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Role of Calcium in the Polar Secretion of Indoleacetic Acid[1]

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ABSTRACT

The rate of auxin transport in sunflower hypocotyls (*Helianthus annuus* L. cv 'Russian mammoth') or corn coleoptiles (*Zea mays* L. cv 'WF9 × 38') was less in seedlings grown in Ca-deficient medium than in controls. The rate of IAA transport depended on the concentration of Ca in the root medium up to 1 millimolar. Further increases in auxin transport were observed when the isolated segments were incubated in medium containing up to 30 millimolar Ca. We suggest that the rate of auxin transport in plant tissue is dependent on the pool of ionic Ca in the extracellular space.

Segments from Ca-deficient seedlings exhibited a high specific requirement for Ca^{2+} in auxin transport. Magnesium, strontium, and several other divalent cations tested for their ability to replace Ca^{2+} in restoring auxin transport showed no effect; partial replacement by lanthanum was observed.

Auxin transport, or auxin flux through the segment, which is the result of IAA secretion by individual cells, was reduced in the low Ca^{2+} segments due both to lowered velocity and to reduced capacity of transport. The requirement for Ca^{2+} in the secretion of auxin is believed to be equivalent to the phenomenon observed in animal cell secretion, where the influx of Ca^{2+} serves as a link between an external stimulus and the secretion response.

In shoots, IAA is thought to be synthesized primarily in the apical cells and secreted or transported polarly to subjacent target cells where this hormone exerts its effects on cell elongation or differentiation. Recently, the chemiosmotic hypothesis of IAA transport has provided a model by which polar auxin transport could occur (12, 25, 28). Since IAA has a pK = 4.7, the amount of the unionized form (IAAH) is appreciable in the acidic extracellular space. In the cytoplasm, where the pH is neutral, the ionized species (IAA^-) will predominate. The permeability of the plasmalemma to the unionized species is at least three orders of magnitude higher than the ionized species (25). Thus, the metabolic activity that maintains the pH gradient at the plasmamembrane can be considered as the driving force for the continuous influx of IAA into the cytoplasm. IAA^- carriers at the basal end of the cells, which have long been hypothesized to exist (4, 14, 19) still provide the only mechanism accounting for the basipetal direction of auxin transport. The driving force for the basal efflux may be provided by both the concentration gradient for the anion and the electronegative potential across the cell membrane which is also maintained by metabolic activity.

Compared to what is known about animal hormone secretion, there is very little known about how IAA secretion is modulated by the cellular environment. We do not have an explanation as to how the effect of light or gravity modifies either the rate or the direction of IAA transport.

Within the last three decades much has been learned about the secretion of substances in animal cells. Studies have revealed a common mechanism for activation of various secretory systems, endocrine or exocrine, neurosecretions, secretion of enzymes and other proteins, ions, water, etc. The chemical or electrical stimulation of the secretory cell seems always to be coupled to an increase in the concentration of Ca^{2+} in the cytoplasm just prior to the actual secretion (29). When the cell is at rest, the cytoplasmic concentration of Ca^{2+} is some three to six orders of magnitude lower than that of the extracellular space (24, 29). A stimulus brings about a change in the permeability of the plasmalemma to Ca^{2+}, and the passive influx of Ca^{2+} ensues. Only a small amount of Ca^{2+} influx is needed to raise the cytosolic concentration several fold, and this perturbation is thought to trigger such processes as secretion, muscle contraction, enzyme activation, etc. (24).

This paper presents evidence showing that polar IAA transport or IAA secretion at the basal end of individual cells (8) into the free space is probably modulated by Ca^{2+}. In the accompanying paper (7), data will be presented showing that concomitant to the basipetal secretion of IAA is an acropetal movement of endogenous Ca^{2+}.

MATERIALS AND METHODS

Culture of Seedlings. Several methods of culturing sunflower (*Helianthus annuus* L. cv 'Russian mammoth') and corn (*Zea mays* L. cv 'WF9 × 38') seedlings were used during the course of the experiments. In many of the earlier experiments, solid supports such as sand, fiber glass, or shredded polyurethane foam were used. In later experiments, the seedlings were grown on top of plastic netting stretched over plastic containers holding about 450 ml solution. One-fifth strength Hoagland solution (15) with or without Ca was used in the early experiments; in later experiments, deionized distilled H_2O, supplemented only with Ca and B, was used.

