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Sites of Auxin Action

REGULATION OF GEOTROPISM, GROWTH, AND ETHYLENE PRODUCTION BY INHIBITORS OF AUXIN TRANSPORT

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ABSTRACT

The inhibitors of auxin transport—NPA (N-1-naphthylphthalamic acid), DPX1840 (3,3a-dihydro-2-(p-methoxyphenyl)-8*H*-pyrazolo[5,1-*a*]isoindol-8-one), and TIBA (2,3,5-triiodobenzoic acid)—inhibited geotropism in roots of intact *Pisum sativum* L. seedlings. NPA and DPX1840 also caused cellular swelling in the roots. The swelling was due to a greater inhibition of elongation than increase in weight and looked identical to the one caused by ethylene. However, ethylene did not act as an intermediate in the action of auxin transport inhibitors because all three failed to stimulate ethylene production and some of their growth-inhibiting effect was retained in the presence of saturating levels of ethylene. In the presence of 10 μM indoleacetic acid the growth-inhibiting effect of auxin transport inhibitors was lost after 18 hours. On the other hand, auxin transport inhibitors did not interfere with the ability of auxin to promote ethylene production. Growth inhibition caused by auxin transport inhibitors was reversible. Pea root sections resumed normal growth following flushing of treated sections with inhibitor-free solutions. Experiments with ^{14}C-2,4-dichlorophenoxyacetic acid revealed that the herbicide and auxin transport inhibitors may have the same binding site. It was concluded that a class of structurally dissimilar compounds may share a similar physiological role since they all appear to compete with endogenous auxin for certain binding sites and they all have similar growth-regulating activities.

NPA[2] (3, 5, 18, 23, 27, 28), DPX1840 (6, 7, 21), and TIBA (12, 16, 17, 20) have been shown to inhibit auxin transport. NPA and CFM, another auxin transport inhibitor (8, 23), were found to have a common binding site in homogenates from coleoptiles (28) and this site was separate from the one that binds auxins (13, 26, 27). TIBA was found to compete with auxin for binding sites in a suspension of crown gall cells (22), in a particulate fraction from coleoptiles (27), and pea seedling buds (14). DPX1840 also altered some responses known to involve auxin: abscission (19), apical dominance, epinasty, tropism (6), and parthenogenesis (7, 21). An earlier report (11) demonstrated that CFM inhibition of pea root geotropism could be due to the disruption of auxin transport at the level of auxin binding.

Auxin binding to specific receptor sites is thought to play an important role in auxin action (13, 16). Does auxin transport, auxin-regulated growth, and auxin-induced ethylene production involve the same auxin-binding site? We have used the auxin transport inhibitors to study this possibility in pea roots. In addition, we have studied the nature of auxin transport inhibitor binding by measuring the ability of the compounds to be flushed from root sections and to displace ^{14}C-2,4-D from root sections.

MATERIALS AND METHODS

Plant Material. Seeds of *Pisum sativum* L. var. Alaska were imbibed for 6 hr, sown in moist vermiculite, and grown in the dark at 23 C.

Studies with Intact Seedlings. Two-day-old seedlings (roots 2–3 cm long) were soaked in a solution of water of 10 μM NPA, DPX1840, or TIBA for 5 min, then placed horizontally in Petri dishes containing vermiculite moistened with the treatment solution. The experiment was then conducted in 10-liter desiccators as described elsewhere (9). Downward curvature of the root tips was measured at 1 and 4 hr.

Growth Studies and Ethylene Evolution. Ten terminal 5-mm sections from 3-day-old seedlings (roots 5–6 cm long) were placed in 125-ml Erlenmeyer flasks containing 6 ml of a pH 5.2 buffer composed of 5 mM dibasic potassium phosphate, 2 mM citric acid, 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1% sucrose (w/v), and appropriate concentrations of growth regulators or IAA. The flasks were sealed with vaccine caps and shaken gently in the dark at 23 C. After incubation the roots were blotted, weighed, and measured. Ethylene evolution was assayed by gas chromatography using a flame ionization detector (1).

