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Active auxin uptake by zucchini membrane vesicles: Quantitation using ESR volume and ΔpH determinations

(auxin transport/symport/plant membrane vesicles/proton cotransport/pH gradient)

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ABSTRACT **Closed and pH-tight membrane vesicles prepared from hypocotyls of 5-day-old dark-grown seedlings of *Cucurbita pepo* accumulate the plant growth hormone indole-3-acetic acid along an imposed proton gradient (pH low outside, high inside). The use of electron paramagnetic spin probes permitted quantitation both of apparent vesicle volume and magnitude of the pH gradient. Under the experimental conditions used, hormone accumulation was at minimum 20-fold, a value 4 times larger than what one would predict if accumulation reflected only diffusional equilibrium at the measured pH gradient. It is concluded that hormone uptake is an active process, with each protonated molecule of hormone accompanied by an additional proton. Experiments with ionophores confirm that it is the pH gradient itself which drives the uptake.**

It is postulated that uptake of the plant hormone, auxin (indole-3-acetic acid, IAA), by plant cells is via an uptake IAA–H^+ symport and that efflux occurs by an anion efflux carrier as well as by diffusion, all of which are dependent upon the size and direction of the pH gradient (1–5). Intact plant cells have previously been shown to have a pH gradient and membrane potential across the plasma membrane, with the wall space being acidic (pH 5–6) and positive and the cytoplasm being basic or neutral (pH 7) (6) and having a negative potential. Recently, zucchini membrane vesicles having a pH gradient of the same direction have been used to study *in vitro* IAA transport, defined here as [^{14}C]IAA accumulation that is sensitive to the electrogenic H^+-carrier FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone) and that can be stimulated by auxin transport inhibitors such as 2,3,5-triiodobenzoic acid (TIBA). These vesicles have been shown to perform the essential functions of auxin transport according to the chemiosmotic theory of auxin transport (7). Thus, they provide an excellent system for studying the various transport components of the plasma membrane without the complications of other cellular compartments (4, 8).

Although the zucchini vesicles have been shown to maintain an ionophore-sensitive pH gradient for an extended period of time, they are most likely not as tightly sealed as is an intact plant cell, and the gradient is not actively maintained. To understand the dynamics of auxin transport and to establish whether the transport is an active or simply passive accumulation down a pH gradient, direct measurements both of vesicle volume and of the size of the pH gradient were needed to compare with the measurements of auxin uptake. This information was obtained with electron spin resonance (ESR).

Nitroxide spin labels are used to measure a variety of biological parameters, including cell volumes, pH and electrical gradients, and membrane and surface potentials (9). The measurements require only a very small amount of material (determinations can be made with only 40 μl of a suspension containing about 1 mg of protein per ml). Recently, we have demonstrated the nitroxide spin-probe ESR technique to be applicable to zucchini membrane vesicle preparations identical to those used for auxin uptake studies (10). According to the chemiosmotic coupling hypothesis (11), the membrane is an integral part of the coupling mechanism and must be intact, forming a continuous closed vesicle, in order for the protonmotive force to drive the transport of other solutes. Since the zucchini membrane vesicles used here have been shown to be osmotically sealed and relatively impermeable to protons (7, 10) and the uptake of auxin has been shown to be by the specific, saturable, and pH-dependent transport of auxin into plasma membrane vesicles (12), they are well suited to elucidate in greater detail the energetics of auxin transport. Using the nitroxide spin-probe technique, we provide direct measurements of the magnitude and persistence of pH gradients across the vesicles and correlate these data with [^{14}C]IAA uptake measurements. The results are consistent with active, electrogenic transport of IAA into the sealed, plasma-membrane vesicle space via IAAH/H^+ cotransport.

MATERIALS AND METHODS

Seeds of zucchini squash (*Cucurbita pepo* Linnaeus cv. Dark Green; Ferry Morse Seed, Mountain View, CA) were planted in moist vermiculite and grown in plastic boxes at 26°C and 95% relative humidity for 5 days in total darkness.

Membrane particles were prepared as described (10). The uptake of [2-^{14}C]IAA (55 mCi/mM, Amersham; 1 Ci = 37 GBq) was measured as described in Hertel *et al.* (7). Vesicle volume and pH gradient determinations were made and ΔpH was calculated as described by Lomax and Mehlhorn (10). All determinations were made by using vesicles prepared exactly as for the *in vitro* auxin transport measurements, except where noted (e.g., greater vesicle concentration).

