EFFECT OF SOME SUBSTITUTED BENZOIC ACIDS AND RELATED COMPOUNDS ON THE DISTRIBUTION OF CALLUS GROWTH IN TOBACCO STEM EXPLANTS [1, 2]

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It has been observed in this laboratory (15) that the normally polar distribution of callus growth on tobacco stem explants becomes non-polar in the presence of low levels of 2,3,5-triiodobenzoic acid (TIBA), while the amount of callus growth is not diminished. It was thought that this phenomenon might reflect a specific inhibition by TIBA of the polar transport of auxin. Subsequent studies (10, 11, 13) were carried out to test this hypothesis. TIBA was found (13) to inhibit transport of indole-3-acetic acid (IAA) in the classical Went (23) transport test and in tobacco stem segments as determined directly by chemical and bio-assay of IAA previously introduced into the plants and then extracted from the apical and basal portions of the stem segments. Other compounds found to cause a redistribution of callus growth and also to inhibit transport in the Went assay were 2-bromo-3,5-dichlorobenzoic acid, γ-phenylbutyric acid, 2,4-dichlorophenoxy acetic acid (2,4-D), and phenylacetic acid (10). The inhibitory effects on polar IAA transport by TIBA and 2,4-D have been independently confirmed by Hay (4) and by Zwar and Rijven (24). Another instance of apparent inhibition of IAA transport by TIBA has been reported by Kuse (6), who measured axillary bud growth and petiole extension in Ipomoea explants (consisting of a section of stem containing 1 node and its leaf) after 7 days' growth in nutrient solution. Bud growth was inhibited by the intact leaf, or by IAA applied to the cut end of a debladed petiole. TIBA applied midway along the petiole permitted bud growth on explants having either auxin source. The portion of the petiole distal to the TIBA was reported to have grown more than the proximal part. Kuse suggested that both effects resulted from accumulation of auxin above the point of TIBA treatment, as though transport had been prevented.

Because two substituted benzoic acids had been shown to prevent auxin transport at concentrations less than those required to inhibit growth, and because these compounds have been studied extensively for auxin activity (8, 9, 20), it was thought to be of interest to screen a number of them for this apparently specific effect on polar transport, as shown by their influence on the growth and distribution of callus on tobacco stem explants.

As two of the TIBA analogs (2-hydroxy- and 2-amino-3,5-diiodobenzoic acids) have been found to uncouple phosphorylation in animal tissue homogenates (H. A. Lardy, personal communication), it was decided to test also 2 other highly active uncouplers, 2,6-dibromo-4-nitrophenol, and 3,5-diiodo-L-tyrosine.

In an attempt to improve the sensitivity of the method of Niedergang-Kamien and Skoog (13) for testing transport inhibition by direct chemical analysis of the distribution of added auxin in treated and untreated tobacco stem sections before and after transport, the distribution of added IAA was measured after a 2-hour transport interval.

Although many experiments (10, 11, 12, 13) indicate that TIBA inhibits the polar movement of IAA, they also show that TIBA drastically reduces the level of extractable IAA, which complicates the assay. The reduction was so great that even with chromatographic purification, not enough added IAA was recoverable from TIBA-treated tobacco stem tissue to permit colorimetric assays. For this and other reasons it was desirable to test the effect of TIBA on the movement of radioactive IAA, the distribution of which in the tissue could be determined without extraction.

METHODS AND MATERIALS

Stem tissue of *Nicotiana tabacum* L. var. Wisconsin 38 was cultured in 125-ml Erlenmeyer flasks each containing 50 ml of medium, as described by Niedergang-Kamien and Skoog (13). The external portion was stripped off down to the cambium in order to expose aseptic tissue; thus the cultured pieces contained part of the cambium, xylem, internal phloem, and pith. Into each flask was placed either a single cylinder or 3 segments obtained by cutting the cylinders tangentially.