Studies with ^{14}C-2,4-D and ^{14}C-CFM. Tissue sections were incubated with growth regulators as described above except that the buffer also contained ^{14}C-2,4-D (1-^{14}C-2,4-D, 3.03 mCi/mmole, Tracerlab) or ^{14}C-CFM (9-^{14}C-CFM, 10.4 mCi/mmole, Cela Merck, Ingelheim, Germany). After incubation for 16 hr, 0.1 ml of the incubation medium was counted by liquid scintillation. The solution was then filtered. The tissue was rinsed three times with 100 ml of ice water. After blotting and weighing, the sections were placed in a scintillation vial containing 2 ml of

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[2] Abbreviations: NPA: N-1-naphthylphthalamic acid; CFM: methyl-2-chloro-9-hydroxyfluorene-9-carboxylate; DPX1840: 3,3a-dihydro-2-(*p*-methoxyphenyl)-8*H*-pyrazolo[5,1-a]isoindol-8-one; TIBA: 2,3,5-triiodobenzoic acid.

NCS tissue solubilizer. The vial was shaken at 45 C at least 4 hr. The radioactivity which had accumulated in the sections was then determined by liquid scintillation counting.

The sodium salt of NPA was a gift from UniRoyal, Inc., Naugatuck, Conn. CFM was a gift from Cela Merck, Ingelheim, Germany. DPX1840 was a gift from Dr. C. W. Bingeman of E. I. du Pont de Nemours and Co., Wilmington, Del.

RESULTS

NPA, DPX1840, and TIBA rapidly inhibited the positive geotropic response in roots of intact pea seedlings (Table I). By 24 hr, the elongation zone of intact roots treated with NPA and

Table I. *Inhibition of Geotropism of Pea Roots by 10 μM NPA, DPX1840, and TIBA*

Treatment	Degrees Curvature[1]	
	1 hr	4 hr
Control	+7 ± 3.8	+26.4 ± 1.8
NPA	−5.2 ± 3.3[2]	−6.2 ± 3.7
Control	+8 ± 2.6	+31.1 ± 3.2
DPX1840	−5.7 ± 3.1	−8.9 ± 3.9
Control	+8.2 ± 1.7	+25.5 ± 1.4
TIBA	−4.3 ± 2.5	−7.4 ± 3.2

[1] Mean ± confidence limit ($P < 5\%$) from 24 seedlings per treatment.

[2] Upward curvature.

