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The action of specific inhibitors of auxin transport on uptake of auxin and binding of N-1-naphthylphthalamic acid to a membrane site in maize coleoptiles

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Abstract. Using both 1-mm segments of corn (*Zea mays* L.) coleoptiles and a preparation of membranes isolated from the same source, we have compared the effectiveness of several inhibitors of geotropism and polar transport in stimulating uptake of auxin (indole-3-acetic acid, IAA) into the tissue and in competing with N-1-naphthylphthalamic acid (NPA) for a membrane-bound site. Low concentrations of 2,3,5-triiodobenzoic acid (TIBA), NPA, 2-chloro-9-hydroxyfluorene-9-carboxylic acid (morphactin), and fluorescein, eosin, and mercurochrome all stimulated net uptake of [^{3}H]IAA by corn coleoptile tissues while higher concentrations reduced the uptake of both [^{3}H]IAA and another lipophilic weak acid, [^{14}C]benzoic acid. Since low concentrations of fluorescein and its derivatives competed for the same membrane-bound site in vitro as did morphactin and NPA, the basis for both the specific stimulation of auxin accumulation and the inhibition of polar auxin transport by all these compounds may be their ability to interfere with the carrier-mediated efflux of auxin anions from cells. At higher concentrations, the decrease in accumulation of weak acids was nonspecific and thus may be the result of acidification of the cytoplasm and a general decrease in the driving force for uptake of the weak acids. Triiodobenzoic acid was an exception. Low concentration of TIBA (0.1–1 μM) were much less effective than NPA in competing for the NPA receptor in vitro, but little different from NPA in ability to stimulate auxin uptake. One possibility is that TIBA, a substance which is polarly transported, may compete with auxin for the polar transport site while NPA, morphactin, and the fluorescein derivatives may render this site inactive.

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Abbreviations: Cl_4-NPA, 2,3,4,5-tetrachloro-N-1-naphthylphthalamic acid; IAA, indole-3-acetic acid; α-NAA, α-naphthaleneacetic acid; β-NAA, β-naphthaleneacetic acid; NPA, N-1-naphthylphthalamic acid; TIBA, 2,3,5-triiodobenzoic acid

Key words: Auxin uptake – Coleoptile – Fluorescein – Morphactin – Naphthylphthalamic acid – Triiodobenzoic acid – *Zea*.

Introduction

In a previous article (Sussman and Goldsmith 1981), we reported that N-1-naphthylphthalamic acid (NPA), an inhibitor of polar auxin transport, stimulated the uptake of auxin by 1-mm slices of maize coleoptiles. Naphthylphthalamic acid also binds with high affinity to a receptor in a membrane fraction isolated from maize (Lembi et al. 1971; Thomson 1972; Thomson et al. 1973). 2-chloro-9-hydroxyfluorene-9-carboxylic acid, a morphactin also competes for the NPA binding site, and occupation of this site may be responsible for inhibition of auxin transport by both NPA and morphactin (Thomson et al. 1973; Thomson and Leopold 1974). On the other hand, 2,3,5-triiodobenzoic acid (TIBA) also stimulates auxin uptake (Rubery and Sheldrake 1974; Rubery, 1977) and inhibits polar transport but is much less effective than NPA in competing for the membrane-bound receptor (Thomson 1972).

Fluorescein and eosin also inhibit auxin transport in the micromolar range (Katekar and Geissler 1975), but information on their affinity for the NPA receptor has been lacking. Conceivably fluorescein or one of its derivatives might be a useful fluorescent probe for the NPA site. Therefore, we have compared the effectiveness of NPA, TIBA, morphactin, fluorescein and two of its derivatives in stimulating the uptake of IAA by maize coleoptile cells with their effectiveness in displacing [^{3}H]NPA from a receptor site assayed in vitro using a membrane fraction obtained from the same tissue.

We also examined the effects of these substances on the uptake of another lipophilic weak acid, benzoic

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acid, that is neither an auxin nor polarly transported. In this way, we hoped to discriminate a specific action of the transport inhibitors at the auxin transport site from nonspecific changes in the driving forces for the uptake of a weak acid.