The basal medium was the modified White's mineral salts-sucrose-agar medium described by Skoog and Tsui (16). The concentration of KH_2PO_4 was tripled (37.5 mg/l), and the iron source was NaFe-Sequestrene (25 mg/l NaFe-ethylenediaminetetraacetic acid). The chemicals to be tested were prepared as alkaline 7.5×10^{-3} M stock solutions, and diluted to give final test concentrations of 5×10^{-3}, 1×10^{-4}, 1×10^{-5}, and 1×10^{-6} M. Each diluted solution was adjusted to pH 6.1 with HCl before autoclaving. To detect auxin inhibitors, the 1×10^{-4} and 1×10^{-5} M concentrations were tested also in the presence of 1×10^{-5} M IAA, and in most experiments cultures supplied with 2×10^{-5} M TIBA were included as checks. In order to avoid thermal decomposition, the 2,5-diiodobenzoic acid and 2,4-dihydroxybenzoic acid were filter-sterilized and added aseptically to the autoclaved basal medium.

As a standard procedure, 7 replicate flasks with segments and 8 flasks with cylinders were used for each concentration of the test compound. Observa-

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tions were made periodically and sample tissue pieces were photographed after 3 to 8 weeks. A record was made of the amount and distribution of callus growth, and the amount of pith enlargement obtained. Cylinders sometimes reveal subtler effects on polarity than do segments (13); however, in the present work, the cylinders yielded no information not also observed in the segments, hence only the latter are illustrated. The effects of active chemicals on callus growth and distribution were striking, and were clearly seen in visual comparisons between treatments, as is evident from figure 3. Data for inactive compounds are summarized here in tabular form only; detailed photographic records of all experiments are found in (5).

The distribution of added IAA in tobacco stem sections after transport was determined by a modification of the method of Niedergang-Kamien and Skoog (13). Tobacco stems, decapitated and stripped of all but the upper 3 well-expanded leaves, were placed in a large beaker containing a 10 mg/l solution of IAA, set before an electric fan, and uptake via the transpiration stream was allowed for 1 hour. The stems were then cut into 1-cm sections and placed in a moist chamber for 2 hours to permit transport. The sections rested across shelves made of two 3-mm glass rods cemented together at the ends. This arrangement permitted the sections to touch the glass tangentially at only 2 points and thus prevented formation of a continuous moisture film between the glass and the tissue which might serve as a pathway for auxin diffusion. After transport, the apical half of each segment was removed and cut into 5 parts. These were pooled and extracted for 3 hours with ether. The basal half was cut into 1-cm sections which were pooled separately by position and also extracted as above. The extracts were chromatographed in isopropanol : ammonia : water, 10 : 1 : 1 (18), eluted with water, and the concentration of IAA in the eluate determined colorimetrically with the $FeCl_3$-$HClO_4$ modified Salkowski reagent (2).

The experiments with IAA labelled in the methylene carbon with C^{14} were carried out as follows. Seedlings of *Phaseolus vulgaris* L. var. Kentucky Wonder were grown in the light for 6 to 8 days. From each hypocotyl a 2-cm section was removed, the apical end being 5 mm below the cotyledonary node. The apical half of the section was marked with an India ink dot applied 3 mm apically from the center. Forty sections per treatment were immersed in a layer of solution about 2 mm deep (4 ml in a 5-cm Petri dish) and uptake was permitted for 3 hours. To avoid complications from surface contamination and handling, the sections were rinsed upon removal from the solution and blotted dry, and a 5-mm section was removed from each end, so that only the central 1-cm portion was used for measurements. Half of the 1-cm sections were cut at once into 2-mm lengths, and half were placed in a moist chamber for 2 hours to permit transport before cutting. In the moist chamber the tissues were placed on glass rod shelves as described above for the tobacco experiment. After cutting, the sections from each position (apex to base) were pooled in a vial.

In early experiments the sections were simply dried, placed in a planchet, and counted whole. Although statistically valid results were obtained which showed that transport had occurred in the controls but not in the presence of TIBA (5), the changes were not great, and the variance was relatively high. An improved technique consisted of adding 0.2 ml of 20 % KOH to each vial and macerating the fresh sections with a glass rod. Then several drops of CS_2 were added and further trituration performed. The macerate was kept at room temperature overnight while the xanthate formed. This procedure solubilized the cellulose to a large degree and gave an adequately homogeneous material for plancheting on stainless steel cupped planchets. After drying in an oven the planchets were counted with a mylar-window gas flow counter. The control solution contained 31 μg/ml of IAA-a-C^{14} having a specific activity of 0.9 mC/mM. This gave the solution an activity of ca. 3.66×10^5 disintegrations/min/ml. The test solution contained in addition 5×10^{-4} M TIBA. In the experiment described below, 3 replicate dishes of each solution were used.

