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Carrier-mediated Auxin Transport*

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Summary. 1. Auxin (IAA) transport was investigated using crown gall suspension tissue culture cells. We have shown that auxin can cross the plasmalemma both by transport of IAA anions on a saturable carrier and by passive (not carrier-mediated) diffusion of the lipid-soluble undissociated IAA molecules (pK = 4.7). The pH optimum of the carrier for auxin influx is about pH 6 and it is half-saturated by auxin concentrations in the region of 1–5 μM. We found that the synthetic auxin 2,4D specifically inhibited carrier-mediated IAA anion influx, and possibly also efflux. Other lipid-soluble weak acids which are not auxins, such as 3,4-dichlorobenzoic acid, had no effect on auxin transport. By contrast, we found that TIBA, an inhibitor of polar auxin transport in intact tissues inhibited only the carrier-mediated efflux of IAA.

2. When the pH outside the cells is maintained below that of the cytoplasm (pH 7), auxin can be accumulated by the cells: In the initial phase of uptake, the direction of the auxin concentration gradient allows both passive carrier-mediated anion influx (inhibited by 2,4D) and a passive diffusion of undissociated acid molecules into the cells. Once inside the cytoplasm, the undissociated molecules ionise, producing IAA anions, to a greater extent than in the more acidic extracellular environment. Uptake by passive diffusion continues as long as the extracellular concentration of undissociated acid remains higher than its intra-cellular concentration. Thus, the direction of the auxin anion concentration gradient is reversed after a short period of uptake and auxin accumulates within the cells. The carrier is now able to mediate passive IAA anion efflux (inhibited by TIBA) down this concentration gradient even though net uptake still proceeds because the carrier is saturable whereas passive diffusion is not.

3. Auxin "secretion" from cells is regarded as a critical step in polar auxin transport. The evidence which we present is consistent with the view that auxin "secretion" depends on a passive carrier-mediated efflux of auxin anions which accumulate within the cells when the extra-cellular pH is below that of the cytoplasm. The implications of this view for theories of polar auxin transport are discussed.

Introduction

The polar transport of auxin through shoot tissues probably involves a passive uptake of auxin into the cells and a preferential "secretion" of

* *Abbreviations:* IAA, indol-3-yl acetic acid; 2,4D-dichlorophenoxyacetic acid; TIBA, 2,3,5-tri-iodobenzoic acid; PIPES, piperazine-NN′-bis-2-ethane sulphonic acid.

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auxin from the basal ends of the cells (eg. Hertel and Leopold, 1963; Hertel and Flory, 1968; Goldsmith and Ray, 1973). Indirect evidence and theoretical arguements suggest that this "secretion" of auxin may depend on a specific auxin carrier within the plasmalemma (Hertel and Leopold, 1963; Hertel *et al.*, 1969).

It is difficult to investigate the uptake and efflux of auxin in any detail at the cellular level in the intact tissues and tissue segments used for the study of polar auxin transport. Cells grown in suspension culture provide a much more convenient experimental system (Rubery and Sheldrake, 1973) and, although there can be no net polar transport of auxin by such cells, the mechanisms of auxin uptake into and of the efflux from these cells may be similar to those involved in the polar transport of auxin by organised tissues.

We have shown previously (Rubery and Sheldrake, 1973) that there is a passive, non-metabolic accumulation of IAA by plant cells that occurs by diffusion of the lipid-soluble undissociated acid across the plasmalemma when the pH outside the cells is less than that of the cytoplasm; the uptake of IAA into the cells is greatly enhanced as the external pH is lowered. If undissociated molecules are the only permeant species considered, the equilibrium distribution, when equal concentrations of undissociated acid are present in the cells and in the extracellular solution, is given by:

$$\frac{\text{Total concentration IAA inside}}{\text{Total concentration IAA outside}} = \frac{1+\text{antilog}_{10}(\text{pH}_{\text{inside}}\text{-pK})}{1+\text{antilog}_{10}(\text{pH}_{\text{outside}}\text{-pK})}. \quad \text{(Eq. 1)}$$

The "pH outside" referred to in this equation is the pH in the immediate environment of the plasmalemma, which is lower than that of the bulk extracellular solution because of the presence of negatively charged polymers in the cell wall (Rubery and Sheldrake, 1973).

In this paper we now describe the properties of a saturable carrier system which mediates the passive transport of IAA anions into and out of the cells of suspension cultures of Virginia Creeper crown gall tissue. We have shown that the synthetic auxin 2,4D inhibits carrier-mediated IAA influx and possibly also, to a lesser extent, efflux. By contrast, TIBA which is an inhibitor of polar auxin transport, although not itself an auxin, inhibits only the efflux of IAA anions and not their uptake. The evidence presented below is consistent with the idea that auxin "secretion" depends on a passive carrier-mediated efflux of auxin anions which accumulate within the cells when the extracellular pH is below that of the cytoplasm.

Materials and Methods

PIPES, 2,4D and IAA were obtained from British Drug Houses Ltd., and the IAA was twice recrystallised from water before use; [1-^{14}C]IAA ammonium salt

(Specific activity 46 mCi m mol^{-1}) from the Radiochemical Centre, Amersham, Bucks., England; TIBA from the Eastman Kodak chemical company; 2,4-, 2,6-, 3,4-, and 3,5-dichlorobenzoic acids from Ralph N. Emmanuel Ltd. Morphactin (9-hydroxy-9-carboxymethylfluorene) was a generous gift from Dr. G. Mohr, Celamerck GmbH & Co. KG. 6507 Ingelheim/Rhein. Stock solutions of these compounds and their potassium salts were kept frozen. pH measurements were made with a Beckman Research pH meter.

The growth and maintainance of the *Parthenocissus tricuspidata* crown gall suspension culture has been described elsewhere (Rubery, 1972). Crown gall cells were chosen for this investigation because, unlike most tissue cultures, they do not depend on a supply of exogenous auxin for their growth. 2–3 week old cultures were used, the cells being washed with distilled water, suspended in fresh growth medium (30–80 mg fresh wt ml^{-1}) and pre-incubated for lh at 25° in a water bath fitted with a reciprocal shaker (120 strokes min^{-1}) before samples were taken with a wide-ended pipette for transport studies. Experiments were performed as follows: (A) as described previously (Rubery and Sheldrake, 1973) in which each individual measurement was obtained from a single incubation (total vol 6 m) in a boiling tube kept in the shaking water bath. Individual incubations forming part of a series were performed in random order and, where appropriate, statistical significance of the results was evaluated by "Students" t test. (B) When the time-course of uptake was followed, it was usually more convenient sequentially to remove 2 ml aliquots for measurement of radioactivity from an incubation in a 25 ml conical flask whose contents were stirred magnetically and maintained at 25° in a water bath. Because of the greater agitation, method B gave higher rates of uptake than method A. In either case, the incubation mixture consisted of stock cell suspension (or crown gall growth medium in experiments which involved the use of cells which had been pre-loaded with 2,4D or TIBA and then filtered under reduced pressure through Whatman Glass-fibre discs), buffer (0.2 M Na_2HPO_4 + 0.1 M citric acid which when appropriately mixed covers the pH range 2.8–8.2) and distilled water or test solution (4:1:1 by vol). After a further 3 min pre-incubation, radioactive IAA was added from a stock solution (10 μCi ml^{-1}) together with any test substance whose presence was not required during the pre-incubation. Termination of uptake by rapid filtration of the cells, weighing and measurement of radioactivity were performed as previously described. Over the periods used for the incubations, (20 s–10 min) very little IAA is metabolized (Rubery and Sheldrake, 1973).