RESULTS

IAA Transport in the Presence and Absence of a pH Gradient. Previous evidence for a pH gradient-dependent uptake of IAA across sealed vesicles in the zucchini membrane preparations used here includes the reduction of IAA accumulation by osmotic shock, by sonication or deep-freezing, or by the ionophores FCCP and nigericin (7). In order to confirm the pH-driven nature of the auxin transport by sealed vesicles, the accumulation of [^{14}C]IAA was measured in the presence and absence of an imposed pH gradient. The

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Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; IAA, indole-3-acetic acid; TIBA, 2,3,5-triiodobenzoic acid; tempone, 2,2,6,6-tetramethyl-4-oxopiperidine-*N*-oxyl; tempacid, 2,2,6,6-tetramethyl-4-carboxylpiperidine-*N*-oxyl.

internal volume was set to a high pH by preparation in pH 7.9 buffer as described. The vesicles then were resuspended in medium of either pH 7.6 (the final pH of the homogenates before resuspension; see ref. 7) or pH 5.5. Fig. 1 illustrates a typical time course for the accumulation of radioactively labeled IAA by zucchini hypocotyl vesicles. With an external buffer of pH 5.5, considerable FCCP-sensitive IAA accumulation was seen. After an initial rapid uptake phase in the presence of a pH gradient, the vesicles achieved a point at which the influx and efflux of [^{14}C]IAA were approximately in balance—by 10–30 min, as can be seen in Fig. 1. Thereafter, the amount of IAA associated with the vesicles gradually diminished, presumably resulting from a slow decline in the protonmotive force across the membrane because of the gradual decrease in ΔpH. The specific inhibitor of auxin transport, TIBA, blocks the movement of IAA through tissue by blocking the efflux carrier (13). With the zucchini membrane vesicles used here, the presence of TIBA resulted in a higher level of [^{14}C]IAA accumulation (Fig. 1) (7). However, the kinetics of uptake and release of IAA association, appeared to be similar either to the control sample or in the presence of TIBA.

In the absence of a pH gradient (Fig. 1), there was no FCCP-sensitive IAA uptake and only little IAA association with the membranes. In pH 5.5 medium, where a gradient was present, FCCP reduced IAA association to the same level as that found with an external pH of pH 7.6, where no gradient existed. This result is consistent with the dissipation by FCCP of the entire protonmotive force (see *Discussion*). The addition of FCCP after 1 hr to vesicles not previously exposed to the protonophore resulted in a more rapid release of [^{14}C]IAA. However, additional FCCP did not change the level of IAA association for vesicles that had already been maintained in FCCP.

In other similar [^{14}C]IAA uptake experiments (14), when [^{14}C]IAA instead was added only after the vesicles were incubated at 4°C for 60 min, the same levels of IAA accumulation were reached as those found after 60 min incubation with [^{14}C]IAA added at 0 min. This result suggests that the amount of auxin accumulation is a function of the pH gradient. When additional [^{14}C]IAA was added after 2.5 hr, the same fraction of total dpm accumulated was reached as in experiments without the addition, indicating that the

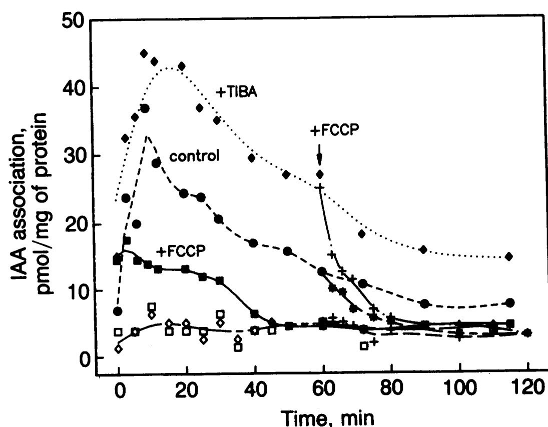

FIG. 1. IAA accumulation in the presence and absence of a pH gradient. Each filtration assay sample contained 2 g fresh weight of zucchini membrane vesicles per ml prepared in pH 7.9 buffer. One portion of the vesicles were resuspended in pH 7.6 medium (□, ◇) and the rest in pH 5.5 medium (■, ◆, ●). IAA accumulation was measured in the presence of 10 μM FCCP (□, ■), 3 μM TIBA (◇, ◆), or no additions (●). Immediately after resuspension at 0 min, 0.2 μM [^{14}C]IAA (6500 dpm per sample) was added. Additional FCCP (10 μM) was added to portions of each treatment at 1 h (+ and *).