Results and Discussion

It has previously been shown (10, 13) that added IAA is transported to the basal halves of 1-cm tobacco stem segments in 3 hours, in quantities many times the endogenous level. It was necessary to shorten the transport interval for reasons of convenience, so an experiment was performed to measure the distribution after 2 hours to see if measurable transport had occurred. Figure 1 shows that nearly complete transport

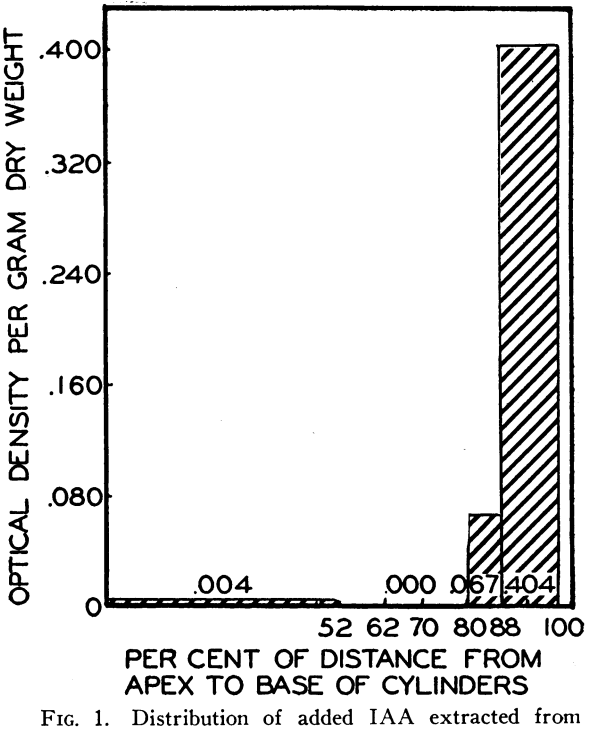


FIG. 1. Distribution of added IAA extracted from different portions of 1-cm tobacco stem segments after 2 hours of transport. The distance along the abscissa is proportional to the dry weight of the tissue in each position as the cuts were not precisely spaced.

of the IAA to the basal 2 mm had occurred. The optical densities were not converted to μg-equivalents of IAA before graphing in this experiment because it was found later that one-dimensional chromatography did not remove all color test inhibitors; however, the purpose of the experiment was primarily to show the distribution. An approximate minimum value based on standards run at the same time is 4 μg/g dry wt. To determine if continuous auxin production occurs in cultured tobacco stem sections, the basal ends of 2 groups of peeled segments were removed after 3 hours; from 1 group, an additional basal portion was removed after 6 hours. All the pieces were subsequently cultured for 36 days. The results are shown in figure 2. If callus growth depended solely on endogenous auxin initially present, one would expect callus growth only on the basal ends which were cut off, as they should have contained all the auxin after 2 to 3 hours. However, callus growth occurred on all pieces, and the amount on the pieces cut after 6 hours was no less than that on the pieces removed after 3 hours. These results argue for a continuous auxin synthesis in the cultured tissues. Therefore any compound which causes apolar callus distribution but does not inhibit callus growth may interfere rather specifically with the transport of this endogenous auxin. Because it is also possible that callus redistribution results from an auxinic action of the non-transported test chemical itself, confirmatory experiments of a more direct nature are needed for full interpretation.