Results and Discussion

The Effect of pH on IAA Uptake into Crown Gall Cells

The existence of a saturable carrier which could mediate IAA anion transport into crown gall cells was suggested by the observed non-linear relationship at pH 6.5 between low external concentrations of IAA (0–2 μM) and the rate of IAA uptake. At higher IAA concentrations, the relationship was linear (Rubery and Sheldrake, 1973). Also, above pH 5.8, the pH dependence of IAA uptake from a 1 μM solution deviated from the titration-like curve expected if diffusion of undissociated acid molecules across the membrane were the only means of uptake. No such deviation was seen at 50 μM-IAA and the curve in this case ran more nearly parallel to the dissociation curve of IAA (Rubery and Sheldrake, 1973); a carrier which was saturated by 50 μM-IAA would leave diffusion as the major uptake mechanism.

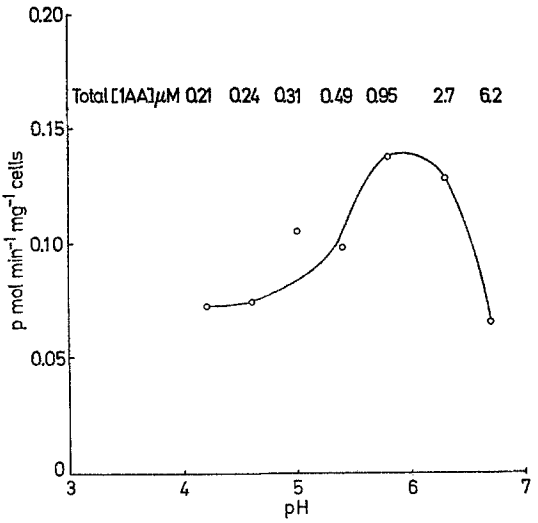


Fig. 1. The effect of pH on the rate of IAA uptake from solutions containing a constant concentration of undissociated IAA (0.19 μM). Uptake was measured using method A. 0.06 μCi radioactivity was present at each pH, appropriate amounts of non-radioactive IAA being added to maintain the concentration of undissociated acid at 0.19 μM. The total concentration of IAA present at each pH is shown on the figure

The effect of pH on IAA uptake was also studied in an experiment in which the concentration of the undissociated form of IAA was held constant at 0.19 μM (calculated using a value for the effective pK in the wall of 5.2) so that uptake due to simple diffusion would be the same at each pH tested (Fig. 1). The operation of the anion carrier is apparent between pH 5 and pH 6.7 with a maximum at about pH 6 suggesting that the pH optimum is in this region. At pH 4.3, where IAA is only slightly ionised, the relationship between IAA uptake and IAA concentration was linear up to 500 μM because of the ineffectiveness of the carrier at this pH (Rubery and Sheldrake, 1973).

The Effect of 2,4D on the Relationship between IAA Concentration and Uptake into Crown Gall Cells

When the effect of simultaneous addition of 2,4D (42 μM) on IAA uptake (0–200 μM concentration range) was studied at pH 6.4 (Fig. 2), it was found that inhibition of the initial rate of IAA uptake (see also Fig. 4) occurred up to 15 μM-IAA but that 2,4D was not inhibitory above this concentration. Similar curves were obtained at pH 5, although the inhibitory effect of 2,4D below 15 μM-IAA was less than at pH 6.4 (see

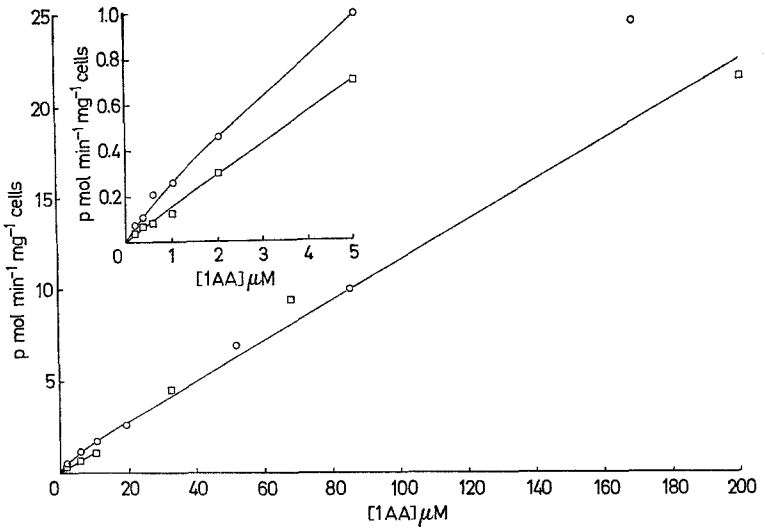


Fig. 2. The effect of 2,4D (42 μM) on the relationship between IAA concentration (0–200 μM) and the initial rate of IAA uptake at pH 6.5. Method A (2 min incubations) was used. The inset shows the range 0–5 μM-IAA on an expanded scale. —○— IAA alone; —□— IAA+2,4D

below). This is again consistent with the operation of a saturable carrier whose transport of IAA anions can be inhibited by 2,4D anions. Once the carrier is saturated, diffusion of the undissociated acid is quantitatively the major uptake mechanism on which 2,4D has no effect, provided that the pH gradient between cells and medium is not altered by a change in cytoplasmic pH (see p. 106). The concentration of IAA which half-saturates the carrier at pH 6.4 may be estimated as about 1–5 μM by extrapolating the linear portion of the uptake curve to zero IAA concentration in order to obtain the diffusion component of IAA uptake which is then subtracted from the total uptake (a curve of this type is plotted in Fig. 6a). (It is not possible to deduce from these data whether the 2,4D inhibition is kinetically competitive or non-competitive because of the errors inherent in the extrapolation procedure.)

