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Influence of 2,3,5-Triiodobenzoic Acid on the Transport and Metabolism of IAA in Lupin Hypocotyls

Juana M. Botía, Ana Ortuño, Manuel Acosta, Francisco Sabater, and José Sánchez-Bravo

Departamento de Biología Vegetal, Facultad de Biología, Universidad de Murcia, 30001 Murcia, Spain

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Abstract. The influence of 2,3,5-triiodobenzoic acid (TIBA) on the transport and metabolism of indolyl-3-acetic acid (IAA) was studied in etiolated lupin (*Lupinus albus* L) hypocotyls. Double isotope-labeled IAA [(5-^{3}H)-IAA plus (1-^{14}C)-IAA] was applied to the cut surface of decapitated seedlings. This confirmed that the species mobilized was unaltered IAA and permitted us to measure the in vivo decarboxylation of applied IAA. A pretreatment with TIBA applied to the cut surface produced a partial or drastic inhibition in the basipetal IAA movement at 0.5 or 100 μM, respectively. Since TIBA inhibits auxin polar transport by interfering with the efflux carrier, the above results suggest that 100 μM TIBA is sufficient to saturate the binding sites in the transporting cells. Compared to the control plants, in vivo decarboxylation of IAA was enhanced in 0.5 μM TIBA-treated plants, while no decarboxylation was detected after treatment with 100 μM TIBA. The in vitro decarboxylation of (1-^{14}C)-IAA catalyzed by purified peroxidase was moderately activated by 100 μM and unaffected by 0.5 μM TIBA. The paradoxical effect of TIBA in vivo vs in vitro assays suggests that the in vivo effect of TIBA on IAA oxidation might be the consequence of the action of TIBA on the auxin transport system. Thus, transport reduction by 0.5 μM TIBA caused a temporary accumulation of IAA in that apical region of the hypocotyl which has the highest capacity to decarboxylate IAA. In the presence of 100 μM TIBA, a concentration which presumably saturates the efflux carriers, most of the added IAA can be expected to be located in the transporting cells where, according to the present data, IAA decarboxylation cannot take place.

The metabolism and transport of indolyl-3-acetic acid (IAA) are usually considered as the main processes in the control of auxin concentration in plant tissues and, consequently, in the regulation of IAA-mediated growth and morphogenesis. It has frequently been proposed that many synthetic or naturally occurring substances which produce growth modifications might act through their influence on these processes. Thus, monophenols should produce growth inhibition in stems by activation of IAA oxidation, while diphenols might have the converse effect (see reviews of Galston and Hillman 1961, Pilet and Gaspar 1968). Furthermore, 2,3,5-triiodobenzoic acid (TIBA) and phytotropines, such as naphthylphthalamic acid (NPA), can either stimulate shoot growth or inhibit root elongation by inhibition of IAA transport and subsequent IAA accummulation (Beffa et al. 1987a, Vesper et al. 1987).

Some recent reports suggest that IAA catabolism and IAA transport are not independent processes. Thus, the in vitro oxidation of IAA by soluble enzymes from maize roots was inhibited in the presence of polar transport inhibitors, such as TIBA or NPA (Beffa et al. 1990). Kaempferol, quercetin, and other such flavonoids, which have long been known to inhibit IAA oxidation (Mumford et al. 1961; see review of Pilet and Gaspar 1968), also inhibited IAA polar transport (Jacobs and Rubery 1988). IAA decarboxylation occurred during basipetal IAA transport along decapitated lupin hypocotyls either when IAA was applied to the complete cut surface (Sánchez-Bravo et al. 1988) or to the stele, where, unlike in the cortex + epidermis, polar transport occurred (Sánchez-Bravo et al. 1991). Although these data might merely represent circumstantial evidence, the existence of an interaction be-

tween IAA catabolism and transport obviously has a physiological relevance for the regulation of plant growth.

The aim of this paper is to analyze the nature of this possible interaction. We studied the influence of TIBA, a well-known inhibitor of IAA transport, on the in vivo metabolism of IAA during the polar transport of auxin in decapitated lupin hypocotyls. The use of IAA labeled with double isotope [(1-^{14}C)-IAA plus (5-^{3}H)-IAA] allows confirmation that the species mobilized is IAA and not an IAA metabolite. In addition, the in vivo decarboxylation of IAA can be measured by the dual-isotope method (Sánchez-Bravo et al. 1988, 1990).