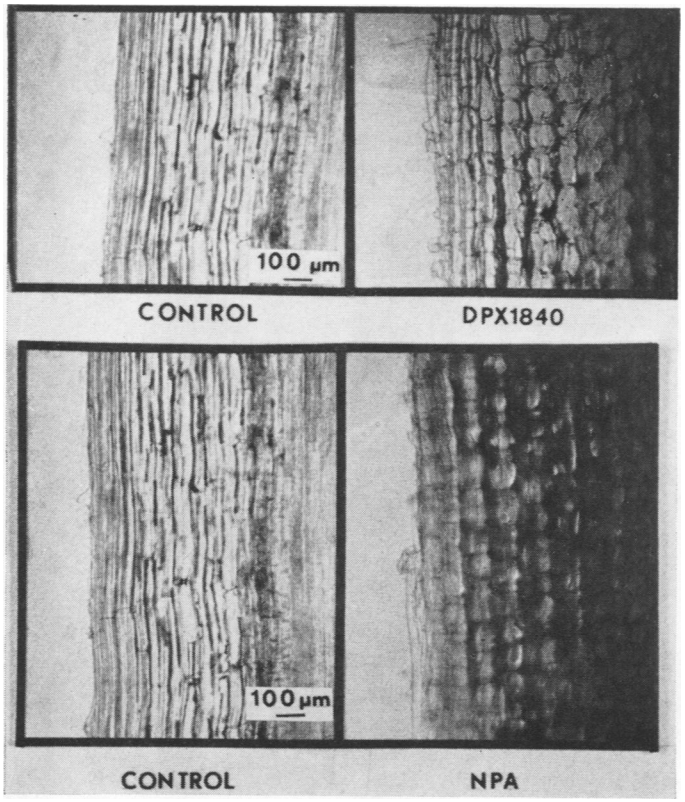

FIG. 1. Freehand longitudinal sections from the elongation zone of roots from pea seedling exposed for 24 hr to water or to 10 μM NPA or 10 μM DPX1840. Seedlings were 2 days old when initially treated.

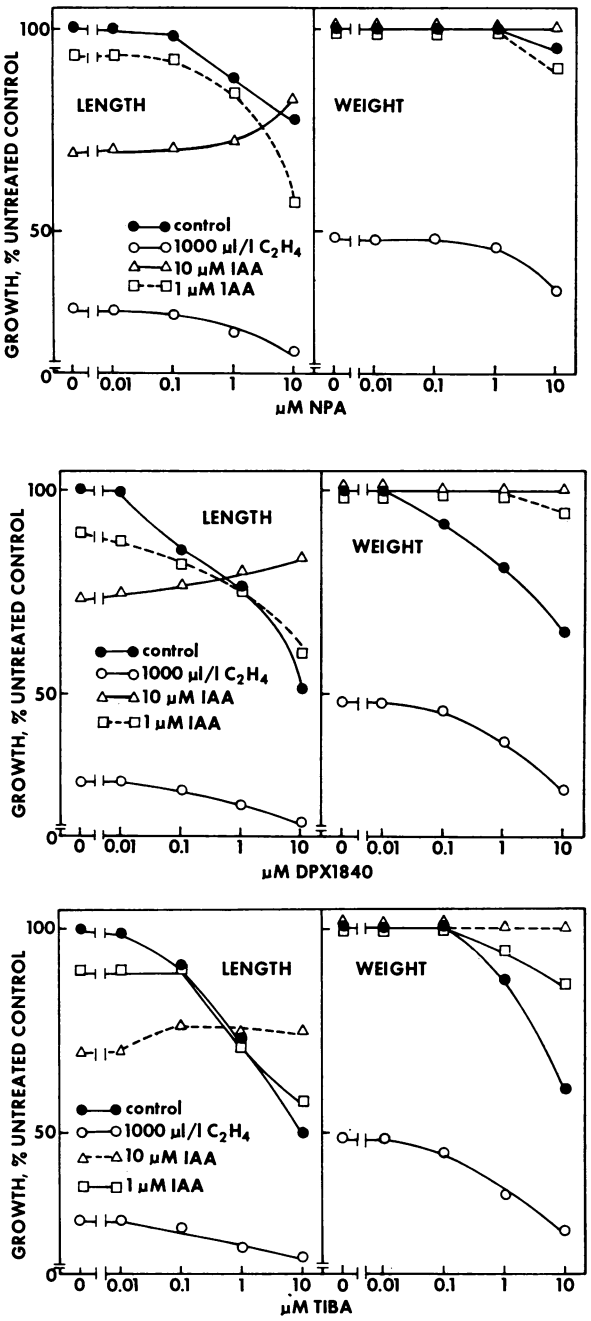

FIG. 2. Effect of IAA and ethylene on NPA-, DPX1840-, and TIBA-induced inhibition of growth in excised pea root tips. Incubation time was 24 hr. Controls grew 142% in length and 186% in weight.

DPX1840 had swollen epidermal and cortical cells (Fig. 1). Roots treated with TIBA did not show this effect.

Dose response data for the growth of excised pea root tips (Fig. 2) showed that the inhibitors had a greater effect on elongation than on the increase in fresh weight. At a concentration of 10 μM NPA and TIBA, the ratio between the increase in fresh weight and the increase in length was 1.2, and in the case of 10 μM DPX1840, 1.3.