The Effect of Varying 2,4D Concentration on IAA Uptake into Crown Gall Cells at Different pHs

The effect of the simultaneous addition of 2,4D on IAA uptake from a 1 μM solution at different external pHs is shown in Fig. 3. At pH 6.5, the maximum inhibition of the rate of IAA uptake is 50%; at pH 5 it is 25% and at pH 4, 20%. The per-cent inhibition approaches a constant

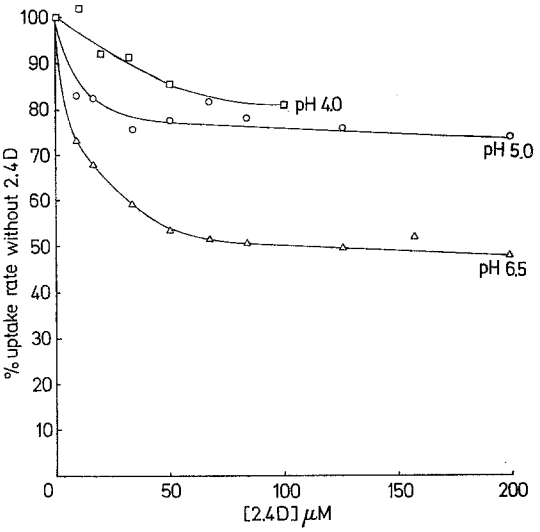


Fig. 3. The effect of 2,4D (0–200 μM) on initial rate of IAA uptake from a 1 μM solution at different pHs. Method A (2 min incubations) was used —△— pH 6.5; —○— pH 5.0; —□— pH 4.0

plateau value with increasing concentrations of 2,4D, at least at the two higher pHs. The proportion of total IAA uptake which is *not* inhibited by 2,4D thus increases as the external pH is lowered and may be identified with diffusion of undissociated IAA molecules. The failure of Sabnis and Audus (1967) to observe 2,4D-inhibition of IAA uptake into maize mesocotyl segments may perhaps be attributable to their use of an incubation medium buffered at pH 5.0 containing 10 μM-IAA as the lowest concentration tested.

The 2,4D-sensitive component of uptake is probably carrier-mediated anion influx. However, a complication in the interpretation of such experiments is the possibility that the entry of 2,4D (pK = 2.8), a stronger acid than IAA (pK = 4.7), may overcome the intra-cellular buffering capacity and lower the cytoplasmic pH. This would reduce the pH gradient between medium and cytoplasm and thus lower the net uptake of undissociated acid by the diffusion mechanism. Such an effect would become increasingly significant at more acidic external pHs because of increased diffusion uptake of 2,4D. Clearly not all inhibition of IAA uptake by 2,4D can be due to this mechanism as *less* inhibition is obtained at pH 5 than at pH 6.5 which is closer to the likely pH optimum of the carrier (Fig. 1) and where a higher relative anion concentration is present. At pH 4, although Fig. 1 indicates that the carrier is inoperative,

nevertheless there is still upto 20% inhibition by 2,4D (Fig. 3) which could be due to a lowering of the internal pH. This effect may also account for the slight inhibition (up to 15% of IAA uptake at pH 6.5) by monochloroacetic acid (pK = 2.85) which has no auxin activity (Fawcett *et al.*, 1956). Method B, which gives higher uptake rates than method A, was used and the inhibition was only apparent at monochloroacetic acid concentrations in excess of 60 μM.

The Specificity of 2,4D Inhibition of IAA Uptake into Crown Gall Cells

In order to examine the specificity of the 2,4D inhibition, the effects of 2,4-, 2,6-, 3,4- and 3,5-dichlorobenzoic acids were tested under the same experimental conditions (Method A; 42 μM at pH 6.5) as used for the studies with 2,4D. None of these substituted benzoic acids inhibited IAA uptake under these circumstances showing that the effect of 2,4D is not simply due to non-specific consequences of the entry of a lipid soluble acid into the cells (Table 1). 2,4- and 3,4-dichlorobenzoic acids have no auxin activity; the 2,6-derivative is moderately active in bioassays although less active than 2,4D (Jönsson, 1961; Zimmerman *et al.*, 1952). The 3,5-derivative has been reported to be slightly toxic to germinating seedlings (Jones *et al.*, 1951).

Table 1. The effect of the simultaneous addition of chlorinated benzoic acids and of 2,4D on the uptake of 1 μM [1-^{14}C]IAA by crown gall cells at pH 6.5. Method A was used. The uptake period was 3 min

Addition	Counts $min^{-1} \cdot mg^{-1} \cdot$ cells min^{-1} ± SE (4 determinations)	
Control	20.41 ± 1.13	
2,4D	13.59 ± 0.24	($p < 0.01$)
2,4-dichlorobenzoic acid	22.46 ± 0.60	(ns)
2,6-dichlorobenzoic acid	22.16 ± 1.21	(ns)
3,4-dichlorobenzoic acid	22.16 ± 1.77	(ns)
3,5-dichlorobenzoic acid	24.35 ± 0.36	($p < 0.02$)

The Effect of 2,4D Addition during the Course of IAA Uptake into Crown Gall Cells

When uptake is initiated by addition of IAA to a cell suspension at a pH below that of the cytoplasm, the concentrations of both undissociated acid and of auxin anions are greater outside the cells than inside; the undissociated acid will enter by diffusion down the concentration gradient and if an anion carrier is present, there will also be a passive influx of anions. As uptake proceeds, the internal concentration of anions will rise above the external concentration because of the continuing

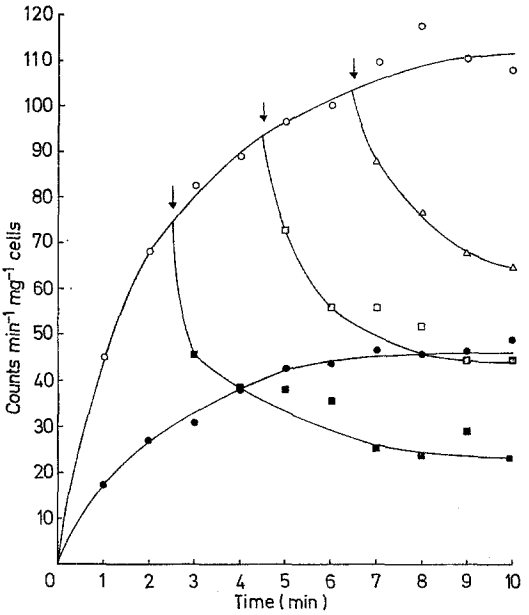


Fig. 4. The effect of "In flight" additions of 2,4D (50 μM final concentration) on the time course of IAA uptake from a 1 μM solution at pH 6.2. Method B was used. —○— Complete time course without 2,4D; the arrows indicate times of 2,4D addition (10 μl or less). —●— at time 0; —■— after $2^1/_2$ min; —□— after $4^1/_2$ min; —△— after $6^1/_2$ min. 2 ml aliquots were removed for each measurement

passive uptake of the undissociated acid which will ionise once it is in the cytoplasm (pH 7–7.2; Caldwell, 1956) to a greater extent than in the more acidic extra-cellular environment. Thus if the carrier were reversible, *efflux* of anions could now take place down the anion concentration gradient which has been established in this way. Such an efflux would reduce, but not overcome, the pH-dependent accumulation of IAA because diffusion is not saturable whereas carrier-mediated transport is. Hence, carrier-mediated anion efflux should be possible while net accumulation of IAA by the diffusion mechanism is still proceeding. A consequence of this efflux would be a greater availability of carrier sites on the outer face of the plasmalemma than on the cytoplasmic face. "In-flight" addition of 2,4D to the incubation medium during net IAA accumulation would allow carrier-mediated uptake of 2,4D anions down a concentration gradient which would alter this distribution of the carrier by increasing its availability to IAA anions on the cytoplasmic face of the plasmalemma and thus stimulate the efflux of IAA.