Materials and Methods

Plant Material

Seeds of lupin (Lupinus albus L cv Multolupa) were imbibed for 24 h and grown in damp vermiculite at 25°C in darkness. Uniform samples of 6-day-old seedlings (65 ± 5 mm in length) were used. Decapitated plants were obtained by cutting the hypocotyls starting 5 mm from the cotyledons, using a safelight at 550 nm.

Application of IAA and TIBA and Extraction of Radioactivity

A drop (10 μl) of aqueous solution containing 1.54 KBq (5-^{3}H)-IAA (specific activity 925 GBq mmol^{-1}, 0.17 μM) and 0.78 KBq (1-^{14}C)-IAA (specific activity 2.18 GBq mmol^{-1}, 36 μM) was deposited on the cut surface of decapitated seedlings. In parallel experiments, decapitated seedlings were pretreated with a drop (10 μl) of 0.5 or 100 μM TIBA solution. To ensure complete uptake of the TIBA solution by the tissues, it was applied to the cut surface of decapitated plants 15–20 min before the application of IAA. The plants were kept in culture trays at 25°C in darkness during treatment. At specific times after the IAA application, hypocotyls from four plants were divided into 5-mm sections. Sections with the same location along the hypocotyls were simultaneously extracted with acetonitrile as described by Sánchez-Bravo et al. (1990). The radioactivity extracted in bulked samples of four sections was expressed as a percentage of radioactivity recovered from the whole hypocotyl.

IAA Transport

From the distribution of radioactivity along the hypocotyl, the velocity of basipetal IAA movement was calculated from the position of the radioactive front at each period of transport. The amount of IAA transported during different periods of time was calculated by adding the radioactivity recovered from sections located below the first centimeter starting from the cut surface. No radioactivity was detected in the roots at different sampling times (up to 6 h). In a previous study (Sánchez-Bravo et al. 1991), we demonstrated that polar transport of IAA occurs in the stele but not in the outer (cortex + epidermis) tissues of lupin hypocotyls. On the other hand, local application of labeled IAA to the outer tissues resulted in little basipetal movement of radioactivity, about 96% of this being located in the first apical centimeter. Bearing in mind that the area of the outer tissues represents 85% of the cut surface, the first apical centimeter can be considered as the loading zone, in which radioactivity applied to, or diffusing into the outer tissues can move to the stele because of the IAA sink capacity of this tissue (Sánchez-Bravo et al. 1991).

IAA Decarboxylation

The in vivo decarboxylation of IAA by decapitated plants treated with radioactive IAA was measured by the dual-isotope method previously described (Sánchez-Bravo et al. 1988). This method permits the estimation of the absolute decarboxylation index (I, in Bq of (1-^{14}C)–IAA decarboxylated) and the relative decarboxylation index (Ir, as a percentage of IAA decarboxylated compared to ^{3}H activity recovered). In a previous paper (Sánchez-Bravo et al. 1990), we demonstrated that the absolute decarboxylation index corresponds to the IAA decarboxylation products present in the radioactive HPLC profiles obtained from acetonitrile extract. The decarboxylation indices were calculated in TIBA-treated and control plants.

The influence of TIBA on in vitro decarboxylation of IAA was studied by using horseradish peroxidase (HRP). The reaction media (3 ml) contained potassium phosphate buffer (10 mM pH 6.3), 1 μM HRP, 4.4 KBq (1-^{14}C)IAA, and unlabeled IAA to give a final concentration of 35 μM, and variable concentrations of TIBA. Acidic and basic isoenzymes of HRP were assayed. The reaction was carried out in open vials with constant stirring, at 30°C in darkness. At different times, up to 2 h after the beginning of the reaction (see Fig. 3), 50-μl aliquots of the media were taken and ^{14}C activity measured by liquid scintillation. Decarboxylation of IAA was calculated by subtracting the radioactivity at the different times from the initial value of radioactivity in the media (without HRP). Results were expressed as a percentage of the initial IAA. In the reaction conditions, the amount of $^{14}CO_2$ retained in the media was undetectable (Acosta et al. 1988). Since halogenated monophenols activate the HRP-catalyzed IAA oxidation (Robert et al. 1976, Sabater et al. 1983), TIBA was recrystallized before use to eliminate any contamination by these products. The purity of TIBA was checked by HPLC. Reverse-phase chromatography was performed with a C_{18} analytical μ-Bondapak column using isocratic elution with an ethanol–water (1:3) pH 3.5 (phosphoric acid) solution at a flow rate of 1 ml min^{-1}. Absorbance at 280 nm was monitored with a Waters LC Spectrophotometer. No contamination was detected in either original or recrystallized TIBA.