Materials and methods

Maize seedlings (*Zea mays* L. cv. Golden Cross Bantam; Agway, Syracuse, N.Y., USA) were grown in the dark and the coleoptiles harvested after 96 h. The apex and primary leaf were removed and each coleoptile sliced into ten 1-mm segments as described in Edwards and Goldsmith (1980). [5^{-3}H]Indole-3-acetic acid [5^{-3}H]IAA; $8.7 \cdot 10^{14}$ Bq mol^{-1}) was purchased from CEA-IRE-SORIN (Gif-sur-Yvette, France) and purified before use by partitioning into ether from bicarbonate buffers (pH 8–9) or by Sephadex LH-20 chromatography, as described in Sussman and Goldsmith (1981). [2,3,4,5-^{3}H]N-1-naphthylphthalamic acid ($1.2 \cdot 10^{15}$ Bq mol^{-1}) was purchased from Research Products International (Elk Grove Village, Ill., USA) and [^{14}C]benzoic acid ($2.2 \cdot 10^{12}$ Bq mol^{-1}) from Amersham (Arlington Heights, Ill., USA). 2,3,4,5-Tetrachloro-N-1-naphthylphthalamic acid (Cl_4-NPA) was a gift from Uniroyal Chemical Co. (Bethany, Conn., USA), and 2-chloro-9-hydroxyfluorene-9-carboxylic acid from Dr. Hans Kende, Michigan State University (East Lansing, Mich., USA).

The uptake of radioactivity from media buffered at pH 5 with either 10mM citrate or 50mM (N-morpholino)ethanesulfonic acid (MES) adjusted with KOH was similar and measured as described previously (Sussman and Goldsmith 1981). The transport inhibitors were added simultaneously with the radioactive compounds at the start of the uptake period.

A centrifugation assay (Thomson et al. 1973; Thomson and Leopold 1974) was used to measure [^{3}H]NPA bound in vitro by a membrane fraction that sedimented between 5,000 and 40,000 *g*. To isolate membranes, 40 g (fresh weight) of 5-d-old coleoptiles of etiolated corn seedlings were homogenized with a mortar and pestle in 80 ml of homogenizing buffer (0.25 M sucrose, 0.1 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, 50 mM 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris) adjusted to pH 7.5 with HCl). After filtering through cheesecloth, the brei was re-extracted with another 80 ml of homogenization buffer. The combined filtrates were then centrifuged for 10 min at 5,000 *g*. The pellets were discarded and the supernatant centrifuged at 40,000 *g* for 2 h. The pellet was then resuspended in 60 ml of binding-assay buffer (0.25 M sucrose, 0.5 mM $MgCl_2$, 10 mM citric acid, adjusted to pH 5 with NaOH), to which [^{3}H]NPA (ca. $3.33 \cdot 10^{10}$ Bq) and varying concentrations of nonradioactive competitor were added. After 30 min at 2–5° C, the tubes were centrifuged at 40,000 *g* for 2 h. Radioactivity in the pellets was determined by scintillation counting after overnight extraction (Edwards and Goldsmith 1980).