Results consistent with such a "counter-flow" were obtained (Fig. 4): There was an immediate loss of radioactivity from the cells following 2,4D addition during IAA uptake.

With the aid of Eq. 1 (Introduction), assuming a cytoplasmic pH = 7 and a vacuolar pH = 5.8, and assuming that the vacuole occupies 95% of the cells' internal volume, it can be calculated that both the concentration of IAA anions and the total concentration of IAA is greater in the cytoplasm than in the medium after 1 min uptake. The concentration of undissociated IAA molecules remains greater in the medium than in the cytoplasm (see Fig. 10). After 10 min, when uptake had almost ceased, the calculated concentrations of undissociated acid on either side of the plasmalemma were about equal indicating that the system was close to equilibrium (Fig. 10). The results of this experiment are consistent with the operation of a reversible carrier which can mediate both IAA influx and efflux. The effect of 2,4D is specific and is not due to internal pH drop as "in-flight" additions of 50 μM-3,4 dichlorobenzoic acid, which is not an auxin (Jönsson, 1961), has no effect under these conditions. Also 2,4D has no effect on uptake at pH 4, rather than pH 6.2, or if an initial concentration of 50 μM-IAA is used (when diffusion is the major uptake mechanism) rather than 1 μM. More direct evidence for a carrier-mediated efflux of IAA is presented below.

The Effect of TIBA on Net IAA Uptake into Crown Gall Cells at pH 6.5

The effects of the inhibitor of polar transport, TIBA, are quite distinct from those of 2,4D. TIBA appears to inhibit auxin anion export but does not interfere with anion influx. Cells pre-incubated for 3 min in 42 μM-TIBA do not differ from controls in their *initial rate* of IAA uptake from a 1 μM solution at pH 6.5 but, after one minute-or-so, increasing stimulation of *net* IAA entry into the cells occurs (Fig. 5). The first three time-points represent triplicate determinations. The stimulation of net uptake by TIBA is significant (p less than 0.01) after 1 minute. The pre-incubation time is not critical. The same stimulation of net uptake occurs if the cells are pre-loaded with TIBA and then resuspended in a TIBA-free medium (Table 2) or if phosphate/citrate buffer is replaced with PIPES buffer. In preliminary experiments, we found that the only other compound which significantly stimulated net uptake was morphactin which is also an inhibitor of polar auxin transport although its mechanism of action may be different to that of TIBA (Thomson and Leopold, 1974). The effect on net uptake of including TIBA in the pre-incubation medium over a range of low concentrations of IAA (0–1.6 μM) at pH 6.4 is shown in Fig. 6a; this figure also shows the curve obtained by subtraction of the diffusion component from the total uptake observed in the absence of

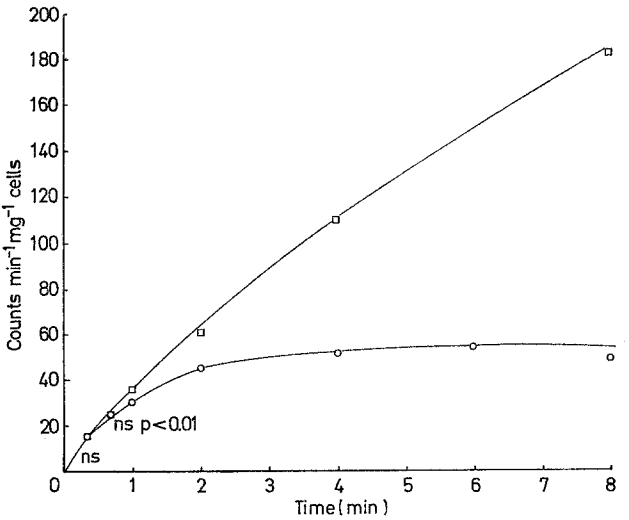


Fig. 5. The effect of TIBA (42 μM) on the time course of IAA uptake from a 1 μM solution at pH 6.6 Method A was used. The cells were pre-incubated for 3 min in the presence and absence of TIBA before radioactive IAA was added. —○— IAA alone; —□— IAA+42 μM-TIBA

Table 2. The effect of pre-loading with TIBA on the uptake of 1 μM [1-^{14}C]IAA by crown gall cells at pH 6.5. Cells were pre-incubated for 3 min in the presence and absence of TIBA, rapidly filtered, and then transferred to radioactive incubation media which contained no TIBA. Uptake was terminated after 3 min. In the control experiment, the radioactive IAA was added directly to the cell suspensions at the end of the 3 min pre-incubation, Method A was used

	Counts min^{-1} mg^{-1} cells after 3 min uptake ± Se (4 determinations)
Control experiment	
1 μM [1-^{14}C]IAA	51.72±0.51
1 μM [1-^{14}C]IAA+42 μM-TIBA	81.0 ±0.90 ($p<0.01$)
Pre-loading experiment	
1 μM [1-^{14}C]IAA	49.68±0.96
1 μM [1-^{14}C]IAA+42 μM-TIBA	90.18±1.35 ($p<0.01$)

TIBA. In a separate experiment, it was found that the amount of radioactivity in the cells at the time at which stimulation of net uptake by TIBA was first evident, appeared to be a linear function of the initial external concentration of IAA (Fig. 6b). At this time, the corresponding