Application of auxin to pea roots increases ethylene production and causes swelling (9, 10). Chadwick and Burg (9, 10) proposed that effect of physiological levels of auxin was due primarily to the increase in ethylene production. When NPA, DPX1840, and TIBA were added to roots exposed to a physio-

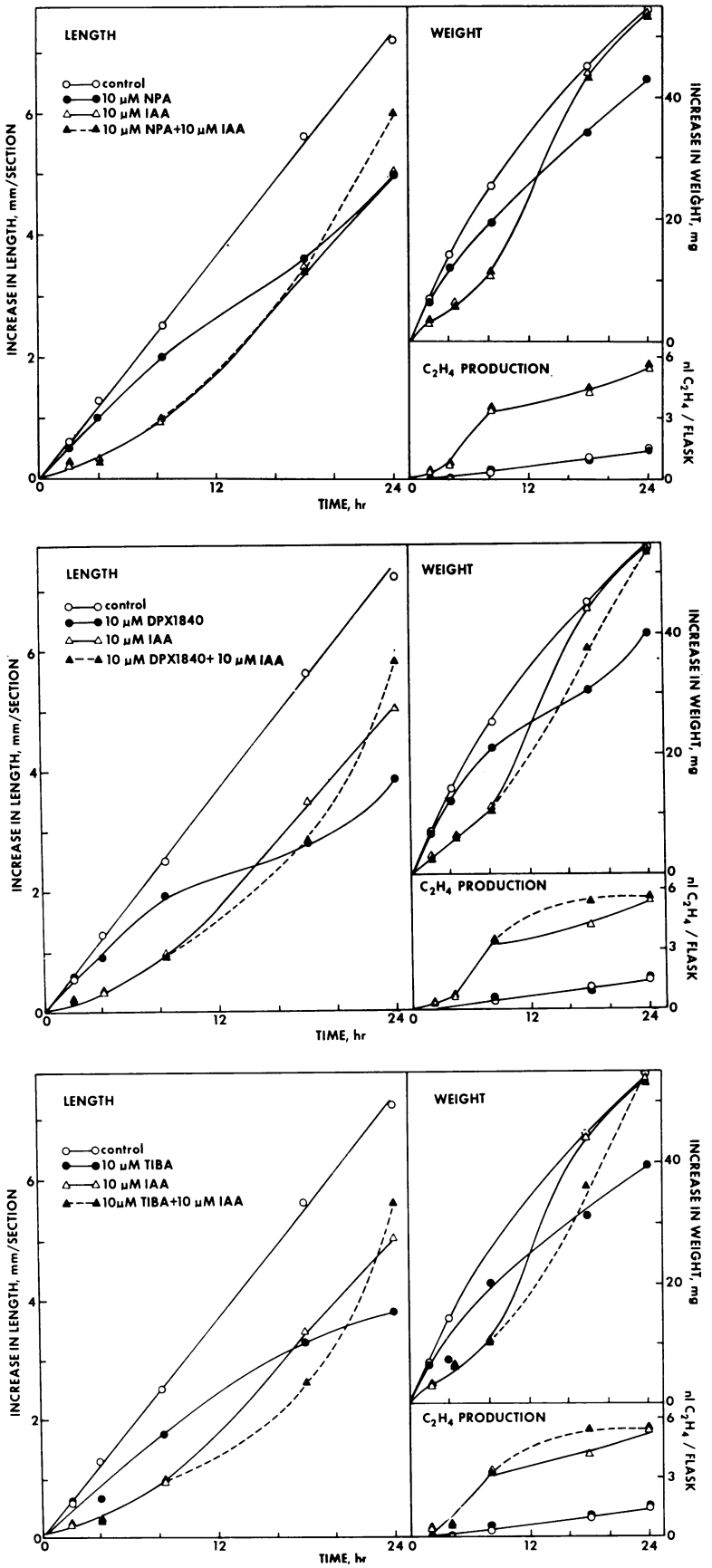

FIG. 3. Time course for growth inhibition of, and ethylene production by, excised pea root tips treated with NPA (top), DPX1840 (center), and TIBA (bottom).