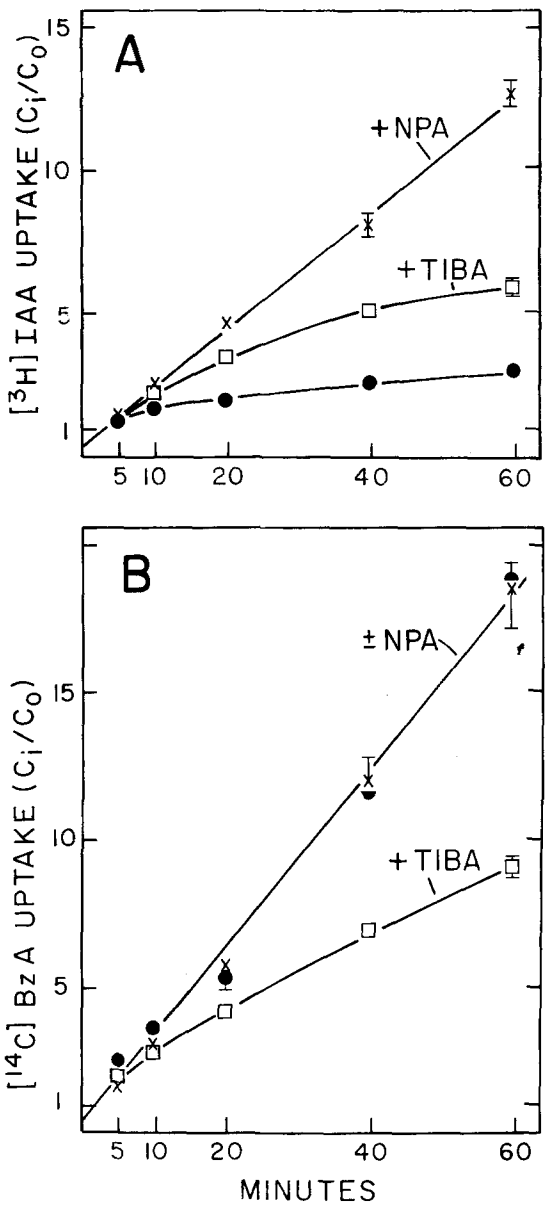

Fig. 1A, B. Comparison of the effect of 10 μM TIBA (□) and 10 μM NPA (×) on the time course of uptake of [^{3}H]IAA (0.04 μM) (**A**) and [^{14}C]benzoic acid (BzA, 0.4 μM) (**B**) by 1-mm segments of maize coleoptiles. Buffer was 10 mM citrate-KOH at pH 5. ● Uptake in the absence of inhibitor. In this and the subsequent figures, the vertical bars indicate the standard error of the mean values

Results

The specificity of the action of inhibitors of polar transport on uptake of auxin by thin slices of maize coleoptile tissue was examined by comparing their effects on uptake of the auxin [^{3}H]IAA and the non-auxin [^{14}C]benzoic acid. At 10 μM, NPA was more effective than TIBA in stimulating IAA uptake (Fig. 1A). Furthermore, 10 μM NPA had no effect on benzoic acid uptake, whereas 10 μM TIBA was inhibitory (Fig. 1 B). These different effects of NPA and TIBA resulted from the rather different sensitivity of the uptake of IAA and benzoic acid to the concentration of these two inhibitors. Note that while NPA (Fig. 2) and TIBA (Fig. 3) exerted similar effects at low concentrations where they both promoted IAA uptake with no appreciable effect on benzoic-acid uptake, at higher concentrations, these two inhibitors had different effects. At 100 μM, NPA was still able to stimulate auxin uptake, but was without effect on

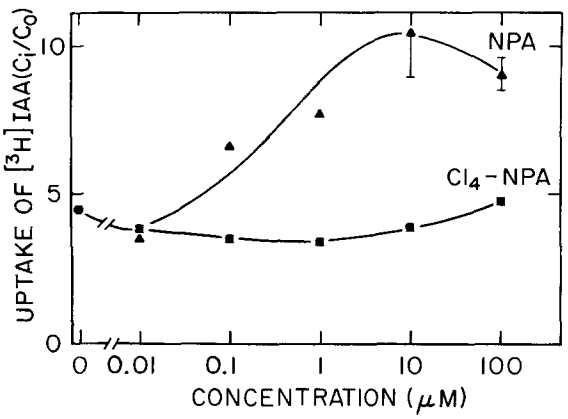

Fig. 2. Effect of concentration of NPA and Cl_4NPA on the uptake of [^{3}H]IAA (8 nM) by 1-mm segments of maize coleoptiles. Uptake was for 1 h in 50 mM MES-KOH at pH 5. Three replicates at each concentration and treatment. Same experiment as Fig. 4

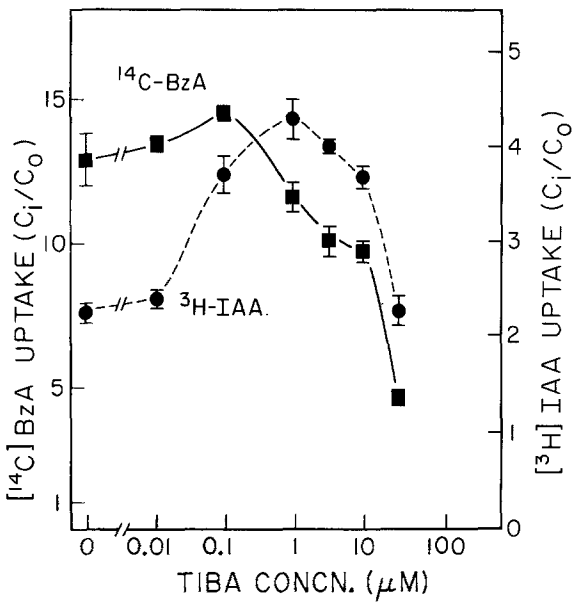

Fig. 3. Effect of TIBA concentration on uptake of [^{3}H]IAA (0.05 μM; ●) and [^{14}C]benzoic acid (BzA, 0.4 μM, ■) by 1-mm segments of maize coleoptiles. Uptake period was 40 min, in 10 mM citrate-KOH at pH 5.