The seeds were germinated in trays lined with paper towels moistened with distilled H_2O. After about 24 h, seeds with well-developed radicles were transplanted to the different solutions. The corn seedlings were grown in the dark. The sunflower seedlings were grown under a 16-h photoperiod under fluorescent and incandescent lamps with an irradiance of about 16 klux at 30°C during the day and 25°C when the lights were turned off.

Pretreatment of Plant Segments. The sunflower seedlings at 4 or 5 d old, or corn seedlings at 3.5 to 4 d old, were harvested and cut with a cutter with four blades spaced 5 mm apart. In this way, three consecutive segments were obtained beginning 3 to 5 mm from the cotyledonary node or the coleoptile tip. Unless otherwise indicated, the segments were immediately used to measure auxin transport. In some cases, the segments were incubated in 20 mM Tris buffer adjusted to pH 7 with HCl with or without the chloride salt of several divalent cations and lanthanum.

Measurement of Auxin Transport. Auxin transport was meas-

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ured by the classical agar donor/receiver method. The donor contained 10 μM [1-^{14}C]IAA (13.5 mCi/mmol). A large donor (2.5 cm diameter), about 0.8 to 0.9 ml in volume, was used in some of the early experiments; a smaller version (0.2 ml; 14 mm diameter) was used in later experiments. The large donor accomodated 20 to 30 segments; only 9 to 12 were used with the small donor. A blank agar disc of the same diameter as the donor was used as the receiver for the auxin reaching the basal end of the segments. After the transport period, the receiver agar was transferred to a piece of filter paper and dried inside a scintillation vial at 80°C before counting in a standard toluene-POPOP-PPO mixture, using a Packard Tricarb Scintillation Spectrometer.

RESULTS

Auxin Transport in Segments with Different Levels of Calcium. The effect of the concentration of Ca^{2+} in the root medium on the rate of auxin transport is shown in Figure 1. Both sunflower hypocotyl and corn coleoptile segments from seedlings cultured in medium with low Ca concentrations (0.05 or 0.25 mM) showed a depressed rate of auxin transport relative to plants cultured in 5 mM Ca. Growth was visibly lessened in the low Ca medium starting about the 5th or 6th d from germination; the observed reduction in auxin transport, however, precedes growth reduction by about 24 to 48 h.

The development of the root during Ca deprivation was even more markedly affected than the hypocotyls. Abnormalities were detected as early as 2 d after transfer to a solution with low Ca. By about 4 d, the roots of the low Ca seedlings were brownish and did not exhibit the extensive lateral root development observed with plants in the high Ca medium.

To determine whether the depressed rate of auxin transport was the result of permanent injury because of the prolonged Ca deficiency or was a direct effect of low Ca, segments were incubated in Ca solutions prior to the measurement of auxin transport. Results shown in Figure 2 reveal two important points. First, the rate of auxin transport is strongly influenced by the Ca concentration in the root medium. Second, incubation of segments from seedlings grown in low Ca medium in Ca solution restored the rate of auxin transport close to the level of the segments grown in high Ca. These results indicate that the depressed rate of auxin transport is largely, if not wholly, attributable to the direct effect of the low Ca^{2+} levels in the tissue and not to secondary effects.

The other experiments that deserve mention (data not presented) support the role of Ca^{2+} in auxin transport. First, segments from seedlings cultured only in deionized H_2O, which were incubated in a series of modified Hoagland solution each deficient in a single macroelement, responded with an increased rate of auxin transport only when the segment incubation medium contained Ca. Second, seedlings cultured in a series of modified Hoagland solution each deficient in a single essential element showed no reduction in the rate of auxin transport except in segments taken from seedlings grown in solutions deficient in Ca or B. The role of B in auxin transport (30) will be subsequently communicated in more detail.