reduction in [^{14}C]IAA levels associated with the membranes over time is not due to the metabolism or degradation of the auxin (14).

pH Gradient and Volume Determinations with ESR. To ascertain that the loss of IAA association was indeed the result of a decline in the proton gradient and to analyze further kinetic data and concentration ratios for the accumulation of auxin, quantitative data were needed about the sealed vesicle volume and the magnitude and duration of the pH gradient across the vesicle membranes. ESR spectroscopy of nitroxide spin probes provides a convenient, accurate, and sensitive method for the quantitation of ΔpH and sealed membrane volume. We recently have shown the method to be effective for the zucchini membrane preparations used here (10). The volume of sealed vesicles within a solution can be determined by quantitating the ESR signal of tempone (2,2,6,6-tetramethyl-4-oxopiperidine-*N*-oxyl; TK in Eq. **1**), a membrane-permeable yet water-soluble nitroxide probe, in the presence and absence of an impermeable paramagnetic quenching agent. The ratio of quenched to unquenched signal is a linear function of the total intravesicular volume, which is sealed and inaccessible to the quencher. A weak-acid nitroxide probe, tempacid (2,2,6,6-tetramethyl-4-carboxylpiperidine-*N*-oxyl; TC in Eq. **1**), partitions preferentially into basic environments (15). This difference can be used to calculate the ΔpH by using Eq. **1**:

$$\Delta pH = \log[vol_{TC}(1 - vol_{TK})/(1 - vol_{TC})vol_{TK}]. \quad [1]$$

In the studies described here, these ESR techniques were used to determine both the vesicle volume and the size of the pH gradient in zucchini vesicles made with either 50 mM or 200 mM Tris buffer at pH 7.9. All vesicle preparations were resuspended in pH 5.5 buffer exactly as for the auxin uptake assays described previously. Fig. 2 *Inset* shows that in both cases, as anticipated above, the pH gradient decays slowly (≈0.003 pH units/min) after an initial rapid phase. The decay in pH is approximately linear with respect to time during these experiments and longer experiments (see Fig. 4*A*; see also ref. 10) and, therefore, represents a negative exponential decline in H^+ gradient. However, the size of the pH gradient in both cases is only about 0.8 pH units directly after resuspension—smaller than the expected value of about 2.4 pH units. This discrepancy has been shown to be the result of both (*i*) a reduction of the pH gradient as a result of residual pH 7.9 buffer in the pellet and vesicle breakage during resuspension and (*ii*) a dissipating phenomenon that causes these vesicles to discharge some of the imposed pH gradient (10). This is a characteristic of the zucchini vesicles because synthetic liposomes prepared from soybean lectin under identical conditions can maintain a pH gradient of >2 pH units (10). Nevertheless, the magnitude of the pH gradient observed here is substantial and approximates that found in the intact plant.

Fig. 2 presents the data for accumulation of [^{14}C]IAA under the same conditions as those used for the quantitation of ΔpH and vesicle volume by ESR. Vesicles prepared with 200 mM buffer internally accumulate higher levels of IAA and maintain accumulation for a greater period of time than those prepared with 50 mM buffer. The duration of accumulation in vesicles loaded with 50 mM buffer (≈4 hr) is in good accord with the time that would be necessary for complete decay of the pH gradient at the rate estimated here, 0.003 pH units/min. With the greater buffer strength (200 mM), more protons are available to maintain the pH gradient, prolonging the IAA accumulation up to 6 hr; however, accumulation should be similar with both buffer strengths because the magnitude of ΔpH is the same in both cases (Fig. 2 *Inset*).