uptake of benzoic acid (Table 1), whereas at TIBA concentrations above 1 μM, stimulation of IAA uptake decreased and disappeared entirely at about 10 μM TIBA, where benzoic-acid uptake was also inhibited (Fig. 3).

Several other inhibitors of auxin transport, namely, morphactin, fluorescein and its derivatives, mercurochrome and eosin, also stimulated IAA uptake when their concentration was 1 μM or below but inhibited uptake of both IAA and benzoic acid at higher concentrations (Fig. 4; Table 1). The effects of all the treatments in Fig. 4 were highly significantly different from NPA (Fig. 2). Furthermore with the exception

Table 1. Effect of high concentration of auxin transport inhibitors on the uptake of [^{14}C]benzoic acid in maize coleoptile slices. Inhibitors were supplied at a concentration of 100 μM in 50 mM MES-KOH at pH 5; [^{14}C]benzoic acid was supplied at 1 μM. The accumulation of radioactivity in the tissue (C_i) relative to that outside (C_o) ± the standard error of the mean was determined after 1 h

Compound	C_i/C_o
None	26.4 ± 1.7
NPA	24.2 ± 1.9
Cl_4NPA	20.7 ± 2.0
Morphactin	7.1 ± 0.2
Fluorescein	3.9 ± 0.3
Eosin	10.9 ± 0.2
Mercurochrome	6.1 ± 1.0

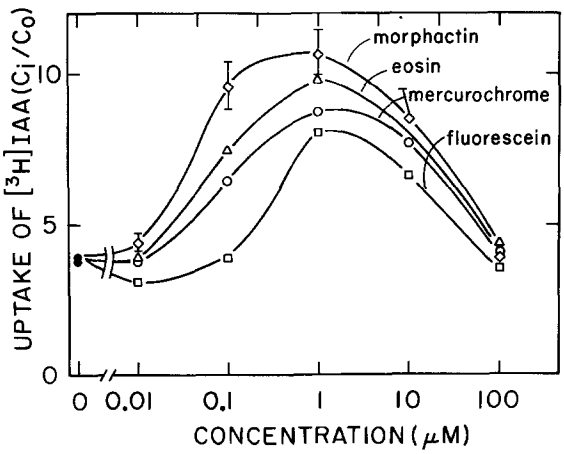

Fig. 4. Effect of concentration of morphactin and fluorescein derivatives on the uptake of [^{3}H]IAA by 1-mm segments of maize coleoptiles. Same experiment and conditions as in Fig. 2. Error bars shown for morphactin only

of eosin and mercurochrome, the difference in the magnitude of adjacent pairs of curves in Fig. 4 was also highly significant. These statements are based on a two-way analysis of variance (Snedecor and Cochran 1967, 299–338) with $P<0.01$ for main or treatment effects. Although the main (treatment) effect of NPA (Fig. 2) was not significantly different from morphactin (Fig. 4) at the 0.05 level, there was still a highly significant difference in the shape of the curves for net auxin uptake as a function of the concentration of these two inhibitors ($P<0.01$ for the interaction effect). In other words, the stimulation of net auxin uptake because of the presence of NPA increased with concentration up to about 10 μM (Fig. 2) without showing the optimum at 1 μM that was seen with the other substances (Figs. 3, 4). Although morphactin and the fluorescein derivatives were all maximally effective at 1 μM, the difference in the magnitude of their stimulation of IAA uptake