Chemicals and Radiochemicals

Radioactive IAA [(1-^{14}C)–IAA and (5-^{3}H)–IAA] was obtained from Amersham International (Amersham, Buckinghamshire, UK). Aqueous solutions were prepared according to Sánchez-Bravo et al. (1988). The radiochemical purity was checked by TLC and only unaltered solutions were used. Other reagents used were unlabeled IAA (Merck), TIBA (Calbiochem), and HRP type VI (two basic isoenzymes, RZ = A_{403}/A_{275} = 3.1) and type VIII (one acidic isoenzyme, RZ = 3.4) (Sigma).

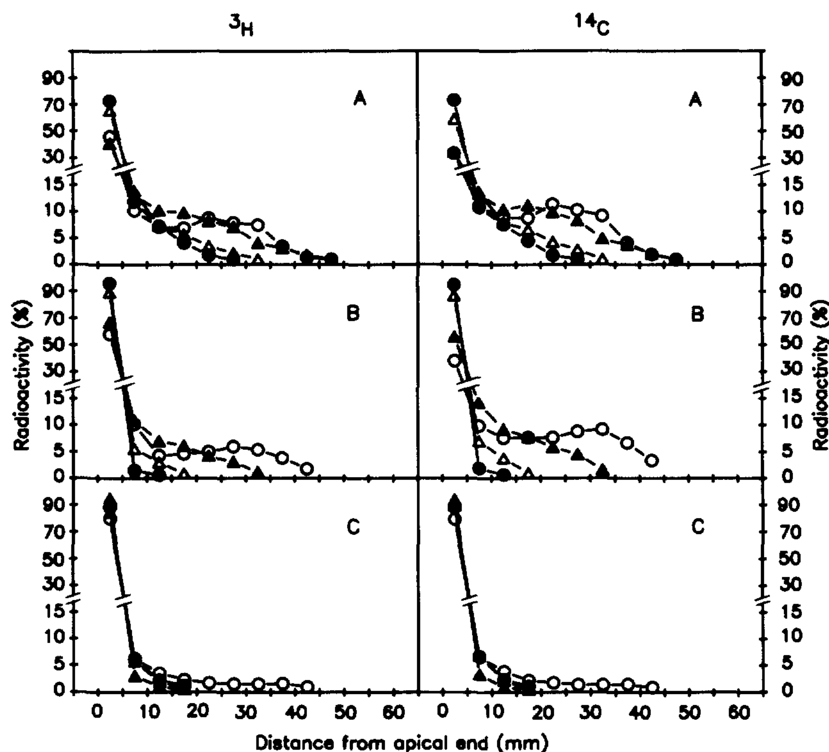

Fig. 1. Time-course of ^{3}H and ^{14}C movement in control and TIBA-treated lupin hypocotyls. Longitudinal distribution of ^{3}H (left) and ^{14}C (right) measured in the acetonitrile fraction after the application of (5-^{3}H)-IAA + (1-^{14}C)-IAA to the cut surface of decapitated seedlings obtained at different times: 1 h (●—●); 2 h (△—△); 4 h (▲—▲); 6 h (○—○). **(A)** Control plants; **(B, C)** decapitated seedlings pretreated with TIBA, 0.5 or 100 μM. Data correspond to bulked samples from four plants and are expressed as a percentage of ^{3}H and ^{14}C extracted with acetonitrile from the whole hypocotyl at each time.

Radioactivity Measurement

The activities of ^{14}C and ^{3}H were measured in a Rack Beta model 1211 liquid scintillation counter (LKB, Turku, Finland). The scintillation solution, and the channels used for the simultaneous counting of both isotopes in the acetonitrile extracts, were as described previously (Sánchez-Bravo et al. 1988). ^{14}C in the aliquots of the reaction media was measured using the standard channel (50–165).

