>
<td>141 ± 6</td>
</tr>
</tbody>
</table>

$^a$ Mean ± se, (n = 3).

Table III. In Vitro Effectiveness of Ethylene, TIBA, or Flurenol on Binding of [3H]NPA to Microsomes Isolated from Etiolated Pea Epicotyls

Microsomes were isolated from untreated, etiolated pea epicotyls and were directly exposed to the test compounds during the 1 h binding assay. Details are given in the text.
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