Tissue Ca^{2+} Level and the Components of Auxin Transport. The flux of auxin through plant axes or auxin transport intensity (mass·time^{-1}), is dependent on the total auxin in the segment or transport density (mass·length^{-1}) and the velocity (length·time^{-1}) (18). The effects of the Ca concentration in which the seedlings were grown on these parameters were determined using 20-mm segments provided with pulses of radioactive auxin (Fig. 3). Following a 40-min pulse, radioactive auxin began to appear in the basal receiver after 120 min for the high Ca^{2+} segments but not until 150 min for the low Ca^{2+} segments. The rate at which radioactivity entered the agar receivers peaked at 200 min

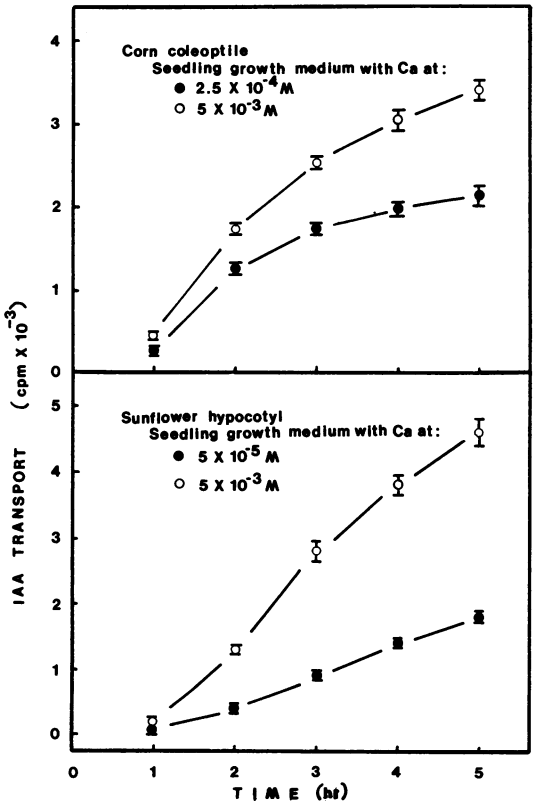

FIG. 1. Effect of Ca concentration in the seedling growth medium on auxin transport in corn and sunflower segments. The seedlings were cultured in one-fifth strength Hoagland solution with the indicated concentration of Ca. Each point is the mean of three replications each with 20, 5-mm segments. Bars, SE.

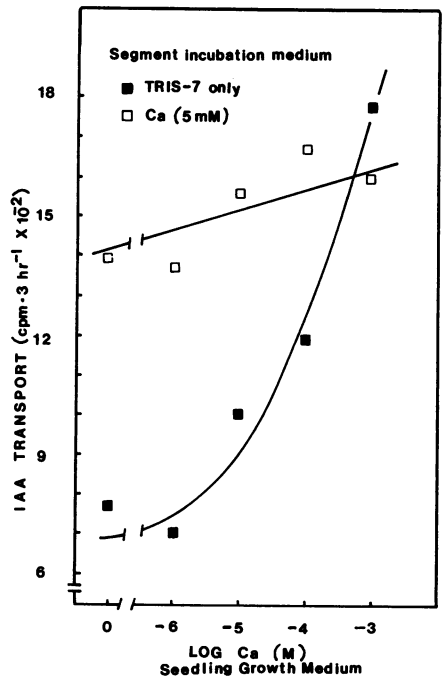

FIG. 2. Effects of Ca concentration in the seedling growth medium and the incubation of segments in Ca solution on auxin transport. Sunflower seedlings were grown in deionized distilled H_2O with 5 ppm B and various concentrations of Ca. The 5-mm segments were incubated for 2 h in Tris buffer (pH 7) alone or in buffer with 5 mM Ca. Each point is the transport by 24 segments for 3 h.