Results and Discussion

Influence of TIBA on Basipetal IAA Transport

The distribution of ^{3}H and ^{14}C at different sampling times showed that the velocity of basipetal IAA transport decreased in the presence of TIBA (Fig. 1). In the absence of TIBA the velocity of IAA transport, as calculated from the position of radioactive fronts, was 30, 17.5, 12.5, and 8.3 mm h^{-1} at 1, 2, 4, and 6 h, respectively (Fig. 1A). In plants treated with 0.5 μM TIBA, the velocity was 15, 10, 8.7, and 7.5 mm h^{-1} over the same periods (Fig. 1B). Treatment with 100 μM TIBA produced a strong reduction in IAA transport during the first period (from 1–4 h) since the radioactive fronts did not move (Fig. 1C).

IAA transport intensity was also reduced by TIBA. Figure 1 shows that the percentage of both isotopes extracted at the same times from the first apical centimeter (assumed to be the zone in which radioactivity in the cortex is loaded into the transport system in the stele) was greater in the presence of TIBA as compared with the control, with 100 μM TIBA causing the highest retention of radioactivity in this zone. In accordance with the above, the amount of ^{3}H and ^{14}C exported at different times from the first apical centimeter was reduced in TIBA-treated plants (Fig. 2). Thus, in the control plants, the transport of both isotopes increased up to 4 h, thereafter ceasing. In TIBA-treated plants almost no radioactivity transport was measured during the first 2 and 4 h for treatment with 0.5 and 100 μM TIBA, respectively, the transport starting afterwards. During these lag periods, the radioactivity recovered below the first centimeter was less than 6% (for ^{3}H) and 5% (for ^{14}C) of that recovered from the whole hypocotyl. In 0.5 μM TIBA-treated plants, the radioactivity mobilized from the first centimeter at 4 h was smaller to that mobilized at 2 h in the control plants, while the radioactivity mobilized at 6 h after 100 μM TIBA treatment was similar to that mobilized at 1 h in control plants. From Fig. 2 it can be deduced that the $^{14}C/^{3}H$ ratio exported from the first centimeter at different times was unaffected by TIBA and varied between 0.51 and 0.54, very close to the ratio in the applied solution (0.51). This fact suggests that the species mo-

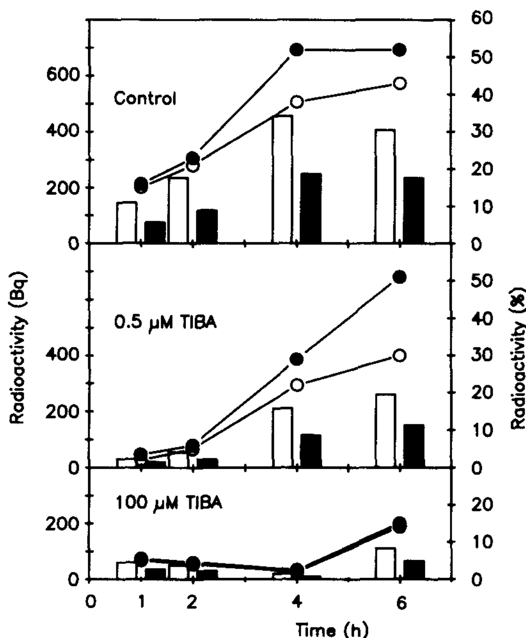

Fig. 2. Influence of TIBA on the intensity of IAA transport. The activity of ^{3}H and ^{14}C exported from the first apical centimeter at different time periods, calculated by adding the activity extracted with acetonitrile below this zone, is indicated for control and TIBA-treated plants. Data are expressed in absolute values (Bq per plant, histogram bars) and as a percentage of activity extracted in the whole hypocotyl (lines). ^{3}H, open; ^{14}C, closed bars and symbols. Data were calculated from the results obtained in the experiments in Fig. 1A (control), 1B (0.5 μM TIBA), and 1C (100 μM TIBA).

bilized down the hypocotyl was mainly unaltered IAA.