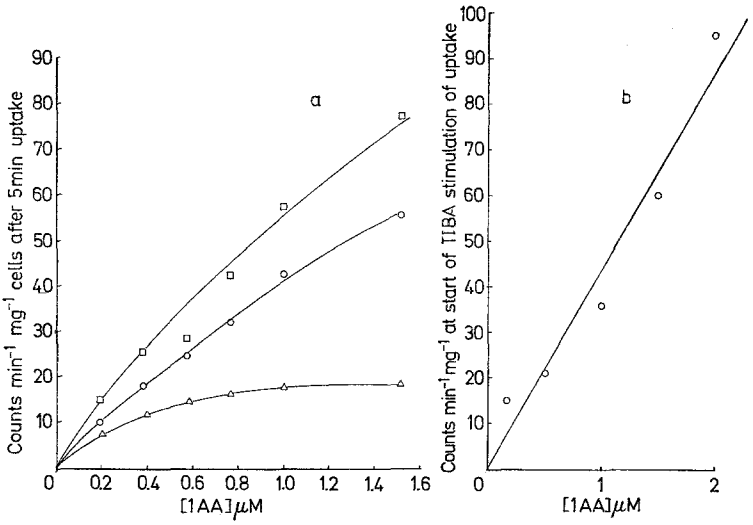


Fig. 6. a) The effect of TIBA (42 μM) on the relationship between IAA concentration (0–1.6 μM) and the total uptake of IAA after 5 min incubation at pH 6.4. Method A was used. —○— IAA alone; —□— IAA+TIBA; —△— Derivative curve obtained by subtraction of diffusion component from the uptake observed in the absence of TIBA. b) The relationship between the amount of radioactivity in the cells at the time at which TIBA-stimulation of net uptake starts and the initial external concentration of IAA. Method A was used

estimated concentration of IAA in the cytoplasm is always greater than the external concentration of IAA. Since TIBA has no effect on the extent to which IAA is metabolised in the course of these experiments, these results suggest that TIBA is exerting its effect via inhibition of passive efflux of IAA anions. The kinetic data suggest that TIBA can interfere with IAA binding to the carrier at the cytoplasmic face of the membrane but that it is not itself exported on the same carrier; if TIBA were leaving the cells on the carrier, the increased availability of the carrier on the outside of the membrane should lead to increases in the *initial* rate of IAA uptake.

If an initial external concentration of 50 μM-IAA rather than 1 μM is used, no stimulation of net uptake by TIBA is seen because effects on the transport of IAA anions are now small in comparison with the high uptake of undissociated acid by diffusion (see Fig. 8). No stimulation of net uptake, such as that which could be observed with TIBA, was obtained using inhibitors of ATP synthesis (Antimycin A, Na_2HAsO_4, NaF). Such stimulation might occur if IAA efflux were active secretion against a concentration gradient. The use of acidic inhibitors such as

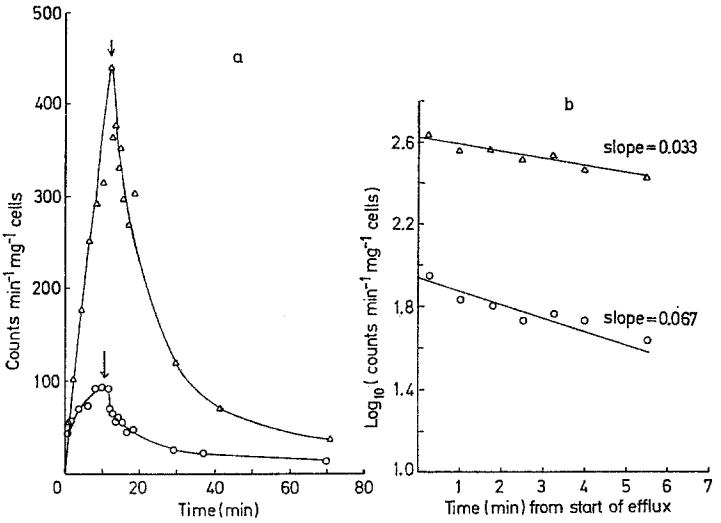


Fig. 7a and b. The effect of TIBA (42 μM) on IAA efflux. a) Time course of influx from a 1 μM-IAA solution at pH 6.5 and efflux into a non-radioactive medium at pH 6.5; —○— IAA alone; —△— IAA+TIBA. b) Relationship between $\log_{10}$ radioactivity in cells and time after start of efflux. —○— IAA alone; —△— IAA+TIBA. Method B was used

azide was avoided because of possible complications due to cytoplasmic pH changes.

The Effect of TIBA on IAA Efflux from Crown Gall Cells

The inhibition of efflux by TIBA has also been demonstrated directly (Fig. 7a). Cells were loaded with radioactive IAA from a 1 μM solution at pH 6.5 in the presence or absence of 42 μM-TIBA for 10 min and then filtered and rapidly transferred to a non-radioactive medium at the same pH plus or minus TIBA. Samples of cells were removed for measurement of radioactivity during both uptake and efflux. The loss of radioactivity from the TIBA-treated cells is much slower than from the control. This effect is particularly striking because there is more IAA in the TIBA-treated cells than in the control at the time at which efflux starts. In both cases, the efflux is first order for at least 5 minutes (Fig. 7b).

The Effect of Varying TIBA Concentration on Net IAA Uptake into Crown Gall Cells at Different pHs

If TIBA interacts with the carrier only at the cytoplasmic face of the plasmalemma which is exposed to a bulk phase at pH 7, then the

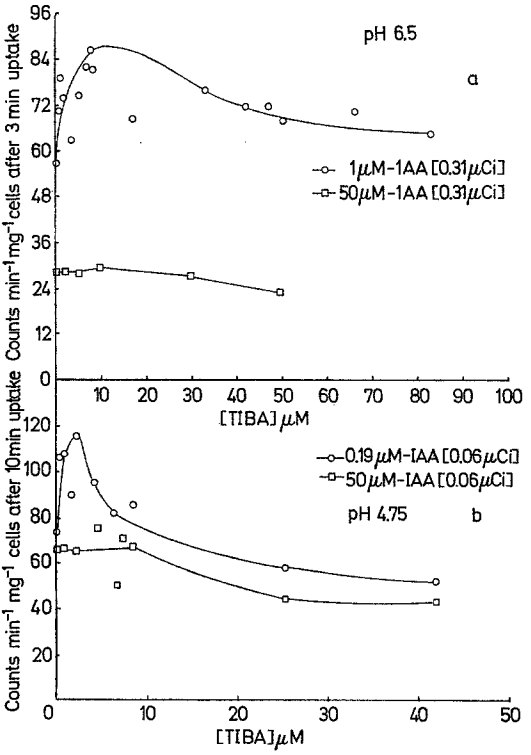


Fig. 8. The relationship between TIBA concentration and the net uptake of IAA from 0.19 μM and 50 μM solutions at pH 4.75 (8b) and from 1.0 μM and 50 μM solutions at pH 6.5 (8a). Method A was used. At pH 4.75, 0.06 μCi radioactivity was present and at pH 6.5, 0.31 μCi

stimulation of the net IAA uptake brought about by diffusion of TIBA into the cells during pre-incubation at pH 6.5 should also occur at more acidic external pHs, below the likely pH optimum of the carrier for uptake (Fig. 1), where little carrier-mediated anion uptake occurs and where the effect of 2,4D is correspondingly small (Figs. 1 and 3). We found this to be the case (Fig.8). At pH 4.75, TIBA stimulates net IAA uptake from a 0.19 μM solution containing 0.06 μCi radioactivity (maximum stimulation at 2.5 μM-TIBA) but not from a 50 μM-IAA solution containing the same amount of radioactivity. In the latter case, a small decrease in IAA efflux would not be detectable against a high background of diffusion uptake. Higher concentrations of TIBA in the pre-incubation medium inhibit the initial rate of uptake irrespective of IAA concentration (Fig. 8a); the level of TIBA (pK = 2.6) inside the cells may be