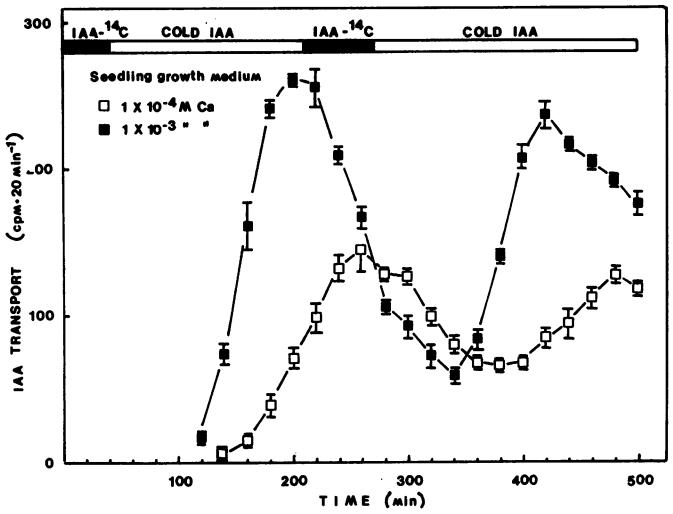

FIG. 3. Effect of Ca concentration in the seedling growth medium on the velocity of auxin transport. The hypocotyl segments (20 mm long beginning 5 mm below the cotyledonary node) were used in each of three replications per treatment. Both radioactive and cold donors contained 0.8 ml of 10 mM IAA. The first pulse of radioactive IAA was applied at the start of the experiment and then replaced with cold IAA donor 40 min later. Another pulse was applied at 210 min from start of the experiment. The receiver agar was replaced every 20 min and the radioactivity was determined as mentioned in the text. Each point is the mean of three replications. Bars, SE.

for the former and 260 min for the latter. The calculated velocity was slower in the low Ca^{2+} segments; both the velocity and the maximum peak height method were less than at higher Ca concentration. The transport density, obtained by dividing the total transport due to the first pulse by the length of the segment, was found to be 37% less for the low Ca^{2+} segments. The transport intensity or flux (transport density × velocity) was inhibited about 50% for the low Ca^{2+} segments.

One of the possible reasons for the reduced IAA transport in tissue with low Ca^{2+} could be inactivation of auxin either by decarboxylation or binding to a cellular component that does not move in the polar transport system. A specific effect of Ca^{2+} on the subcellular distribution of peroxidase in etiolated zucchini seedlings has been observed (22); in the presence of EGTA, there was an increase in the soluble peroxidase, while the addition of Ca^{2+} (but not Mg^{2+}) reversed the effect of the Ca chelator. Thus, it is possible that low levels of Ca^{2+} in the cell brought about an increase in soluble peroxidase which in turn could have caused a higher rate of IAA degradation, thus less IAA transport.

To test this hypothesis, decarboxylation of [1-^{14}C]IAA was measured during the course of transport by enclosure of the entire transport set-up with 20 hypocotyl segments inside a 50-ml beaker. A piece of filter paper saturated with 1 N NaOH was suspended inside the air-tight system. After 3 h, five representative segments were ground with 1 ml absolute ethanol; then, 10 ml Aquasol (New England Nuclear) was added to the mixture and radioactivity determined. No attempt was made to measure quenching or the nature of the radioactive compounds present in the mixture. Low concentrations of auxin (10^{-6} and 10^{-7} M) in some donors were included to minimize possible interactions of auxin levels with oxidase/peroxidase levels in the cell. The total amount of $^{14}CO_2$ evolved, and the radioactivity in the segments increased as the concentration of auxin in the donor was increased (Table I). These parameters, however, unlike the rate of auxin transport, were not influenced by the level of Ca^{2+} in the tissue.

The Ionic Specificity of the Auxin Transport System. Sunflower hypocotyl segments from seedlings grown in different concentrations of Ca were incubated in Pipes buffer alone or in buffer containing 1 mM of the chloride salts of Ca, La, Mg, or Sr. Segments incubated in buffer alone showed an increase in the rate of auxin transport as the concentration of Ca in the root medium was increased to 1 mM (Fig. 4). No further increase was observed when the concentration of Ca in the root medium was increased to 3 mM. However, when identical segments were incubated in 1 mM Ca (40 ppm), auxin transport was further increased. Incubation in 1 mM La increased auxin transport above that in buffer alone, particularly for segments from seedlings grown in low Ca medium. Incubation of the segments in

Table I. *Effect of Calcium Concentration on the Transport and Decarboxylation of [1-^{14}C]IAA*

Sunflower seedlings were cultured in shredded polyurethane foam saturated with Hoagland solution with either 0.05 or 5.0 mM Ca. Each replication with 20, 5-mm hypocotyl segments was placed inside a 50-ml beaker covered with parafilm. A piece of filter paper saturated with 1 N NaOH was suspended in the beaker for absorption of the $^{14}CO_2$. At the end of the 3-h transport period, five representative segments were ground in 1 ml absolute ethanol and the combined radioactivity of the ground tissue and extract was determined after addition of 10 ml of scintillation cocktail.