Considered in terms of the chemiosmotic theory, TIBA inhibits polar IAA transport by interfering with the efflux carrier in the transporting cells (Depta and Rubery 1984), the partial inhibition of IAA transport observed in plants treated with 0.5 μM TIBA (Figs. 1 and 2) means that this concentration was insufficient to saturate the binding sites of the efflux carrier. The effect produced by a higher concentration of TIBA (100 μM) (Figs. 1 and 2) supported this interpretation, since in this assay most of the radioactivity of the hypocotyl was recovered from the first apical centimeter (Figs. 1 and 2).

The small amount of radioactivity exported from the first centimeter during the lag period of transport induced by TIBA treatment (Fig. 2) was probably mobilized through a system other than the polar transport system. As reported (Sánchez-Bravo et al. 1991), polar transport of IAA occurs in the stele of lupin hypocotyls and the application of labeled IAA to the cortex + epidermis tissues results in a small percentage (<4%) of IAA being transported out the first apical centimeter. Therefore, it seems that the basipetal IAA movement down lupin hypocotyls has two components: (1) a minor component comprising about 5% of added IAA, which represents nonmediated diffusion of IAA, since this component was not inhibited by the highest TIBA concentration used (Figs. 1 and 2); and (2) the main component which is TIBA-sensitive and corresponds to the carrier-mediated IAA polar transport. The existence of these two components in the basipetal movement of IAA has been reported to occur in other plant materials (De la Fuente and Leopold 1972, Goldsmith 1977, Johnson and Morris 1989, Sheldrake 1979, Vardar 1964).

The appearance of a lag period with no transport of radioactivity following TIBA treatment, the duration of which increased with TIBA concentration (Fig. 2), suggests that the effect of TIBA may disappear, at least partially, with time. This might be due to a progressive depletion of TIBA in the application zone since, as has been reported, TIBA itself can be polarly transported (Thomson et al. 1973). Alternatively, TIBA could be metabolized during the lag period of IAA transport. However, though catabolism of TIBA in lupin hypocotyls cannot be discarded, most of the studies on plant metabolism of TIBA have shown that it is not readily metabolized (see review of Lawrence 1984).

Influence of TIBA on In Vivo and In Vitro IAA Decarboxylation

The in vivo decarboxylation of IAA by decapitated lupin seedlings was measured by the dual-isotope method. Table 1 shows the activities of ^{3}H and ^{14}C, from which the absolute and relative decarboxylation indices in the whole hypocotyl at different transport periods were calculated. As previously reported (Sánchez-Bravo et al. 1988, 1991), the variation with time of the decarboxylation index measured in the acetonitrile extract could be caused by several factors: an increase in IAA decarboxylation itself, an increase in acetonitrile-insoluble but NaOH-soluble conjugates of decarboxylation products (which diminish the decarboxylation index in the acetonitrile fraction), and, to a lesser extent, the variability between plant samples. Table 1 clearly indicates that 0.5 μM TIBA increased IAA decarboxylation as compared to the control. However, almost no decarboxylation was detected in 100 μM TIBA-treated plants.

A study of the influence of TIBA on the in vitro decarboxylation of (1-^{14}C)-IAA catalyzed by HRP (Fig. 3) showed that 100 μM activated the reaction

Table 1. Effect of TIBA on *in vivo* IAA decarboxylation by lupin hypocotyls.

	Control				0.5 μM TIBA				100 μM TIBA			
Time (h)	^{3}H (Bq)	^{14}C (Bq)	I (Bq)	Ir (%)	^{3}H (Bq)	^{14}C (Bq)	I (Bq)	Ir (%)	^{3}H (Bq)	^{14}C (Bq)	I (Bq)	Ir (%)
1	974	468	29	5.8	1264	464	181	28.0	1084	550	3	0.5
2	1166	505	90	15.1	1230	526	101	16.1	1298	640	22	3.3
4	1000	466	44	8.6	902	406	85	17.2	993	510	−4[a]	−0.8[a]
6	923	419	52	10.9	850	301	132	30.6	781	407	−9[a]	−2.5[a]

The activity of ^{3}H and ^{14}C extracted with acetonitrile from the whole hypocotyl at different times after IAA application is indicated. Data were obtained from the results of the experiment in Fig. 1A (control), 1B (0.5 μM), and 1C (100 μM TIBA), and are expressed as Bq per plant. From the activities of ^{3}H and ^{14}C, the absolute (I) and relative decarboxylation index (Ir) in the whole hypocotyl was calculated (see Materials and Methods).