The substantially greater auxin accumulation into the vesicles prepared with 200 mM rather than 50 mM buffer is

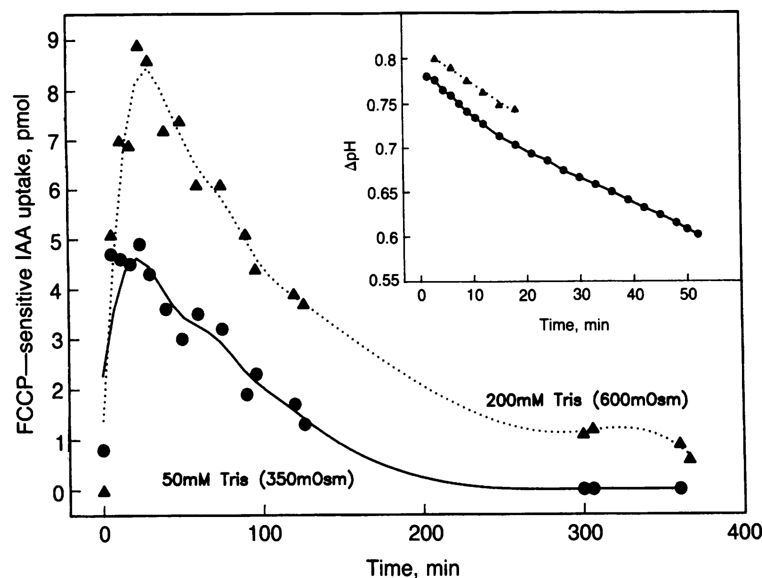

FIG. 2. Time course for ΔpH dissipation and IAA accumulation. Vesicles were prepared as in Fig. 1 with either 200 mM (▲) or 50 mM (●) Tris medium and resuspended at 2 g fresh weight per ml in pH 5.5 buffer. Kinetics of FCCP-sensitive IAA accumulation were measured as described in Fig. 1 in the presence of 3 μM TIBA. ESR determinations of ΔpH were calculated from aqueous line heights of nitroxide spin-probe measurements (*Inset*). Samples contained 1 mM tempone or tempacid in the presence or absence of 0.15 M $Na_2MnEDTA$ and included additions as for the auxin uptake assay.

the predictable result of the greater volume of vesicles with a higher internal osmolarity. After correction for the osmotic effect of external quenching agent on the ESR signal height (10), the sealed vesicles made with 50 mM buffer were found to constitute about 0.6% of the total volume of the membrane resuspension or 7.5 μl per mg of protein. Those prepared with 200 mM were half again as large, 0.9% of the total volume or 11.3 μl per mg of protein. The 50 mM buffer was in osmotic balance with the external buffer while the osmotic concentration of the 200 mM buffer was higher than that of the external buffer (600 mosM vs. 350 mosM), which caused the vesicles to swell. Thus, the difference in vesicle size when loaded with different buffer strengths explains the observed differences in total IAA accumulation.

Calculation of Auxin Concentration Gradients. Perhaps the simplest model for auxin accumulation is one that assumes a passive equilibration of the auxin in response to the pH gradient across the vesicle membrane. This model makes only one assumption: the uncharged auxin molecule is freely permeable while the charged species is impermeable. This assumption is reasonable in view of the structure of the uncharged auxin molecule, which lacks polar residues and would not be expected to interact strongly with water.

The determination of auxin accumulation in response to a pH gradient is analogous to the measurement of the pH-dependent accumulation of a fluorescent probe into membrane vesicles. In both cases the concentration of a species is measured in the bulk aqueous phase, and the remaining probe partitions into both membrane and intravesicular aqueous domains. Neglect of membrane binding of the solute can have a significant effect on data analysis; indeed, the major problem that has been encountered in analyzing the accumulation of fluorescent probes is that membrane binding of the probe complicates that data analysis. However, these complications can be overcome if an operational vesicle volume is employed in relating the probe accumulation to the inferred pH gradient (16).

Knowing the vesicle volume and assuming negligible membrane binding of auxin, we calculated the internal IAA concentration generated by the sealed vesicles (from the IAA uptake and sealed vesicle volume per ml of sample, Fig. 2) as follows:

$$[IAA]_i = \text{pmol of IAA per } \mu l \text{ of sealed vesicle volume.} \quad [2]$$

With internal buffer concentrations of either 50 or 200 mM Tris, the maximal $[IAA]_i$ was found to be 4–5 μM (a correction for membrane binding would increase this figure). Since only 0.1–0.2 μM IAA was present in the external solution, this concentration represents at least a 20-fold accumulation of the auxin over the outside concentration. The actual figure is most likely several-fold higher because we used the total sealed vesicle volume for these calculations, while in reality, only the plasma membrane vesicles transport IAA (12); thus, a considerable portion of that volume most likely does not transport IAA, being either of tonoplast origin (the only other pH-tight vesicles in these preparations into which IAA only partitions; ref. 12) derived from nontransporting cells or of the wrong sidedness. These calculations confirm, therefore, that the vesicles are able to accumulate auxin against a concentration gradient, when driven by a pH gradient.