Donor [1-^{14}C]IAA	Ca in Medium	^{14}C in Receiver	^{14}CO Evolved	^{14}C in the Segment
μM	*mM*	*cpm·h^{-1}*	*cpm·3 h^{-1}*	*cpm·5 segment^{-1}*
0.1	0.05	12 ± 1	21 ± 3	57 ± 4
0.1	5.0	29 ± 2	23 ± 3	43 ± 7
1.0	0.05	132 ± 3	120 ± 4	374 ± 32
1.0	5.0	212 ± 17	136 ± 4	346 ± 33
10.0	0.05	861 ± 36	1039 ± 44	3084 ± 99
10.0	5.0	1312 ± 60	1117 ± 91	3125 ± 136

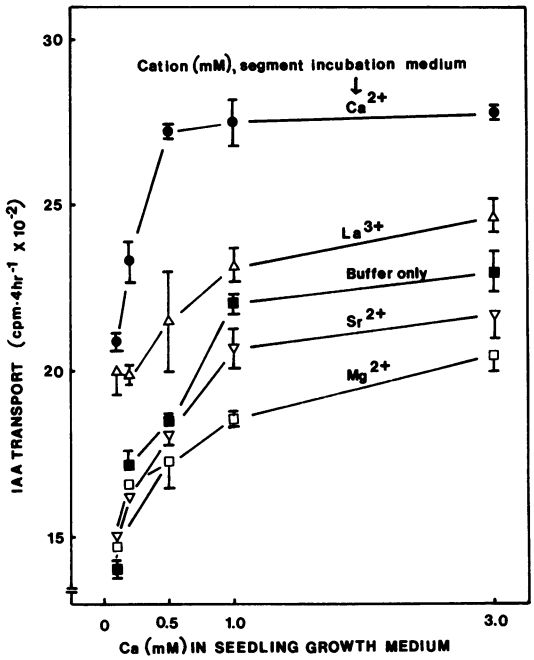

FIG. 4. Effects of Ca concentration in the seedling growth medium and the incubation of segments in solutions of various cations on the rate of auxin transport. Sunflower seedlings were grown in deionized distilled H_2O with 5 ppm B and the indicated concentration of Ca. The 5-mm segments from 5-d-old seedlings were incubated for 2 h in 0.02 M Pipes buffer (pH 7) alone or in buffer with 1 mM of the chloride salts of either Ca, La, Mg, or Sr. Each point is the mean of three replications with 12 segments each. Bars, SE.

either Mg or Sr did not increase IAA transport over that for segments incubated in buffer alone. In fact, Mg and Sr appeared to diminish the promotive effects of high Ca concentrations in the seedling medium.

In the above experiment, the segments were incubated in only one concentration (1 mM) of the element in question. In Figure 5 all the seedlings were grown in 0.1 mM Ca but the segments were incubated in increasing concentrations of the various elements, up to 31.6 mM. Increasing the concentration of Mg and Sr did not increase the rate of auxin transport, and Mg may have been inhibitory at the higher concentrations. Increasing La in the incubation medium up to 3.1 mM increased the rate of auxin transport; the segments lost turgor when incubated at higher concentrations. Other divalent cations tested and found to be without promotive effects on IAA transport were Ba, Co, Fe, Ni, Mn, and Zn (data not shown). Calcium was the only divalent cation found to be able to increase the rate of auxin transport up to 31.6 mM; higher concentrations caused the segments to lose turgor.

DISCUSSION

Calcium Pools and Auxin Transport. Sunflower hypocotyl and corn coleoptile segments with different levels of Ca^{2+} were obtained by growing seedlings with different concentrations of Ca in the root medium. The rate of auxin transport decreased as the concentration of Ca in which the seedlings were grown was lowered. These results are similar to those obtained with segments having normal Ca^{2+} content but washed with the Ca chelator, EDTA (9), before measurement of auxin transport. Although treatment with EDTA lasted only a few hours, the plant materials used in the present experiments grew and developed under severe to moderate Ca deficiency for several days. In both cases, however, incubation in Ca^{2+} solutions for 2 h restored auxin transport to a level close to, if not the same as, that in seedlings that were never subjected to Ca deficiency. These results indicate the absence of permanent damage to the auxin transport system during the period of Ca deprivation and suggest that the observed reduction in the rate of auxin transport was simply due to a limited Ca^{2+} supply.