[a] Negative values arise from the simultaneous counting channels used to calculate the activity of ^{3}H and ^{14}C by the double-isotope method.

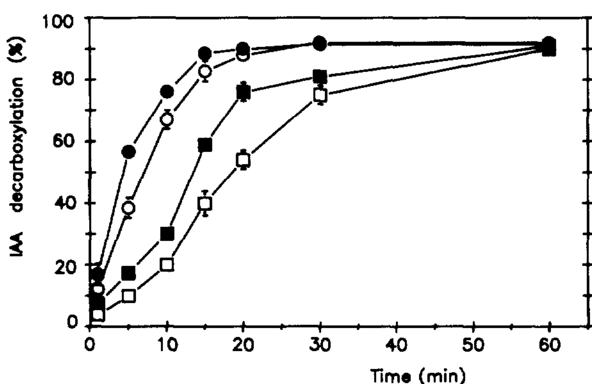

Fig. 3. Effect of TIBA on in vitro IAA oxidation catalyzed by HRP. Time course of IAA decarboxylation in the absence (open symbols) or presence (closed symbols) of 100 μM TIBA. Data obtained with acidic (squares) or basic (circles) isoenzymes of HRP are indicated. No further decarboxylation was detected after 60 min of reaction. Data represent the mean value of triplicate samples. Vertical bars represent ± SD when larger than symbols.

moderately, whereas 0.5 μM TIBA produced an undetectable effect in the assay conditions used. Differences in the time course of the reaction between acidic and basic isoenzymes of HRP are in agreement with other reports (Acosta et al. 1989, Yamazaki and Nakajima 1986) and remained essentially unchanged in the presence of TIBA (Fig. 3). The effect of TIBA on in vitro IAA oxidation catalyzed by enzyme extracts prepared form etiolated lupin hypocotyls according to Sánchez-Bravo and Núñez (1990) was similar to that produced by the HRP-catalyzed reaction (data not shown).

While the influence of TIBA on polar IAA transport is widely documented (see reviews of Goldsmith 1977 and Kaldewey 1984), the effect produced by this and other inhibitors of polar auxin transport on IAA metabolism has been scarcely studied, the results being controversial. Thus, Beffa et al. (1990) reported that auxin transport inhibitors, such as TIBA, 3,5-diiodo-4-hydroxybenzoic acid (DIHB), 3,5-diiodo-2-hydroxybenzoic acid (DIBA), and NPA, reduced in vitro IAA-oxidase activity but not the peroxidase activity of an enzyme preparation purified from maize roots. However, DIHB has been reported to act as a cofactor in the in vitro oxidation of IAA catalyzed by HRP, while it inhibited the in vivo decarboxylation of (1-^{14}C)-IAA by segments excised from cress roots (Robert et al. 1976). The results obtained with 100 μM TIBA in the present experiments are in accordance with this paradoxical effect of the polar transport inhibitors on IAA oxidation first noted by Robert et al. (1976), since TIBA inhibited the in vivo decarboxylation of IAA by decapitated seedlings (Table 1) and stimulated the in vitro decarboxylation of IAA by HRP (Fig. 3).

Two comments can be made with regards to these discrepant results. The first concerns the differences obtained in the in vitro assays. While HRP-catalyzed IAA oxidation was activated by TIBA (Fig. 3) and DIHB (Robert et al. 1976), these and other auxin transport inhibitors inhibited IAA-oxidase activity in maize roots (Beffa et al. 1990). However, TIBA did not inhibit IAA-oxidase in enzyme extracts from lupin hypocotyls (data not shown). Therefore, it seems likely that IAA oxidation catalyzed by enzyme preparations from different plant sources are affected in different ways by these compounds.