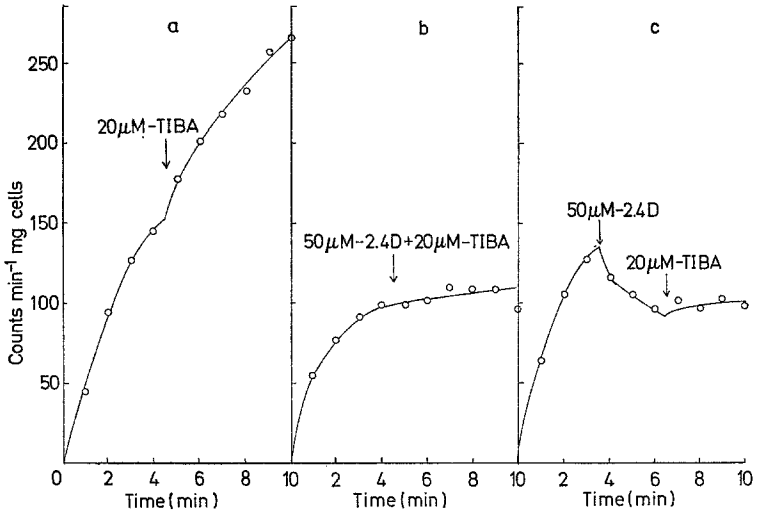


Fig. 9a—c. The effect of "In flight" additions of 2,4D (50 μM final concentration) and TIBA (20 μM final concentration) on the time course of IAA uptake from a 1 μM solution at pH 6.2. Method B was used. a) TIBA added after $4^1/_2$ min; b) 2,4D and TIBA added simultaneously at $4^1/_2$ min; c) 2,4D added at $3^1/_2$ min followed by TIBA at $6^1/_2$ min. 2 ml aliquots were removed for each measurement

sufficient to lower the pH of the cytoplasm. This inhibition also occurs if the cells are pre-loaded with 42 μM-TIBA at pH 4.75 and then re-suspended in a medium, at the same pH, containing radioactive IAA (1 μM) but no TIBA. A similar biphasic effect of TIBA on IAA uptake from a 10 μM solution at pH 5.0 has been reported for maize mesocotyl segments. 10 μM TIBA stimulated IAA uptake whereas higher concentrations were inhibitory (Sabnis and Audus, 1967).

At pH 6.5, using 0.31 μCi radioactivity, similar curves result, but the TIBA concentration in the pre-incubation medium which maximally stimulates uptake from 1 μM-IAA solution is increased from 2.5 μM to 10 μM (Fig. 8b), probably because less TIBA diffuses into the cells at this higher pH. Inhibition of the initial rate of uptake owing to a lowered cytoplasmic pH is less apparent for the same reason. Thus, at external TIBA concentrations upto 80 μM, uptake from 1 μM-IAA at pH 6.5 does not fall below that found in the absence of TIBA (Fig. 8b).

The Effect of TIBA and 2,4D Addition during the Course of IAA Uptake into Crown Gall Cells

"In-flight" additions of TIBA (20 μM-TIBA added after $4^1/_2$ min incubation) increase the rate of uptake from 1 μM-IAA solution at pH 6.2

(Fig. 9a). If increasing concentrations of TIBA, up to 50 μM, are added simultaneously with 42 μM-2,4D after $3^1/_2$ min, the stimulatory effect of 2,4D influx on IAA efflux (see Fig. 4) is reduced and finally overcome (Fig. 9b; the curve obtained with 20 μM-TIBA is shown). Similarly, if TIBA is added 3 min after 42 μM-2,4D the net IAA efflux is again reversed by 20 μM- or higher concentrations of TIBA (Fig. 9c). These observations can be interpreted to provide further evidence for TIBA inhibition of IAA anion efflux on a reversible carrier down a concentration gradient: The 2,4D influx increases carrier availability on the cytoplasmic side of the membrane; TIBA can inhibit the export of IAA on the carrier.

Attempted Demonstration of an Effect of 2,4D on IAA Efflux from Crown Gall Cells

Experiments were carried out in an attempt to detect an effect of 2,4D on IAA efflux. Because the main effect of 2,4D is to inhibit carrier-mediated IAA-anion influx, cells were pre-loaded (using method B) with 2,4D supplied at different external concentrations for 3 min at various pHs and then resuspended in a medium (pH 6) containing 1 μM-IAA but no 2,4D. Clear-cut results have not so far been obtained; there may be a small TIBA-like effect of 2,4D, but this is difficult to analyse because of the probable lowering of the internal pH which is brought about by the concentrations of 2,4D which appear to be required to interact with the carrier at the inner surface of the membrane. Using method A, cells pre-loaded with 42 μM-2,4D at pH 6.5 show no significant difference from controls whereas cells pre-loaded with 42 μM-TIBA show the usual increased net rate of uptake (Table 2) suggesting that the carrier interacts much more readily with TIBA than with 2,4D at the cytoplasmic side of membrane. It is difficult to be sure whether the small increase in IAA uptake observed at low 2,4D pre-loading concentrations reflect a change in the initial rate of uptake or in the net rate as is the case for TIBA. This is an important distinction because an increased initial rate would imply that 2,4D can leave the cells on the carrier. Further experiments are being carried out to clarify this point.

General Discussion

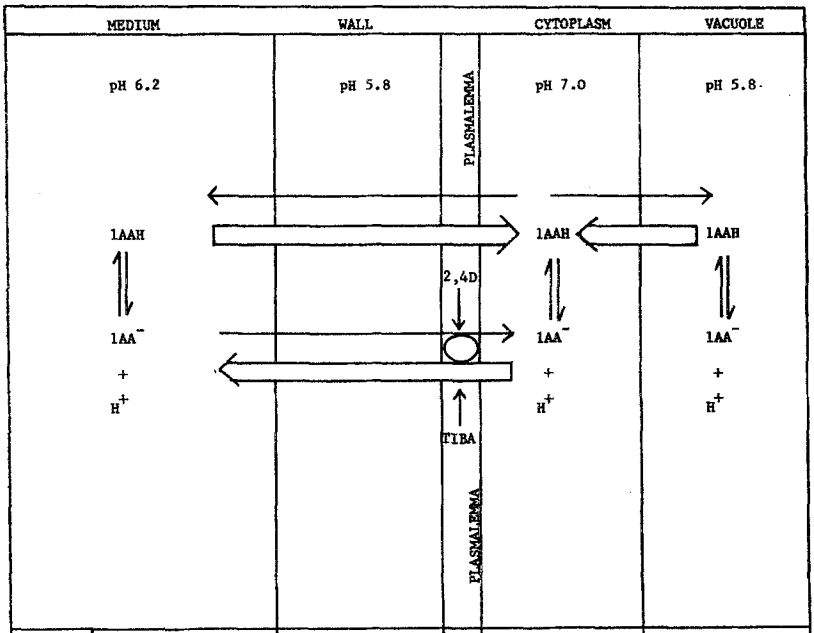
The "secretion" of IAA from cells is recognised to depend on active metabolic processes and is thought to require the participation of a specific carrier (Goldsmith, 1969; Hertel *et al.*, 1969). If this "secretion" involves the movement of IAA anions out of the cells into regions of higher anion concentration, then the transport would have to be coupled to some spontaneous process such as ATP hydrolysis or the movement of