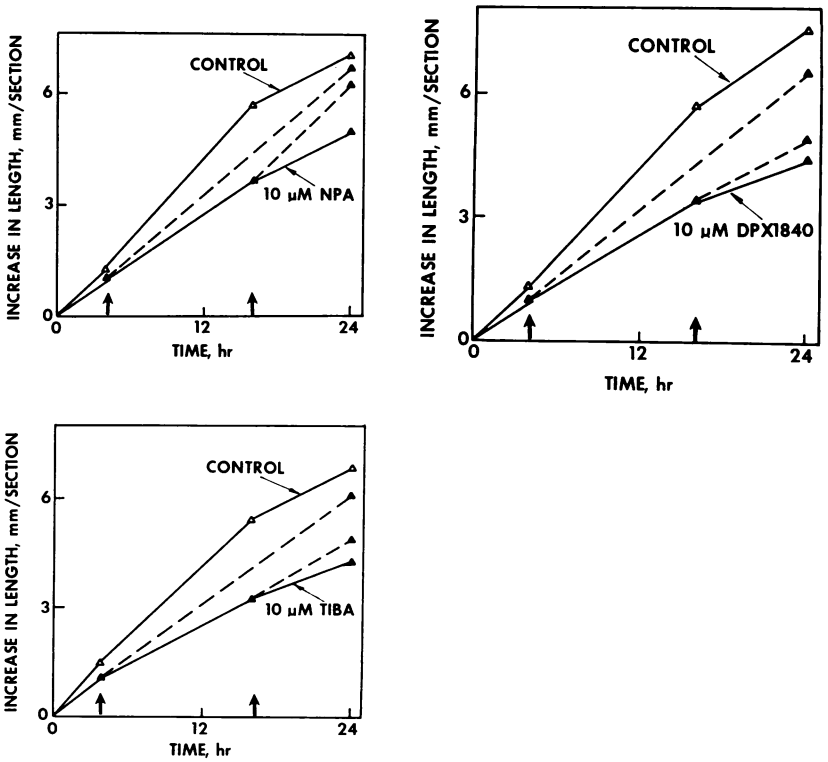

FIG. 4. Time course showing reversibility of NPA (upper left), DPX1840 (upper right), and TIBA (lower left) induced inhibition of elongation of excised pea root tips. Roots in solutions containing inhibitors were transferred to inhibitor free solutions at the times indicated by arrows.

logically saturating dose of ethylene an additional decrease in growth was observed (Fig. 2).

Addition of 1 μM IAA did not overcome the inhibition of length caused by the inhibitors but did overcome the effect on water uptake caused by DPX1840 and TIBA. An anomalous result was obtained when 10 μM IAA was added to roots exposed to the inhibitors. In all cases, auxin blocked the further inhibition of growth and in addition promoted growth as the levels of NPA, DPX1840, and TIBA increased. The effect of the inhibitors on water uptake was completely blocked in the presence of 10 μM IAA.

Time course curves showing the effect of NPA, DPX1840, and TIBA on the growth of excised pea roots are shown in Figure 3. Over a 24-hr period the increase in the length of control sections was linear while the increase in weight decreased slowly with time. As shown earlier by Chadwick and Burg (10), IAA caused an immediate inhibition of elongation followed by the resumption of a normal rate of elongation after 8 hr. Changes in weight were more complex. The initial decrease in water uptake stopped at 8 hr, the same time as the decrease in elongation. After 8 hr, water uptake in auxin-treated tissue was faster than controls until the weight of treated sections approached the weight of control sections. Subsequently, the growth of both groups of root sections remained the same. The increase in ethylene production occurred during the 8-hr period in which elongation and weight was inhibited and then returned to normal as the supply of exogenous IAA was used up.