The maximal accumulation of IAA at diffusional equilibrium with a pH gradient of 0.8 units (Fig. 2 *Inset*) should be similar to that of tempacid (another weak acid with about the same pK_a) and, therefore, would be only 5 times the external concentration of IAA, rather than the 20-fold or greater accumulation measured here. The relationship between the level of IAA accumulation predicted for passive diffusion, whether facilitated or not, and the actual observed IAA accumulation by zucchini membrane vesicles is depicted in greater detail in Fig. 3. The "predicted" curve was generated for passive diffusion of a weak acid with the same pK_a as IAA by using the pH gradient measurements in Fig. 2. The observed accumulation ratios ($[IAA_i]/[IAA_o]$) were calculated from the experiment shown in Fig. 1 by using Eq. 2. In the absence of the auxin transport inhibitor TIBA, the observed IAA accumulation ratios actually reached 15-fold; an even greater (20- to 25-fold) maximal accumulation was found with TIBA present. The equilibrium kinetics of the *in vitro* [^{14}C]IAA transport appeared to be shifted by TIBA. Blocking or slowing the efflux site would favor increased and prolonged accumulation, as was observed. Thus, internal IAA concentrations were enhanced at least 3- to 5-fold over the equilibrium distribution. This difference is convincing evidence that there is indeed active transport of IAA by these zucchini membrane vesicles. Although large, these accumu-

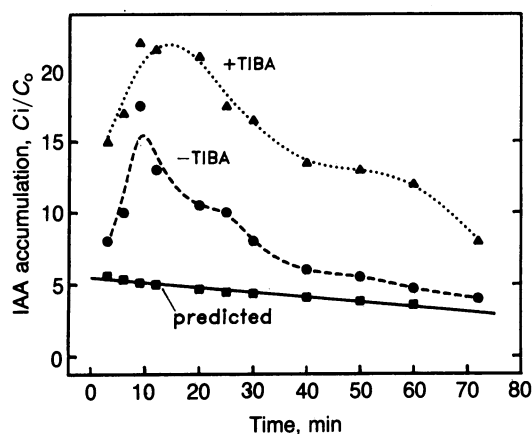

FIG. 3. Predicted passive diffusion values versus observed levels for FCCP-sensitive [14C]IAA accumulation by membrane vesicles from zucchini hypocotyl tissue. Values were calculated as described in the text. C_i/C_o, ratio of inside and outside concentrations.

lation levels are still within the physiological concentration range for IAA.

The Effects of FCCP and Nigericin on IAA Uptake. A comparison of the response of the zucchini membrane vesicles to ionophores in terms of both the pH gradient across the membranes and auxin accumulation is shown in Fig. 4. In both cases the zero time point is equal to the time of resuspension of the vesicles in the lower pH medium, thus creating a pH gradient across any sealed membranes. As seen before (Fig. 2 *Inset*), in the control sample (i.e., without ionophores present) the pH gradient decayed slowly and linearly (Fig. 4*A*). The electrogenic protonophore FCCP should reduce the pH gradient only to the point where the proton-motive force is reduced to zero by the opposite but equal electrical potential. The ESR measurements depicted in Fig. 4*A* show clearly that FCCP indeed did not totally dissipate the ΔpH. Instead, the FCCP sample reached an equilibrium ΔpH to which the control sample eventually declined. By contrast, the electroneutral H^+/K^+ exchanger, nigericin, totally dissipated the pH gradient, as would be expected. Similar results were seen with a different time course and buffer conditions (10).