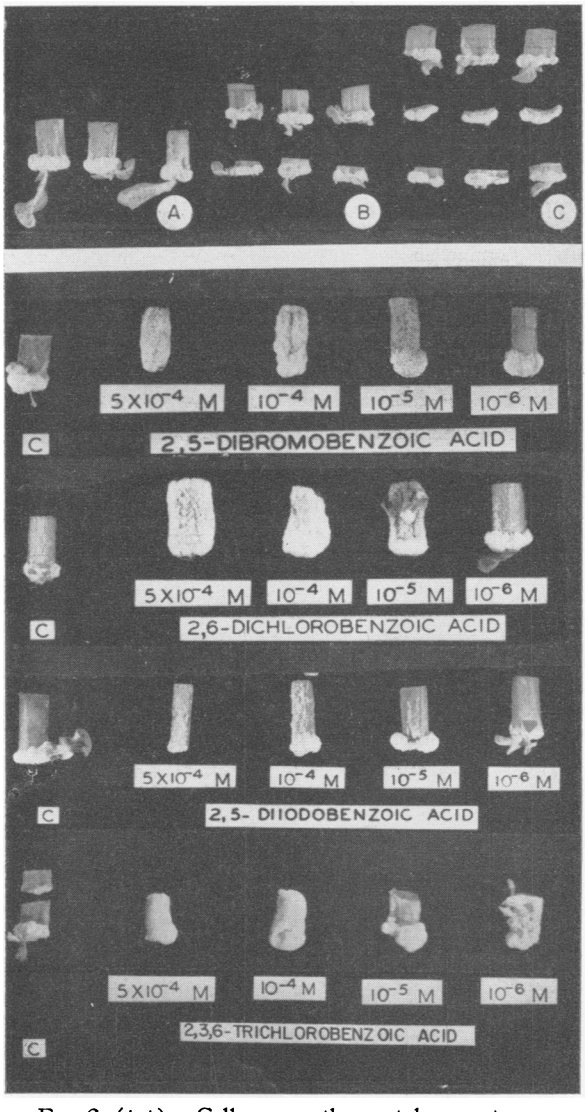


FIG. 2 (*top*). Callus growth on tobacco stem segments with basal ends removed at successive intervals, subsequently cultured 36 days. A: Intact controls. B: "Parent pieces" (upper) and basal ends (lower) removed after 3 hours. C: "Parent pieces" (upper), basal ends removed after 6 hours (center), and basal ends removed after 3 hours (lower). All pieces are oriented with basal ends toward bottom of photograph; basal ends are not necessarily matched to respective "parent pieces".

FIG. 3 (*bottom*). Effect of concentration of 4 benzoic acid derivatives on the distribution of callus growth in tobacco stem segments cultured in vitro. C (left): Controls with polar callus growth (at basal ends). Age of cultures: 37, 47, 42, and 45 days, respectively, top to bottom.

As shown in figure 3 and table I, 2,3,6-trichlorobenzoic acid, 2,6-dichlorobenzoic acid and 2,5-dibromobenzoic acid were highly active in causing apolar distribution of callus growth, and 2,5-diiodobenzoic acid was weakly active in this respect. The 1st 3 com-

TABLE I

ACTIVITY OF SOME SUBSTITUTED BENZOIC ACIDS AND RELATED COMPOUNDS IN CAUSING APOLAR DISTRIBUTION OF CALLUS GROWTH IN TOBACCO STEM SEGMENTS CULTURED IN VITRO.*

Strongly active	
2,5-dibromobenzoic**	2,6-dichlorobenzoic**
2,3,6-trichlorobenzoic***	
Weakly active	
2,5-diiodobenzoic**	
Inactive	
2-bromobenzoic**	2-chloro-4-aminobenzoic†
3-bromobenzoic**	2-chloro-5-nitrobenzoic†
4-bromobenzoic**	2,4-dimethoxybenzoic†
2-iodobenzoic	2,5-dinitrobenzoic
3-iodobenzoic	2,4-dihydroxybenzoic
4-iodobenzoic	2,5-dihydroxybenzoic
2-nitrobenzoic	2-amino-3,5-diiodobenzoic
4-nitrobenzoic	2-hydroxy-3,5-diiodobenzoic
2-acetylaminobenzoic	2-hydroxy-3,5-dinitrobenzoic
4-acetylaminobenzoic	2,4-dichloroanisole
2,4-dichlorobenzoic	3,5-diiodo-L-tyrosine†
3,4-diiodobenzoic**	2,6-dibromo-4-nitrophenol†
potassium iodide††	

* Source of chemicals: Eastman White Label except as indicated below.