The effect of NPA, DPX1840, and TIBA on elongation and weight increase was much the same for all compounds. Compared to IAA, the effect on growth appeared to be more gradual and prolonged. Recovery was observed in roots treated with NPA and DPX1840 but none was observed with roots treated with TIBA during the 24 hr allotted to the experiment. When inhibitors were added at the same time as auxin, the controlling factor appeared to be auxin during the first 14 to 16 hr. However, as the IAA was used up, growth increased so that the final rate of growth exceeded that of the control.

Figure 3 also shows that NPA, DPX1840, and TIBA had no effect on ethylene production themselves, though some enhancement by DPX1840 and TIBA did occur in the presence of IAA.

The effect of NPA, DPX1840, and TIBA on growth was reversible. Figure 4 shows that transferring treated root sections into inhibitor-free buffer resulted in a return to a near normal growth rate.

NPA, DPX1840, and TIBA competitively inhibited the accumulation of 2,4-D in excised pea root tips (Fig. 5). In addition, NPA also inhibited the accumulation of ^{14}C-CFM, another auxin transport inhibitor. Additional experiments with ^{14}C-CFM indicated that, unlike the case for NPA, neither TIBA (10–50 μM) nor DPX1840 (10 μM) had any effect on CFM accumulation (data not shown).

DISCUSSION

The experiments described in this paper were designed to study the nature of the attachment site of a group of auxin transport inhibitors (NPA, DPX1840, and TIBA) in roots. Roots were selected as the experimental system as a means of evaluating the applicability of current concepts concerning the mode of action of auxin transport inhibitors in a tissue which shows a negative response (growth inhibition) to auxin. Experiments were done to evaluate the effect of transport inhibitors on three processes (growth, geotropism, and ethylene production) which are under auxin control. For the purposes of this discussion it is assumed that these processes are either under the control of auxin or auxin dependent. Flushing experiments were done to characterize the nature of the attachment site in terms of its affinity for these inhibitors. The competitive replacement between 2,4-D and auxin transport inhibitors was performed as another way of characterizing the attachment site. The primary purpose of these experi-