a different type of molecule down a concentration gradient in order to achieve an overall decrease in free energy (Hertel and Leopold, 1963; Goldsmith and Ray, 1973). The term "active transport" is usually regarded as applying to such processes. On the other hand, if movement of IAA anions out of the cells is by passive transport down a concentration gradient, it will be accompanied by a decline in free energy. ATP hydrolysis might still be directly involved in the mechanism of transport but in energetic terms, it would be only facilitating a process which is spontaneous in its own right.

If this type of "downhill" transport is to take place, energy must be supplied to create the necessary concentration gradient. We have indicated earlier that the passive entry of IAA that occurs when the external pH is below that of the cytoplasm leads to a higher internal than external concentration of auxin anions (shown diagrammatically in Fig. 10). The energetic drive for this accumulation of IAA is supplied by the difference in chemical potential of H^+ ions across the plasmalemma. On this model, the exit of IAA anions from the cells is not active in itself, but depends indirectly on metabolic processes, including the hydrolysis of ATP, which are concerned with the regulation of cytoplasmic pH and thus with the maintainance of the pH gradient across the plasmalemma. One aspect of this would be the removal of H^+ ions which are liberated in the cytoplasm as a result of the dissociation of newly entered IAA molecules in order that the pH of the cytoplasm is not lowered as a consequence of IAA entry. In this sense, the efflux of IAA anions may be regarded as proton-driven.

The simplest interpretation of the data presented is in terms of the model illustrated in Fig. 10 in which an auxin anion carrier is situated within the plasmalemma and can passively carry anions into or out of the cell down a concentration gradient. However, the properties of the carrier must be different at the external and internal faces of the membrane: IAA uptake into the cells is negligible at external pHs of 7 or greater (Rubery and Sheldrake, 1973) and yet considerable carrier-mediated anion efflux occurs from the cytoplasm (pH 7) into the medium. The inhibitory effects of 2,4D on IAA uptake (Figs. 2 and 3) suggest that 2,4D can bind to the carrier at the external face of the membrane; it may also bind at the internal face. By contrast, TIBA does not appear to affect carrier-mediated IAA influx suggesting that it binds to the carrier only at the internal face of the membrane.

The question remains open of whether there may be active export of IAA against a concentration gradient in certain situations: Circumstances in which the cytoplasmic concentration of auxin anions could be lower than that of the extracellular region include the early stages of IAA uptake and the possibility that local microenvironments might provide a



species	concentration (μM)		concentration (μM)		concentration (μM)		concentration (μM)	
	1 min	10 min	1 min	10 min	1 min	10 min	1 min	10 min
$1AA^-$	0.956	0.936	0.914	0.895	5.88	14.63	0.36	0.895
1AAH	0.03	0.029	0.073	0.07	0.031	0.068	0.031	0.077
total 1AA	0.987	0.965	0.987	0.965	5.919	14.71	0.391	0.972

Fig. 10. A diagrammatic representation of IAA movement into and out of crown gall cells showing diffusion of undissociated acid and carrier-mediated transport of IAA anions. The width of the horizonta llines indicates approximately (not to scale) the relative magnitudes of the different components of the transport. The calculations on the table arise from Fig. 4 and are based on the following assumptions. pH cytoplasm = 7; pH vacuole = 5.8. Volume vacuole/volume cytoplasm = 20. The dry wt of the cells is 5% of the fresh wt. The concentrations in the different compartments are calculated after 1 min (counts min^{-1} mg^{-1} cells = 40) and after 10 min (counts min^{-1} mg^{-1} cells = 100)

reduced concentration if IAA anions in the immediate vicinity of a carrier. The failure of inhibitors of ATP production to modify transport in our experiments suggests that active transport of IAA does not occur to a significant extent in crown gall cells under our experimental conditions.

The polarity of auxin transport in shoot tissues is thought to be explicable by a selective localisation of an "auxin secreting system" in the plasmalemma at the basal ends of the cells (Hertel and Leopold, 1963; Hertel and Flory, 1968; Goldsmith and Ray, 1973). TIBA inhibits the secretion of auxin and leads to an increased immobilisation of auxin within the tissues (Hertel and Leopold, 1963; Christie and Leopold, 1965; Hertel and Flory, 1968). The "auxin secreting system" probably involves an auxin carrier which is specific for active auxins (Hertel *et al.*, 1969). IAA binds specifically to particulate fractions from homogenates of coleoptiles with a binding constant in the range 1–10 μM and this binding is inhibited by TIBA and synthetic auxins (Hertel, Thompson and Russo, 1972).

If it is assumed that the auxin carrier involved in polar auxin transport and the auxin anion-carrier in callus cells are similar or identical, the mechanism of polar auxin transport might be explicable as follows: When the pH within the cell walls is acidic, auxin enters the cells passively and accumulates within the cytoplasm. When the internal auxin anion concentration exceeds the external concentration, auxin efflux takes place on the anion carrier which is preferentially localised in the plasmalemma at the basal end of the cell. Some of this "secreted" auxin may be passively taken up again by the same cell, but some will diffuse through the cell walls and be taken up by neighbouring cells which will in turn "secrete" auxin preferentially through their basal ends leading to a net polar transport of auxin through the tissue. This transport would be active in the sense that it requires the expenditure of metabolic energy on control of the cytoplasmic pH. Polar auxin transport is inhibited in tissues kept in nitrogen or treated with metabolic poisons (Goldsmith, 1969).

This interpretation of the mechanism of polar auxin transport focuses attention on a number of factors which might be expected to affect the uptake of auxin into and its "secretion" from cells, and thus to affect the transport of auxin through a tissue. These factors include the extracellular pH, the extracellular and intracellular auxin concentrations, and pK of the auxin.