The second comment concerns the possible different influences of auxin transport inhibitors on in vitro vs. in vivo IAA decarboxylation. Though some data suggest that IAA-oxidases could be, at least partially, separate from peroxidases in some

plant tissues (Beffa et al. 1990, see review of Sembder et al. 1981), it is generally accepted that plant peroxidases are involved in the in vivo oxidation of IAA. In fact, in enzyme preparations from etiolated lupin hypocotyls, peroxidase activity showed a similar variation to IAA-oxidase activity along the hypocotyl as well as during hypocotyl ageing (Sánchez-Bravo and Núñez 1990). In addition, the same reaction products were identified in the in vitro IAA oxidation catalyzed either by an enzyme from lupin hypocotyls (Cuello et al. 1975) or HRP (Sabater et al. 1983) as in the in vivo IAA oxidation by segments or decapitated lupin seedlings (Sánchez-Bravo et al. 1990), indolyl-3-methanol being the major product in all cases. However, the extrapolation of the results obtained from the in vitro assays to the in vivo situation must be subject to severe scrutiny, since such factors as the presence of endogenous cofactors or the actual concentration of enzyme and IAA in the tissues are largely unknown or unchecked. In addition, in studying the influence of inhibitors on IAA oxidation, it does not appear to have been taken into account that the results obtained in vivo can be an indirect consequence of the effect produced by these compounds on the auxin polar transport system.

From the above, two possible interpretations can be made to explain the results here obtained with 0.5 μM TIBA, which produced no effect on the in vitro IAA decarboxylation in the assay conditions used in the experiment in Fig. 3, but which increased the in vivo IAA decarboxylation by decapitated seedlings (Table 1): (a) the actual concentration of enzyme, TIBA, and IAA in the tissues led to the activation of IAA oxidation in a similar way to that observed in vitro at a higher TIBA concentration (Fig. 3); (b) as a consequence of the transport reduction (Fig. 1), a higher concentration of IAA was maintained for a longer period of time in the first apical centimeter, reported to exhibit the highest decarboxylation capacity in 6-day-old lupin hypocotyls (Sánchez-Bravo et al. 1991). The second interpretation seems to be in accordance with previous results, which showed that IAA decarboxylation decreased as IAA transport increased (Sánchez-Bravo et al. 1988).

However, the effect produced by 100 μM TIBA on in vivo decarboxylation (Table 1) can hardly be explained through its in vitro effect (Fig. 3). Robert et al. (1976) came to the same conclusion in the study performed using DHIB. As reported (Beffa et al. 1987b), DHIB, like TIBA, inhibited auxin polar transport by interfering with the efflux carrier. Bearing in mind that 100 μM TIBA suppressed the polar transport of IAA (Figs. 1 and 2), the most probable explanation for this paradoxical effect of TIBA is that no IAA decarboxylation occurs in the transporting cells, where IAA presumably remains immobilized in the presence of saturating concentrations of transport inhibitors. In fact, according to Rubery (1987), the effect of TIBA on the efflux carrier increases the IAA sink capacity of the transporting cells.

The inability of the transporting cells to oxidize IAA contrasts with the high capacity of the stele and of the cortex + epidermis tissues to metabolize the auxin (Sánchez-Bravo et al. 1991). The existence of a lateral diffusion of polarly transported IAA from the transport system (located in the stele), to the outer tissues (cortex + epidermis) (Sánchez-Bravo et al. 1991), might explain the IAA decarboxylation observed during the polar transport of IAA in lupin hypocotyls (Sánchez-Bravo et al. 1988, 1991).

Though further experiments are needed to confirm that no oxidation of IAA occurs in the transporting cells, the possibility suggests new ways of looking at the intracellular pathway of IAA transport as well as the subcellular localization of peroxidases. The presence in the vacuole of basic peroxidases (Gross and Matile 1980, Mäder et al. 1986), highly efficient in IAA oxidation (Fig. 3), suggests that intracellular movement of IAA in the transporting cells might occur mainly in the cytoplasm, the involvement of the vacuole being irrelevant. On the other hand, the presence of soluble cytoplasmic IAA-oxidizing peroxidases in the transporting cells seems highly improbable. In fact, the pattern of glycosidation exhibited by plant peroxidases and their synthesis route (endoplasmic reticulum, Golgi) (Van Huystee 1987), points to either a vacuolar or an apoplastic localization. This agrees with data reported by Waldrum and Davies (1981), which suggest that little, if any, IAA oxidase is freely soluble in the cytoplasm.

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