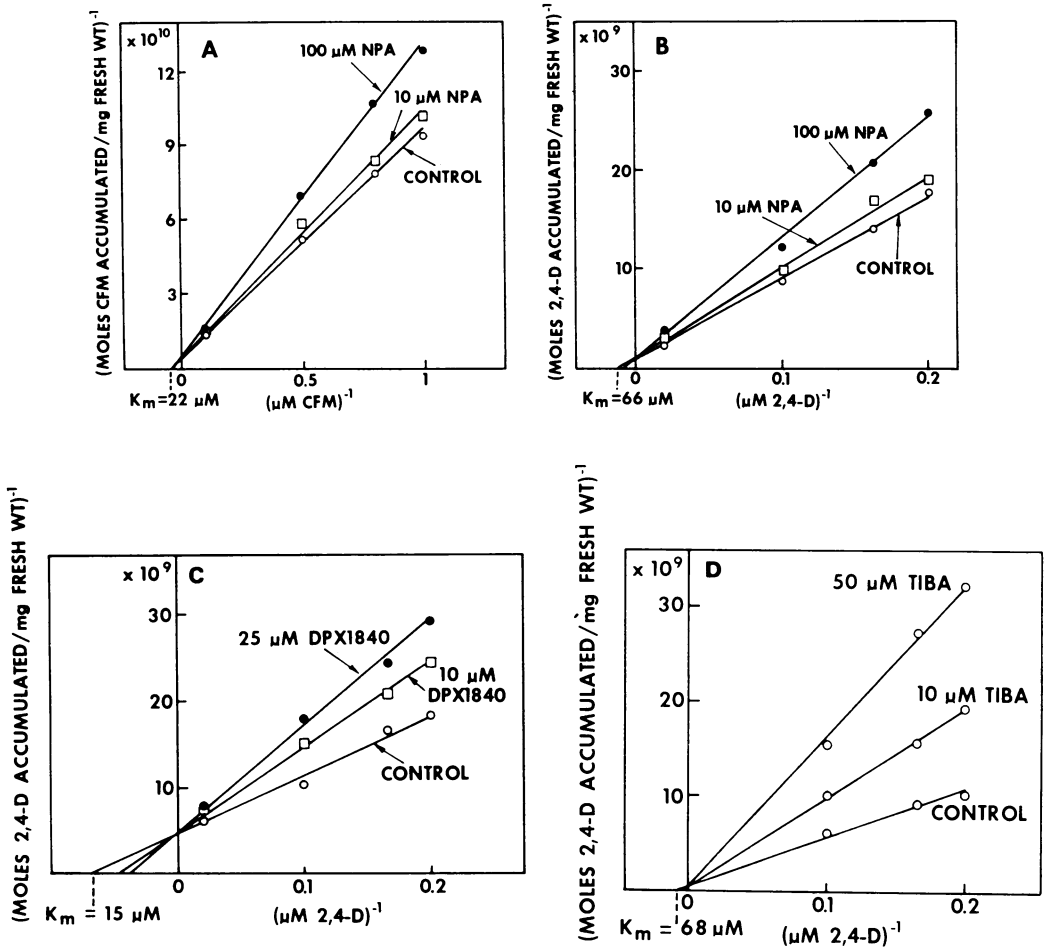

FIG. 5. Competitive inhibition of 2,4-D and CFM accumulation in excised pea root tips by NPA (A and B). DPX1840 (C), and TIBA (D). Incubation times were 4 hr for NPA and 16 hr for DPX1840 and TIBA. Given concentrations of inhibitor were used with 0.41 μM ^{14}C-2,4-D plus unlabeled 2,4-D (1–50 μM) or 0.13 μM ^{14}C-CFM plus unlabeled CFM (1–10 μM).

ments was to provide evidence that the physiological effectiveness of transport inhibitors was due to their ability to bind to an auxin attachment site.

Earlier work has shown that various plant growth regulators, including NPA, TIBA, and CFM, act as auxin transport inhibitors by competing with auxin for binding sites. This data has accumulated from work with cell suspensions (22), excised root sections (11), and particulate fractions from shoot homogenates (13–15, 26–28). Others have recently reviewed the literature in this field and concluded that the primary site of auxin action in roots is probably located at the cell wall or plasma membrane (4, 24).

Geotropism involves the sensing of a change in the orientation of a gravitational field, altered auxin transport, and the modification of growth. While the exact step of geotropism under the control of the inhibitors is not known, it is evident that they rapidly block the downward curvature of pea roots (Table I). On the assumption that the inhibitors do not block the ability of pea roots to sense the direction of a gravitational pull, the data obtained are indicative of an effect on either lateral auxin transport or the resultant asymmetrical growth.

Application of NPA and DPX1840 caused a pronounced swelling of intact pea roots while TIBA did not. Root swelling is the result of a slower rate of elongation compared to the increase in weight. This phenomenon also occurs in excised roots and is shown by the data in Figure 2. All of the inhibitors reduced the elongation of isolated pea roots to a greater extent than weight. This observation is similar to that seen by Chadwick and Burg (9, 10), when isolated roots were treated with ethylene or auxin. They showed that auxin-induced root growth inhibition was due to auxin-stimulated ethylene production. The data in Figure 2 support the idea that the reduction in growth caused by the auxin transport inhibitors was due directly to the compounds themselves and not through an effect on ethylene production. There are two facts which support this interpretation. First, the inhibitors did not promote ethylene production (Fig. 3). Second, when root sections were treated with a physiologically saturating level of ethylene, a further reduction of growth occurred when the auxin transport inhibitors were added (Fig. 2). If the mode of action of auxin transport inhibitors had been through the regulation of ethylene production, then a promotion of ethylene should have occurred and the effect of the inhibitors should have been masked by a saturating concentration of ethylene.