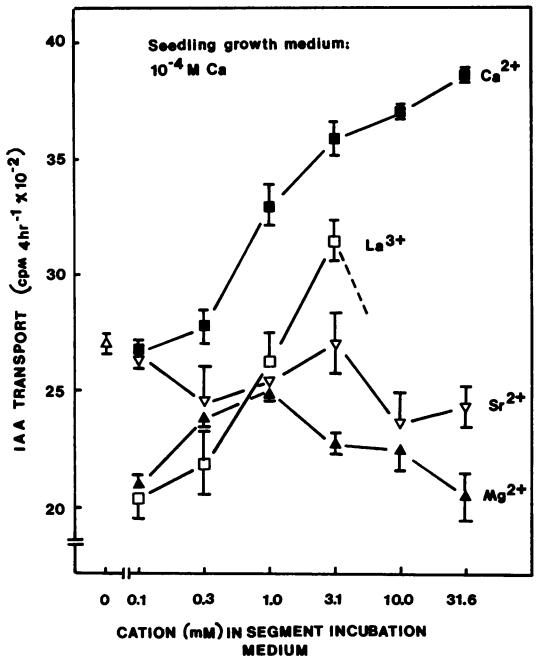

FIG. 5. The effect of incubating segments in increasing concentrations of various cations on auxin transport of segments with suboptimal Ca nutrition. Sunflower seedlings were grown in one-fifth strength Hoagland solution containing 0.1 mM Ca. The hypocotyl segments were incubated for 2 h in 0.02 M Pipes buffer (pH 7) containing the indicated concentrations of the chloride salts of Ca, La, Mg, or Sr. Each point is the mean of three replications with 12 segments each. Bars, SE.

Much of the Ca in plant tissues is extracellular (31), as it is in animal tissues (22, 29). Increasing the concentration of Ca in the root medium above 1 mM did not result in a further increase in auxin transport in the buffer-incubated segments (Fig. 4). However, incubation of identical segments in Ca^{2+} solution further increased the rate of auxin transport. It appears that there are at least two pools of extracellular Ca in the intact plant cell; namely, a small pool of ionic and easily displaceable Ca^{2+} directly involved in auxin transport, and a large pool of insoluble and strongly adsorbed Ca that is of no immediate significance to the auxin transport system. Most likely, the available pool which is present in the free space and plasma membrane, is slowly converted to the unavailable pool which is bound to the cell wall. The low rate of Ca^{2+} ascent in the intact plant and the high capacity of the cell wall to bind Ca^{2+} may prevent the available pool of Ca^{2+} in the hypocotyl from building up. On the other hand, incubation of short hypocotyl segments in Ca solutions probably caused an immediate increase in the available Ca^{2+} in the extracellular space.

The above findings imply that basipetal auxin transport, which is important in development and tropistic responses, is dependent upon a supply of Ca^{2+}, and this supply may come from the very small pool of available Ca^{2+} in the free space.

Cation Specificity in Auxin Transport. So far, two types of Ca-deficient segments have been used. The first may be called EDTA-stressed (9), and the second, which includes those used in the present set of experiments, are those grown in Ca-deficient medium. There is a marked difference between these two types of Ca-stressed segments in terms of their response to the test for cation specificity. The segments from seedlings grown under low Ca conditions showed a fairly high specificity for Ca^{2+} (Figs. 4 and 5). Only La^{3+} at the lower concentrations seemed to be able to substitute for Ca^{2+}. Mg^{2+} and Sr^{2+} in several experiments did not increase IAA transport above the level of the segments incubated in buffer alone.

The segments that were Ca-stressed with EDTA, on the other hand, did not show a high specificity for Ca^{2+}. Even the monovalent cations K^+ and Na^+, although not as effective as the divalent cations, increased auxin transport (9).

The discrepancy in the response of the two types of segments to cation specificity can be explained if we assume that the treatment with EDTA for 2 h (9) mostly affected the cells close to the exposed cut ends of the hypocotyl segments. During the course of auxin transport measurement, Ca^{2+} could have equilibrated out from tissue which was less accessible to the EDTA. Moreover, the specificity of EDTA is not very precise and numerous other essential cations were probably removed by the treatment. Thus, Ca^{2+} homeostasis in the EDTA-stressed segments may not have been attained before auxin transport was measured. The occurrence of such a situation in hypocotyls from seedlings grown under low Ca conditions is unlikely because of the relatively long periods that the plants remained in the treatment. Burström (3) has alluded to the dangers of assuming that EDTA-stressed segments are identical to segments which have developed at a suboptimal supply of Ca in the root medium.