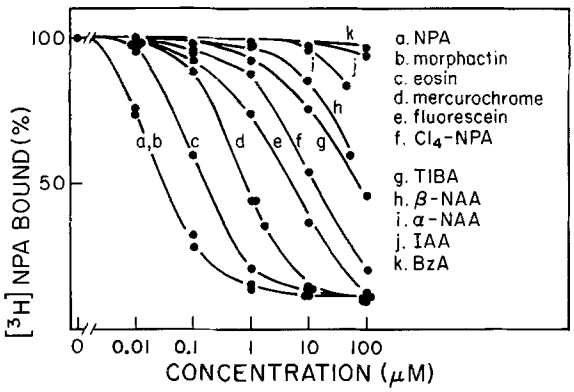

Fig. 5. Competition by auxin transport inhibitors, auxins, and auxin analogues for [^{3}H]NPA that is specificially bound to membranes isolated from maize coleoptiles. The maximum specific binding (100%) represents $1.67 \cdot 10^3$ Bq out of the $4.17 \cdot 10^3$ Bq initially supplied to membranes in 1 ml reaction volume (5 nM [^{3}H]NPA). The assay was conducted with a membrane fraction that sedimented between 5,000 and 40,000 *g* derived from 0.67 g fresh weight of coleoptiles

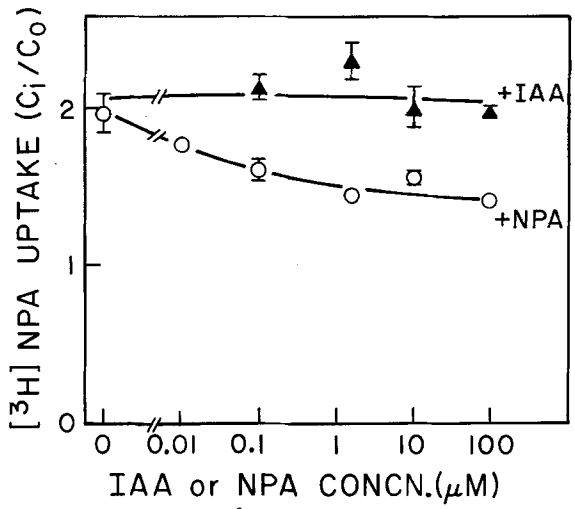

Fig. 6. The uptake of [^{3}H]NPA) (20 nm) by 1-mm segments of maize coleoptiles as a function of increasing concentrations of either unlabeled IAA (▲) or NPA (○). Uptake proceeded for 1 h in medium buffered at pH 5 with 50 mM MES-KOH

indicated that their relative effectiveness in this regard was morphactin > eosin ≧ mercurochrome > fluorescein.

A variety of compounds known to stimulate auxin uptake were also tested for their ability to compete in vitro for the membrane-bound NPA receptor (Fig. 5). NPA and morphactin competed equally well for the NPA site. Morphactin and the fluorescein derivatives maintained the same relative effectiveness (Fig. 5) as in stimulating auxin uptake (Fig. 4). Compared with NPA, a chlorinated analog (Cl_4-NPA) was much less effective in the competitive binding assay (Fig. 5) and was also ineffective in IAA uptake (Fig. 2). TIBA, although at least as active as NPA in stimulating IAA uptake at low concentrations, was relatively inactive in competition for the NPA-site while the auxins, α-NAA and IAA, which also stimulate IAA uptake somewhat (Sussman and Goldsmith 1980) were virtually inactive even at concentrations up to 100 μM. The weak acid, benzoic acid, at 100 μM was also ineffective in competing for the NPA site in vitro.

Previously we reported that the stimulatory effect of NPA on uptake of [^{3}H]IAA from acidic buffers disappeared at high concentrations of IAA (Sussman and Goldsmith 1980). The possibility that high auxin concentrations interfered with uptake of NPA was eliminated by the finding that NPA uptake was independent of IAA concentration (Fig. 6). Interestingly, however, a portion of the uptake of [^{3}H]NPA was sensitive to the concentration of unlabeled NPA. This "saturable" portion was about 25–30% of the total accumulated and decreased as the uptake period extended beyond 15 min (Fig. 7). Although an apparent