1. The extracellular pH will depend in part on the composition of the cell walls, in particular on the acidic pectic substances, and will also be influenced by an efflux of hydrogen ions from the cells. There is evidence that the action of auxin involves a stimulation of hydrogen ion secretion, which can lower the pH of a weakly buffered incubation medium at least to pH 5 (Rayle, 1973). During polar auxin transport, an action of auxin which caused the extracellular pH to be lowered would lead to an increased uptake of auxin by the cells. Thus in an organized, polar tissue the polar transport of auxin provides a supply of auxin to the cells, and

the action of auxin on the cells would in turn affect the polar transport of auxin. Furthermore, there is some indirect evidence which suggests that the receptors for auxin action may be the same as, or closely related to, the auxin carriers in the plasmalemma (Hertel *et al.*, 1969). The suggestion implies that if the latter are distributed in a polar manner, the auxin receptors will also have a polar distribution. A polarization of auxin action would create differences in the extracellular ionic environments at the different ends of the cells which could conceivably maintain the polar distribution of the auxin carrier-receptors. These considerations could help to explain the "autocatalytic" maintainance of the polar auxin transport system by auxin transport itself (for references, see Sheldrake, 1974).

2. The concentration of a substance depends on the amount of substance and the volume in which it is dissolved: the extracellular and cytoplasmic auxin concentrations depend on the amount of auxin present and the volumes of these compartments. The volume of the extracellular compartment increases in tissues subjected to osmotic stress; changes in the volume of this compartment are associated with changes in the rate of polar auxin transport (A. R. Sheldrake, unpublished results). A redistribution of the cytoplasm within vacuolated cells can also be brought about by centrifugation (Cande, Goldsmith and Ray, 1973).

Different volumes of cytoplasm adjacent to the plasmalemma at the different ends or sides of a cell would, for a given amount of auxin moving across the plasmalemma into the cytoplasm, lead to locally different cytoplasmic auxin concentrations and thus affect the relative rates of auxin influx and efflux at different ends or sides of the cell. The asymmetrical distribution of cytoplasm in centrifuged cells could therefore have a direct effect on the rate and pattern of auxin movement through a tissue.

3. The pKs of IAA and α-naphthaleneacetic acid (NAA) are similar (4.7 and 4.2 respectively). Therefore their pH-dependent uptake into cells might be expected to be similar. They are transported with similar velocities, NAA somewhat slower than IAA (Hertel and Flory, 1968). 2,4D and TIBA are both transported with a considerably lower velocity than IAA (Hertel and Flory, 1968; Thomson *et al.*, 1973) and a higher proportion of 2,4D is immobilised within the tissue (Hertel and Flory, 1968). At external pHs of about 5, when IAA and NAA are taken up into the cells rapidly, 2,4D and TIBA are mostly in the anionic form (pK values of 2.8 and 2.6 respectively) and would thus enter the cells more slowly. However, it can be calculated from equation 1 that, if equilibrium were reached, there would be a greater accumulation of 2,4D or TIBA than of IAA or NAA in the cytoplasm. Furthermore, the affinities

of these compounds for the auxin anion export system will also influence the velocities of their polar transport through tissues.

The pH dependent uptake of undissociated auxin and its ionisation within the cytoplasm means that a hydrogen ion influx must accompany the influx of auxin. It would be most interesting to know in what ways this auxin-associated hydrogen ion influx and the carrier-mediated efflux of auxin anions are related to auxin-stimulated hydrogen ion secretion.

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References

Caldwell, P. C.: Intracellular pH. Int. Rev. Cytol. **5**, 229–227 (1956)

Cande, W. Z., Goldsmith, M. H. M., Ray, P. M.: Polar auxin transport and auxin-induced elongation in the absence of cytoplasmic streaming. Planta (Berl.) **111**, 297–314 (1973)

Christie, A. E., Leopold, A. C.: On the manner of triiodobenzoic acid inhibition of auxin transport. Plant & Cell Physiol. **6**, 337–345 (1965)

Fawcett, C. H., Wain, R. L., Wightman, F.: Plant growth-regulating activity in certain carboxylic acids not possessing a ring structure. Nature (Lond.) **178**, 972–974 (1956)

Goldsmith, M. H. M.: Transport of plant growth regulators. In: The physiology of plant growth and development, pp. 127–162, Wilkins, M. B., ed. London: McGraw-Hill 1969

Goldsmith, M. H. M., Ray, P. M.: Intracellular localisation of the active process in polar transport of auxin. Planta (Berl.) **111**, 297–314 (1973)

Hertel, R., Evans, M. L., Leopold, A. C., Sell, H. M.: The specificity of the auxin transport system. Planta (Berl.) **85**, 238–249 (1969)

Hertel, R., Flory, R.: Auxin movement in corn coleoptiles. Planta (Berl.) **82**, 123–144 (1968)

Hertel, R., Leopold, A. C.: Versuche zur Analyse des Auxinstransports in der Koleoptile von *Zea mays* L. Planta (Berl.) **59**, 535–562 (1963)

Hertel, R., Thomson, K.-St., Russo, V. E. A.: *In-vitro* auxin binding to particulate cell fractions from corn coleoptiles. Planta (Berl.) **107**, 325–340 (1972)

Jones, R. L., Metcalfe, T. P., Sexton, W. A.: The relationship between the constitution and the effect of chemical compounds on plant growth. 3. Chlorinated benzaldehydes and benzoic acids. Biochem. J. **48**, 422–425 (1951)

Jönsson, Å.: Chemical structure and growth activity of auxins and antiauxins. In: Encyclopedia of Plant Physiology, vol. XIV, pp. 959–1006, Ruhland, W., ed. Berlin-Heidelberg-New York: Springer 1961

Rayle, D. L.: Auxin-induced hydrogen ion secretion in *Avena* coleoptiles and its implications. Planta (Berl.) **114**, 63–73 (1973)

Rubery, P. H.: Studies on indoleacetic acid oxidation by liquid medium from crown gall tissue culture cells: The role of malic acid and related compounds. Biochim. biophys. Acta (Amst.) **261**, 21–34 (1972)

Rubery, P. H., Sheldrake, A. R.: Effect of pH and surface charge on cell uptake of auxin. Nature (Lond.) New Biol. **244**, 285–288 (1973)

Sabnis, D. D., Audus, L. J.: Growth substances interactions during uptake by mesocotyl sections of *Zea mays* L. Ann. Bot. **31**, 263–281 (1967)

Sheldrake, A. R.: The polarity of auxin transport in inverted cuttings. New Phytologist **73**, 639–644 (1974)

Thomson, K.-S., Leopold, A. C.: *In-vitro* binding of morphactins and 1-N-naphthylphthalamic acid in corn coleoptiles and their effects on auxin transport. Planta (Berl.) **115**, 259–270 (1974)

Thomson, K.-S., Hertel, R., Müller, S.: 1-N-Naphthylphthalamic acid and 2,3,5-triiodobenzoic acid. *In-vitro* binding to particulate cell fractions and action on auxin transport in corn coleoptiles. Planta (Berl.) **109**, 337–352 (1973)

Zimmerman, P. W., Hitchcock, A. E., Prill, E. A.: Substituted benzoic acids as growth regulators. Contr. Boyce Thompson Inst. **16**, 419, 427 (1952)