** Kindly supplied by Dr. R. L. Weintraub, Ft. Detrick, Maryland.

*** Heyden Chemical Co.

† Kindly supplied by Prof. H. A. Lardy, Enzyme Institute, University of Wisconsin (originally from commercial sources).

†† Mallinckrodt Analytical Reagent.

pounds did not inhibit callus growth, whereas the less active 2,5-diiodobenzoic acid did so markedly. Neither 2,3,6-trichlorobenzoic, 2,6-dichlorobenzoic, nor 2,5-dibromobenzoic acid alone had any visible effect on pith enlargement; 2,6-dichlorobenzoic and 2,5-dibromobenzoic acids failed to affect the pith enlargement caused by added IAA. Because the amount of 2,5-diiodobenzoic acid was limited, this compound was not tested in the presence of added IAA; 2,3,6-trichlorobenzoic acid was not tested with added IAA because insufficient plants were available at the time.

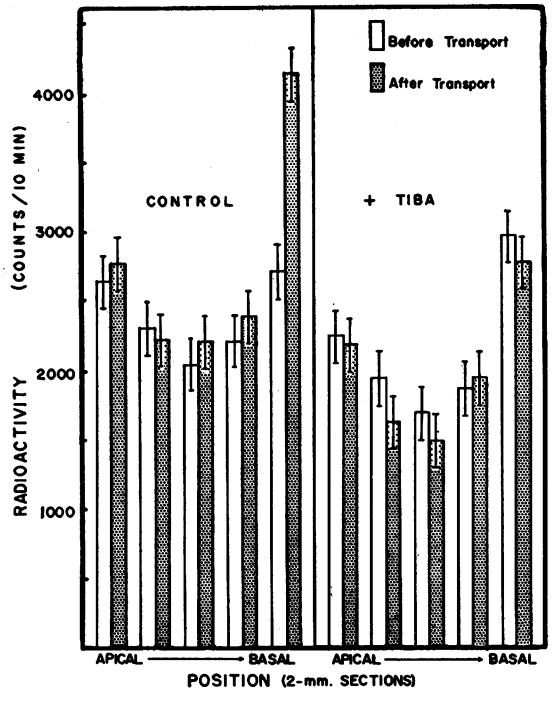
The remaining compounds listed in table I had no effect on callus distribution. As a check on possible effects of the high potassium levels introduced as the excess base used in dissolving the test compounds, and of any iodide resulting from TIBA breakdown, potassium iodide was tested at levels as high as 10^{-3} M. No effect on callus distribution was observed; only a slight inhibition of callus growth resulted, and that only at the highest level of KI tested.

It is noteworthy that several of the substances tested here which are known to be phosphorylation uncouplers did not alter the typically polar distribution of callus growth, although they did severely inhibit the amount. Earlier reports (1, 12) that such uncouplers did inhibit auxin transport in the Went assay, therefore, may reflect a non-specific inhibition due to a general toxic effect, rather than a selective action on polarity. The uncoupling agents did markedly inhibit the cell enlargement of the pith tissue which occurs in the presence of added IAA (table II). The relative inhibition is indicated by the number of (—)

TABLE II

EFFECT OF THE COMPOUNDS LISTED IN TABLE I ON PITH ENLARGEMENT OF TOBACCO STEM TISSUE CULTURED IN VITRO IN THE PRESENCE OF 10^{-5} M IAA

Inactive at 10^{-4} and 10^{-5} M	(0)
3-bromobenzoic	2,6-dichlorobenzoic
4-bromobenzoic	2,5-dibromobenzoic
3-iodobenzoic	2,5-dinitrobenzoic
4-iodobenzoic	2-chloro-5-nitrobenzoic
2-nitrobenzoic	2,4-dihydroxybenzoic
4-nitrobenzoic	2,5-dihydroxybenzoic
4-acetylaminobenzoic	2-hydroxy-3,5-dinitrobenzoic
2,4-dichloroanisole	
Inhibitory at 10^{-4} M, inactive at 10^{-5} M	(—)
2-bromobenzoic	2-acetylaminobenzoic
2-iodobenzoic	2,4-dichlorobenzoic
2,4-dimethoxybenzoic	
Strongly inhibitory at 10^{-4} M, somewhat at 10^{-5} M (— —)	
2-chloro-4-aminobenzoic	2-amino-3,5-diiodobenzoic
2,3,5-triiodobenzoic	3,5-diiodo-L-tyrosine
Strongly inhibitory at both 10^{-4} and 10^{-5} M (— — —)	
3,4-diiodobenzoic	2-hydroxy-3,5-diiodobenzoic
2,6-dibromo-4-nitrophenol (— — — —)	