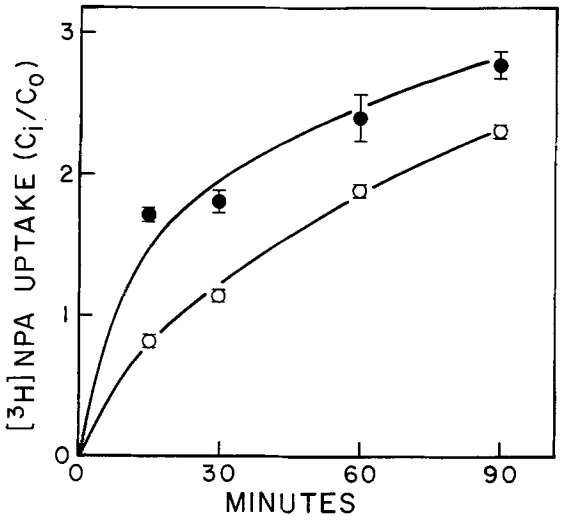

Fig. 7. The time course of uptake of [^{3}H]NPA by 1-mm segments of maize coleoptiles at a concentration of 20 nM (●) or in the presence of a high concentration of nonradioactive NPA (10 μM, ○). The medium was buffered at pH 5 with 50 mM MES-KOH

saturable uptake of benzoic acid in this system was shown previously to be the result of saturating the metabolism of benzoic acid (Sussman and Goldsmith 1981), this was not the case for uptake of [^{3}H]NPA. When the radioactivity in tissues incubated at pH 5 in 20 nM [^{3}H]NPA either with or without addition of 20 μM unlabeled NPA was quantitatively extracted (15 s in boiling 75% ethanol), concentrated and chromatographed on Silica-gel thin layers (see Sussman and Goldsmith 1981, for details) with two different solvent systems (toluene:acetic acid 90:10, $R_f = 0.1$; $CHCl_3$:CH_3OH:acetic acid 10:50:2, $R_f = 0.9$), the

only radioactivity was coincident with authentic NPA. Although the affinity of this saturable site for NPA appears to be quite high, a precise determination of its K_d is not possible based on an uptake experiment with tissue slices. With a K_d of approx. 20 nM (Thomson 1972; see also review by Hertel 1974) and a receptor concentration of 50 pmol g^{-1} fresh weight, we estimate that as much as 40–50% of the [^{3}H]NPA in 1-mm segments of maize coleoptiles could be bound to the NPA receptor. Since uptake of 20 nM [^{3}H]NPA by corn coleoptile segments decreased by 25–30% as the concentration of NPA was raised to 10 μM with nonradioactive NPA (Figs. 6, 7), the magnitude of the concentration-dependent uptake of [^{3}H]NPA by intact cells was about what would be expected on the basis of saturating the NPA binding site in vivo. This saturable site does not seem to be important for transport of NPA across the membrane because after the first 15 min there is no further change in the amount of concentration-dependent uptake (Fig. 7), but the uptake of NPA continues to be linear with time. These observations indicate that under favorable circumstances we may be able to detect binding of NPA to its receptor in intact tissues.

Discussion

Naphthylphthalamic acid is an inhibitor of polar auxin transport (Thomson et al. 1973; Thomson and Leopold 1974; Katekar and Geissler 1975); it also stimulates the net uptake of auxin by maize coleoptile cells (Sussman and Goldsmith 1981). Auxins are lipophilic weak acids that accumulate in plant cells because they dissociate more completely at the alkaline pH of the cytoplasm than in the more acidic cell walls. The more impermeable the auxin anion the greater the accumulation will be (Raven 1975; Rubery and Sheldrake 1974; reviewed by Goldsmith 1977). There is evidence that a component of the efflux of auxin from cells of corn coleoptiles may be carrier-mediated (Edwards and Goldsmith 1980; Sussman and Goldsmith 1981), and it was postulated that inhibition or inactivation of this carrier-mediated efflux by NPA causes both the increased auxin uptake and the inhibition of polar transport (Sussman and Goldsmith 1981).

A membrane-bound receptor site for NPA has been studied in cell-free extracts (Lembi et al. 1971; Thomson 1972; Thomson et al. 1973; Sussman and Gardner 1980). Thomson and Leopold (1974) suggested that binding to this site is involved in the inhibition of polar transport by morphactins and derivatives of NPA. Our results lend further support to their hypothesis. We find that other known inhibitors of auxin transport, including TIBA, morphactin, and fluorescein derivatives, also stimulated the net auxin uptake of thin slices of maize coleoptile tissue, and with the exception of TIBA, all these substances also had an appreciable affinity for the same membrane-bound site as NPA. Presumably occupation of this site in some way inactivates the carrier-mediated polar efflux of auxin anions and this in turn causes the increased total uptake.