Preliminary experiments (7) with single concentrations of the drugs and with auxin uptake measured at a single time point, showed that both FCCP and nigericin reduced IAA accumulation to approximately the same level obtained with concentrations of unlabeled IAA sufficient to saturate uptake. In Fig. 4*B* both FCCP and nigericin are seen to have strikingly similar effects on the kinetics and degree of action on [14C]IAA uptake. All additions were made to the vesicles at 0 min, and the data indicate that FCCP and nigericin each required ≈40 min to bring [14C]IAA association to an equilibrium level. This can be explained by an initial, rapid accumulation of the auxin before the ionophores have a chance to act, followed by the slow efflux or leakage of IAA as the pH gradient (or proton-motive force), which normally maintains the auxin accumulation, is dissipated. In other experiments (10), when potassium was present both within and outside of the vesicles, nigericin fully and immediately abolished the pH gradient. The fact that the IAA association was not reduced to zero in the presence of the drugs (thus in the absence of a proton-motive force) may be explained as the entrapment of IAA in the extravesicular spaces of the filtrate and partitioning into the lipid bilayer (see ref. 17).

In other experiments (12), both FCCP and nigericin were found to reduce the uptake of IAA with a similar concentration dependence. The maximal activity was achieved at 10 μM, the level used in all subsequent experiments. The

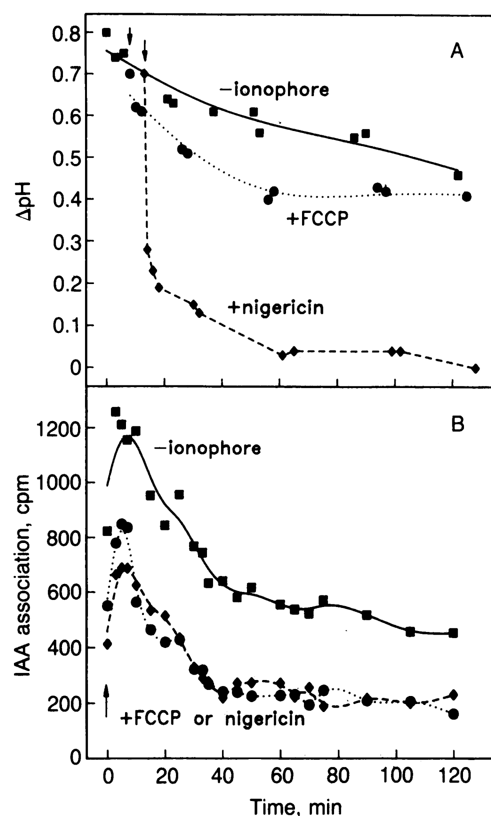

FIG. 4. Time course for the inhibition of ΔpH and IAA association by saturating concentrations of FCCP or nigericin. Membranes were resuspended at 5 g fresh weight per ml and allowed to incubate on ice for 5 min, after which the reaction was started by addition of an equal amount of pH 5.5 medium containing additions as described in Figs. 1 and 2. [14C]IAA was at 0.5 μM; FCCP and nigericin were at 10 μM. (*A*) ΔpH calculated from spin-probe line measurements as described in Fig. 2. (*B*) Accumulation of [14C]IAA measured by filtration assay.

concentration dependence for the inhibition of IAA uptake was very similar to that observed for the reduction of the pH gradient in submitochondrial particles by FCCP (18).

DISCUSSION

In zucchini membrane vesicle preparations, we show here that auxin uptake is driven by the imposed pH gradient since its dissipation by the uncoupler FCCP or the ionophore nigericin causes the release of auxin from the vesicles (Fig. 4); and when no gradient is imposed, there is little or no IAA association with the membrane preparations (Fig. 1), which argues against the association being a result of membrane or receptor binding. Hertel (5) has proposed that auxin uptake occurs via cotransport with two protons—an electrogenic symport and, thus, active transport. While passive transport can be either mediated or nonmediated, active transport is always facilitated, occuring via a saturable, specific carrier (8). The uptake of IAA into zucchini plasma membrane vesicles has been shown to be specific, saturable, and thus indeed facilitated by a carrier (12). Exact knowledge of vesicle volume and ΔpH now allows not only the differentiation between active and passive transport of auxin, but also makes possible a better understanding of previously unexplained components of the uptake data.