It has been speculated (12) that the observed Ca promotion of auxin transport may be due to the promotion of proton secretion by this cation (6). An acidic extracellular space, besides being a requisite for cell wall extension, is a prerequisite for the chemiosmotic hypothesis of IAA transport. Data presented here do not support the above speculation for at least two reasons. First, the promotion of proton secretion exhibits an optimum at 0.3

to 10 mM Ca (6), while promotion of auxin transport by Ca does not show a saturation until about 30 mM $CaCl_2$ (Fig. 5). Second, the ionic requirement for proton secretion is not specific for Ca. Even with the use of 'acid-washed' coleoptiles, Mg^{2+}, Sr^{2+}, and even K^+ showed some promotive effects (6). Ca^{2+} and Mg^{2+} were found to be equally effective in causing IAA-dependent proton secretion (2).

Calcium and Secretion. The requirement for Ca^{2+} in maintaining membrane integrity is well known. Thus, the cytoplasmic efflux of inorganic solutes such as potassium (20, 30) as well as photosynthates (26) increases as the Ca^{2+} in the cell environment is reduced. On the other hand, the efflux of IAA declines as the Ca^{2+} level is reduced. We believe this to be strong, although indirect, evidence that there is a carrier involved in IAA efflux and that this carrier requires Ca^{2+}. Jacobs and Gilbert (16) have recently presented very strong evidence for the presence of an IAA carrier on the basal end of pea cells.

The mechanism for the involvement of Ca^{2+} in auxin transport in plant cells is not clear at present. The role of Ca^{2+} is much more understood in animal systems (23, 29) where more extensive investigations have been conducted, not only in secretion but in the more general process known as stimulus-response coupling mechanism (24). The operation of these Ca^{2+}-dependent processes requires the existence of a transmembrane Ca^{2+} gradient; the increase in cytosolic Ca^{2+} seems to be the common perturbation that ultimately leads to such response as secretion.

There are characteristic similarities between auxin secretion in plant cells and catecholamine secretion in the adrenal medulla (11). Both plant and animal cells exhibit a low rate of secretion when low in Ca^{2+}, and a concomitant increase in the rate of secretion upon the addition of Ca^{2+}. Stimulation of the adrenal medulla with acetylcholine, just as auxin treatment of some plant cells (1, 5), leads to depolarization, which is essential to the influx of Ca^{2+}. The large electrochemical Ca^{2+} gradient in favor of the extracellular space in animal cells is also true for plants (31). The increase in cytosolic Ca^{2+} prior to secretion is well documented in animal cells, but not in plant cells (however, see 27). Thus, we can only hypothesize that the Ca-auxin secretion relationship is, at the moment, only a probable manifestation of the stimulus-secretion coupling mechanism in plants. The only other example that I am aware of, wherein the stimulus-secretion coupling mechanism may be involved, is the secretion of amylase isoenzymes in the barley aleurone layer (17).

To summarize, our hypothesis on the role of Ca^{2+} in the secretion of IAA is based largely on the known role of Ca^{2+} in secretion in animal systems, from *Paramecium* (23) to complex mammalian systems (29). According to the chemiosmotic hypothesis of IAA transport (12, 25, 28), the polar secretion starts with the passive entry of the undissociated IAA (IAAH) mostly at the apical end of the cell. The molecule ionizes in the neutral cytoplasm and then binds to the putative carrier (13, 16) on the inside surface of the basal plasmalemma. Probably, as the internal level of IAA reaches a certain concentration, membrane depolarization ensues followed by a thermodynamically downhill Ca^{2+} influx. The rise in Ca^{2+} in the cytoplasm may lead to the binding of this element to calmodulin which then may activate the IAA-carrier complex causing the secretion of IAA.

The rise in concentration of Ca^{2+} as a 'second messenger' in the cytoplasm is transient since Ca^{2+} itself activates the Ca^{2+} efflux pump (10, 21, 24). We have evidence, as shown in the accompanying paper (7), that there is an acropetal transport of Ca^{2+} in sunflower hypocotyl segments associated with the basipetal transport of IAA. The Ca-ATPase efflux pump together with the absorption of Ca^{2+} into mitochondria and other organelles, may act to restore the cytoplasmic Ca^{2+} level to the low nonsecreting state.

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