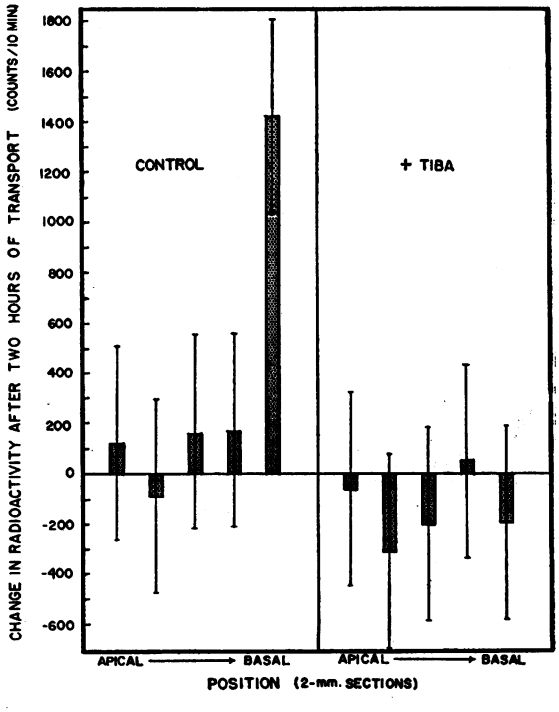


FIG. 4 (*left*). Effect of TIBA on the distribution of IAA-*a*-C^{14} in bean hypocotyl sections before and after a transport interval. The clear bars show the distribution before transport; the shaded bars, after. The vertical lines at the top of each bar indicate the 95 % confidence limits.

FIG. 5 (*right*). Change in radioactivity of different portions of 1-cm bean hypocotyl sections after 2 hours of transport of IAA-*a*-C^{14}. The data are derived from those of figure 4 by difference.

signs and is based on the extent of inhibition at the 2 levels tested. Differences within each group are not considered important, except that 2,6-dibromo-4-nitrophenol appeared to be the most toxic of all the compounds tested.

It is of interest that the 2-bromo- and 2-iodo- derivatives were stronger growth inhibitors than their 3- and 4-substituted isomers, which is consistent with the view of Muir and Hansch (8) that displacement of an electron-attracting substituent in one of the *ortho* positions is involved in the growth reaction induced by benzoic acids. The disubstituted benzoic acids with substituents in the 4-position were growth-inhibiting, except 2,4-dihydroxybenzoic acid. Veldstra (21) has reported that substituents larger than fluorine in the 4-position rendered the benzoic acids inhibitory or inert with respect to coleoptile elongation or pea stem curvature.

Preliminary experiments in which other criteria were used to test for transport inhibition were performed with 2,6-dichloro- and 2,5-dibromobenzoic acids. The results were in agreement with the culture experiments. These tests included the release of apical dominance in bean seedlings, and the direct measurement of IAA extracted from tobacco stem sections before and after transport.

The results of a typical experiment to test the effect of TIBA on the distribution of IAA-*a*-C^{14} in bean hypocotyl sections before and after transport are shown in figures 4 and 5. In figure 4 is shown the total activity in 2-mm sections from each position, before and after transport, and the 95 % confidence limits. The significant feature is the increase in radioactivity in the basal 2 mm of the control sections after transport, and the lack of any such increase in the presence of TIBA. This difference is emphasized in figure 5, in which the activity before transport has been subtracted from that after. No significant change occurred in the TIBA-treated tissue, whereas marked transport occurred in the controls.