It is worth noting that the inhibition of root growth and geotropism and the induction of swelling, which are typical of ethylene action (1), were duplicated by auxin transport inhibitors. This observation can be interpreted in two ways. First, the role of ethylene in the loss of geotropism and the induction of swelling is to block auxin transport. Second, geotropism and growth may involve a number of steps, each of which is sensitive to different agents, with the net result that if one of the points is blocked, the final outcome remains the same. It follows from this that ethylene is not the only compound capable of regulating root growth, though it may be an important one under natural conditions.

Figures 2 and 3 also indicate that the simultaneous addition of

a high (10 μM) IAA concentration and inhibitor gave rise to an anomalous increase in growth after an initial lag period. A similar observation was made earlier with CFM (11).

Prior to 18 hr, the elongation of sections treated with inhibitor and IAA was controlled primarily by IAA-regulated processes as evidenced by the increased rate of ethylene production. According to the interpretation of Chadwick and Burg (9, 10), the high level of ethylene produced by roots treated with IAA (Fig. 3) indicates an increased level of IAA in the root. After 18 hr, the level of IAA in the root returns to normal because of conjugation and other degradative processes and the rate of ethylene production returns to normal. At this point, the growth rate of auxin-treated roots increases and approaches that of control roots. In contrast, when roots were treated with inhibitor and auxin together, the rate of growth has again initially controlled by auxin, and as soon as the auxin was used up, the growth rate exceeded that of the controls. There is no obvious explanation for this effect at this time. However, it is worth noting that a similar stimulation of root growth by auxin was reported by Thimann and Lane (25) after seeds treated with auxin were transferred to an auxin-free medium. Åberg (2) also discussed the phenomenon of auxin-treated roots displaying positive after-effects.

It is unlikely that the mutual antagonism between auxin and the growth inhibitors was due to increased availability of IAA in the tissue (sparing effect), since, except for a few cases where extremely low concentrations of IAA were studied (2), the normal effect of auxin in roots is to decrease growth.

All of the inhibitors used in this study were readily washed out of pea roots. Figure 4 shows that flushing root sections with inhibitor-free buffer resulted in a return to near normal growth rates. From these results we concluded that the inhibitor binding site is characterized as one having a reversible affinity for the inhibitors.

The data shown in Figure 5 are consistent with the idea that NPA, DPX1840, and TIBA have the same binding site. The results indicate that 2,4-D can be dislodged from its binding site in a competitive fashion with each inhibitor studied.

The concentrations of inhibitors used to demonstrate this effect were higher than those used in studies with cell-free homogenates (27, 28). The higher K*m* obtained from root tissue studies may be due to the resistance to diffusion of the inhibitors into intact cells. Our work showed that NPA, CFM, and 2,4-D may have the same binding site in pea roots (Fig. 5). However, Thomson and Leopold (28) reported that the site in the particulate fraction of corn coleoptiles that binds NPA and CFM did not bind auxin. In further studies with DPX1840 and TIBA we failed to observe any competition between these two inhibitors and CFM (data not shown). The reason behind the difference observed between the work reported here and that done earlier by Thomson and his coworkers is not known. It is appropriate to point out that different plant species (monocot *versus* dicot), and different tissues (leaf *versus* root) were used.

The data presented here are consistent with two interpretations. Our results can be accepted as supporting the idea that there may be two or more auxin attachment sites in the cell and one of these sites may be capable of regulating both auxin transport and growth. Specifically, the data obtained suggest that the site for auxin transport is the same as the one involved in growth. Since geotropism contains elements of growth and transport, it would also be affected by the same inhibitors. The site controlling ethylene production was not found to be involved with growth and transport and was not influenced by auxin transport inhibitors. Alternatively, our results can be taken as support for the idea that both growth and geotropism are dependent on auxin transport.

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