A feature of these results was that at low concentrations (0.01–1 μM) of the transport inhibitors, the stimulation of uptake was specific for auxin (e.g. Fig. 3); but at higher concentrations, TIBA, morphactin, and the fluorescein derivatives inhibited uptake of weak acids generally as shown by decreased uptake of both IAA and benzoic acid (Figs. 3, 4, Table 1). Perhaps higher concentrations of these inhibitors, all of which are weak acids, either lower cytoplasmic pH or otherwise interfere with its regulation. Collapse of the cytoplasmic pH would reduce the driving force for the uptake of weak acids generally.

With the exception of NPA, the dependence of IAA uptake on inhibitor concentration peaked at approx. 1 μM (Fig. 4). This optimum probably results from the summation of two different processes with different dependencies on concentration, (a) specific stimulation of auxin uptake by the inhibitor, and (b) nonspecific inhibition of uptake of weak acids. The domination of the latter at concentrations above 1 μM could account for the fact that morphactin, eosin, mercurochrome, and fluorescein all optimally stimulate IAA uptake at approximately the same concentration. Despite similar optimal concentrations, however, the magnitude of their stimulation of net auxin uptake indicated that the half-maximal concentrations were different and that their order of effectiveness was morphactin $>$ eosin $\geqq$ mercurochrome $>$ fluorescein in this regard.

In general, inhibitors that stimulated auxin uptake also competed for a membrane-bound NPA-receptor in vitro. This relationship should be most obvious when a group of inhibitors that is chemically related is compared. For example, the order of affinity for the NPA-binding-site assay seen in vivo (morphactin > eosin > mercurochrome > fluorescein; Fig. 5) was the same as their order of effectiveness in stimulating IAA uptake half-maximally (Fig. 4). Comparing the concentrations in the medium necessary to compete for NPA binding with those needed to stimulate auxin uptake is less likely to be profitable simply because the former assay involves a homogenate while the latter involves intact cells, and the external concentration of an inhibitor is unlikely to be an adequate indication of the concentration at the active site in

tissue sections. This is because the concentration at the active site depends not only on factors influencing permeability, compartmentation, and binding but also on the balance between uptake and metabolism. Furthermore, since the inhibitors are weak acids, both their uptake and binding will depend in part on their pH-dependent dissociation. These considerations could account for discrepancies such as NPA appearing to be as effective as morphactin in the binding assay (Fig. 5) while appearing somewhat less effective than morphactin at low concentrations in stimulating IAA uptake by tissue slices (Figs. 2, 4).

In this context, the observation that the affinity of TIBA for the NPA-receptor (Fig. 5) seems several orders of magnitude too low to account for its effects on auxin transport or uptake (Fig. 3) is more difficult to explain; however, TIBA shares certain properties with the auxins, α-NAA and IAA. Both TIBA and auxins (a) stimulate net uptake of auxin, but (b) have relatively low affinity for the NPA receptor (in vitro), and (c) are polarly transported while NPA is not (Thomson et al. 1973). These observations indicate that there may be two classes of substances capable of inhibiting polar transport and stimulating uptake of auxin: (a) ones like TIBA and auxins, that are themselves polarly transported and compete for the auxin anion efflux site; and (b) substances such as NPA, morphactin, and the fluorescein derivatives, that compete in vitro for the NPA site but in vivo may block or inactivate the polar efflux site without themselves being transported. The two sites, the transport and the regulatory one, are probably closely related in vivo. Our observations are consistent with those of Thomson et al. (1973) who found that TIBA competes with auxin for another binding site, and also concluded that inhibition of auxin transport by TIBA and NPA may involve different receptors. However, a completely different alternative is also consistent with our data. If the NPA receptor were located on the tonoplast and increased the uptake into the vacuole, it would also stimulate uptake and inhibit polar transport. Thus definitive conclusions as to how binding of an inhibitor to the NPA site regulates auxin transport must await more detailed understanding of the relation of this site to the postulated auxin-anion carrier as well as further clarification of the intracellular location of the receptors for the transport inhibitors.

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