Figure 4 also shows that less radioactivity was found in the central 2-mm portion than at the ends, in both treated and control populations. Two possible explanations are: 1), that auxin destruction at the cut surfaces causes non-transportable breakdown products to accumulate at the ends, or 2), that more auxin can enter the tissue via the cut ends than via the epidermis. Although auxin destruction at cut surfaces is a well-known phenomenon (17), the time for such action was limited because in the "before transport" treatment the cut ends which had been in contact with the solution were removed immediately before the central 1-cm sections were cut into 2-mm lengths. Even so, the counts were higher in the apical portion than in the central ones. The second alternative is supported by the findings of Thimann and Schneider (19) that IAA enters Avena coleoptiles more rapidly through cut surfaces than through the epidermis. However, the subject of differential permeability has been much discussed (14, 19) and some indirect evidence has been presented for increased permeability at the intact side of slit pea stems (14).

Various studies have established that the polar auxin transport mechanism expends metabolic energy (1, 3, 22) and is, in fact, inhibited by compounds which interfere with respiration or energy transfer. These findings have been reviewed and extended by Niedergang-Kamien and Leopold (12), who report that polar auxin transport can also be inhibited by certain sulfhydryl-binding compounds at concentrations which do not inhibit growth or respiration. They suggest that the inhibition of transport is of a more specific type. Because TIBA has been reported to bind sulfhydryl groups (7), and other sulfhydryl reagents have been shown to inhibit auxin transport (12), the action of TIBA on transport may indeed involve sulfhydryl binding. However, a number of other auxin-like compounds have been found to inhibit auxin transport. In addition to those reported by Niedergang-Kamien and Skoog (13), and those described here, Zwar and Rijven (24) have reported that 2,4-dichlorophenoxypropionic, 2,4-dichlorophenoxybutyric, 2,4,6-trichlorophenoxyacetic, and indole-3-propionic acids inhibit the transport of IAA through bean hypocotyl segments. Compounds found inactive by Zwar and Rijven were *p*-chlorophenylisobutyric acid, 2,4-dichloroanisole, coumarin, *o*-isopropyl-N-phenylcarbamate, indole, indole-3-acetonitrile, and phenylacetic acid.

Although supporting experiments (e.g., direct analyses) are needed to establish the point, the effects on callus growth and distribution of 2,6-dichlorobenzoic acid, 2,5-dibromobenzoic acid, and 2,3,6-trichlorobenzoic acid so closely resemble those of TIBA as to suggest that inhibition of auxin transport is involved in all cases. The growth of callus indicates that concentrations of these compounds which stop polar distribution do not interfere with respiration or energy transfer. Although further work is needed to determine if these auxin-like transport inhibitors also involve sulfhydryl binding or function by other mechanisms, the high degree of structural specificity found in the experiments reported here suggests that auxin transport has a stereochemical aspect, perhaps involving competition for "carrier" or "transport" sites, as has been postulated in a general sense by Niedergang (10) and by Zwar and Rijven (24) to account for the facts 1) that so few auxins are actively transported, and 2) that none has been found to be transported nearly as rapidly as IAA.

Summary

The capacity of tobacco stem segments to transport polarly many times the endogenous levels of IAA was shown by the distribution in segments after 2 hours of transport. Presumptive evidence for continuous auxin production and transport by cultured tobacco stem explants was obtained.

Twenty-nine compounds, mostly substituted benzoic acids, were tested on tobacco stem explants for their effect on callus growth and distribution. Three compounds, 2,3,6-trichloro-, 2,6-dichloro- and 2,5-dibromobenzoic acids, were highly active in causing apolar distribution of callus growth, and 2,5-diiodobenzoic

acid was weakly active. The remaining 25 compounds were inactive with respect to callus distribution.

When tested alone, the highly active compounds had no inhibitory effect on callus growth; 2,5-diiodobenzoic acid was markedly inhibitory.

None of these compounds alone noticeably stimulated pith cell enlargement. Neither 2,5-dibromo- nor 2,6-dichlorobenzoic acid affected the amount of pith cell enlargement obtained in response to a stimulatory IAA level (10^{-5} M); 2,3,6-trichloro- and 2,5-diiodobenzoic acids were not tested together with IAA.

TIBA was found to inhibit the polar transport of IAA-*a*-C^{14} in bean hypocotyl sections, thus confirming earlier studies by a method not dependent on extraction or bio-assay of IAA.

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