As pointed out by Sussman and Goldsmith (19), the effect of voltage across the plasma membrane on auxin uptake depends on exactly how the auxin anion moves. Membrane voltage would have no effect if IAA^- were cotransported in a neutral form with a single proton, would decrease net

uptake if IAA^- moved in a negatively-charged form, or would increase net uptake if IAA were cotransported with two protons. Our data indicate that the latter is the most likely.

The electrogenic nature of the auxin uptake carrier has been demonstrated in studies with valinomycin, a specific K^+ ionophore (7). When added to zucchini vesicles loaded with 25 mM KCl and resuspended in low K^+ medium, a strong negative potential should be generated inside the vesicles. Hertel *et al.* (7) found that this resulted in a large increase in [^{14}C]IAA accumulation. In a similar experiment, Hertel (5) found that with equimolar K^+ inside and out—i.e., where no potential should have been created—valinomycin did not increase IAA accumulation.

That valinomycin increases IAA uptake in a situation where it also should increase membrane potential substantiates the proposal by Hertel (5) that IAA uptake occurs by an electrogenic symport mechanism involving cotransport of two protons and one auxin molecule. Such an auxin-uptake system will not cease functioning when an uncoupler reduces the proton motive force to zero. To demonstrate this point, consider a carrier of arbitrary charge that binds two protons and an auxin anion (or, equivalently, a proton and a protonated auxin molecule). Let the transmembrane electrical potential be designated as ΔY. Then at equilibrium, the following relationship will govern the distribution of auxin molecules, where i and o refer to the concentrations of species inside and outside of the vesicles, respectively (20):

$$[H^+]_i^2[IAA^-]_i = e^{F\Delta Y/RT}[H^+]_o[IAA^-]_o. \quad [3]$$

The effect of an uncoupler such as FCCP is to conduct protons electrogenically until the electrical potential is balanced exactly by the pH gradient—i.e.,

$$[H^+]_i = e^{F\Delta Y/RT}[H^+]_o. \quad [4]$$

When this condition is met, Eq. **3** reduces to:

$$[H^+]_i[IAA^-]_i = [H^+]_o[IAA^-]_o. \quad [5]$$

Using Eq. **4**, we can relate the pH gradient, as measured with the spin probes, to the auxin accumulation, assuming that the electrical potential is zero. With the pH gradient of 0.8 units, the ratio $[H^+]_i/[H^+]_o$ is 5; hence, the expected accumulation ratio for auxin is 25 (Eq. **4**), in close agreement with the maximum observed ratio (when TIBA was present, Fig. 3). When FCCP is added, the expected accumulation ratio is 5 (Eq. **5**), in good agreement with the observed ratio. Given the approximations involved in this calculation (e.g., that the electrical potential without FCCP is zero), we consider the agreement between the calculated and observed auxin ratios to be reasonable and consistent with the proposed stoichiometry of one auxin molecule and two protons on the transporter.

Although the accumulation ratio of IAA is quite large, the concentration of IAA^- and any H^+ that are cotransported should have little if any effect upon the pH gradient across the membrane because they exist in only micromolar concentrations in the presence of millimolar buffer concentrations. This suggestion is supported by ESR measurements, which show no effect of micromolar IAA (or TIBA) concentrations on the magnitude of the pH gradient (not shown). The micromolar quantities of IAA involved in these experiments are also too small for any efflux out of the vesicles by the permeant species to counteract or "short-circuit" the electrogenic, active transport.

In summary, we consider that Hertel's observation of an electrical potential dependence of auxin uptake (5) together with our demonstration that auxin accumulation substantially exceeds expectations based on weak-acid equilibration to support strongly the hypothesis for an active uptake mechanism for this hormone. The energy required to transport IAA into zucchini membrane vesicles can be furnished by an artificially imposed pH gradient across the membrane, whereas the energy for the IAA movement into an intact plant cell is most likely provided by the metabolically maintained pH and electrical gradients, as proposed by Rubery and Sheldrake (1). A modification of their hypothesis is necessary to accommodate our data. Apparently, a proton is cotransported with IAAH, providing additional free energy. This modification is necessary to explain accumulation above that expected for passive uptake of a weak acid across the pH gradient. Also the evidence indicates that the uptake carrier across the plasma membrane for auxin is electrogenic rather than electroneutral. The striking correlation between the *in vitro* auxin transport system and the *in vivo* polar auxin transport by a variety of plant tissues indicates that this may be regarded as a basic component of polar auxin transport.

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