EFFECT OF AUXIN UPON LOSS OF CALCIUM FROM CELL WALLS[1, 2]

ROBERT CLELAND[3]

DEPARTMENT OF BOTANY, UNIVERSITY OF LONDON KING'S COLLEGE, LONDON

In 1955, Bennet-Clark (2) and Carlier and Buffel (4) independently suggested that auxin induces elongation of stem and coleoptile tissues by removing calcium from the cell wall. According to this calcium-bridge hypothesis, the factor which limits cellular expansion is the number of calcium ions which cross-link the pectin chains of the cell wall. Removing such cross-linkages by auxin would result in elongation. It has been suggested that auxin brings about this removal of calcium by chelation (2, 9, 19) or by methylation of the carboxyl groups to which the calcium is attached (12, 13).

Considerable evidence from work with stems and coleoptiles has been used to support such a theory. Calcium increases the rigidity of cell walls (1, 15) and inhibits elongation (8, 16, 19). Ethylenediamine tetraacetic acid (EDTA), which might be expected to remove calcium from the wall by chelation, promotes elongation (2, 9, 19). Auxin induces an increase in the transfer of methyl groups from methionine to the pectin of Avena (12, 13) and maize (6) coleoptile cell walls.

The fact that calcium promotes root elongation suggests that calcium bridges do not control the elongation of root cells (3).

If this theory is correct, adding auxin to a tissue should result in a loss of calcium from the cell walls. The loss of calcium from whole Avena coleoptile tissues has been examined by Thimann and Takahashi. Initial experiments suggested that the combination of auxin and EDTA enhanced this calcium loss (17) but subsequent experiments have indicated that the increase is not significant (Thimann, personal communic.)

This calcium-bridge hypothesis has now been directly tested. The effect of auxin on the loss of Ca^{45} from cell walls has now been determined in Avena coleoptile and maize mesocotyl tissue.

MATERIALS AND METHODS

Experiments were first performed with mesocotyl sections of *Zea mays* L. and were later repeated with coleoptile sections of *Avena sativa* L. var. Victory in order to compare the results of this study with the methylation studies of Ordin et al (12, 13). Labeling of the cell walls was initially obtained by pretreating sections with Ca^{45}. However, it was thought that the carboxyl-calcium bonds formed in this way might be different from those formed at the time of cell wall synthesis. If this were so, with sections treated with Ca^{45} only the new calcium-carboxyl bonds would involve Ca^{45} and the magnitude of the loss of Ca^{45} would not be a true indication of the amount of loss of total calcium from the walls. Therefore in some experiments Avena seedlings were grown from germination in a solution which contained Ca^{45} so as to be certain that the calcium present in the walls would be uniformly radioactive.

Maize mesocotyl sections were obtained as follows. Maize seeds were germinated in moist sand and allowed to grow in the dark for about 90 hours. Mesocotyls of 2.5 to 3.5 cm in length were then selected and one 5 mm section was excised from each mesocotyl, using a double-bladed cutter. The apical incision was 1 mm below the node. Avena coleoptile sections were obtained in the manner of McRae and Bonner (11).

The sections were incubated for 4 to 10 hours in 20 ml of water containing $Ca^{45}Cl_2$ in a concentration of either 1 or 2.2×10^{-6} M. $Ca^{45}Cl_2$ had a specific activity of 1.4 mC/mg. The sections were then collected, rinsed with distilled water, and placed in distilled water for 1 to 2 hours to remove excess Ca^{45}. One lot of sections was then harvested at this point in order to determine the initial labeling of the cell wall; the remaining sections were ready for the experimental treatment.

Alternatively, Avena seeds were soaked for 18 hours and husked. Four hundred seeds were then placed on dacron gauze mesh which was stretched over a frame and suspended so that the surface of the mesh barely touched the surface of 1 liter of 2×10^{-7} M $Ca^{45}Cl_2$ solution. After about 70 hours, coleoptiles of 2.5 to 3.0 cm in length were selected and a 10 mm section was excised from each coleoptile. The apical cut was 3 mm below the tip. The central leaf was removed from the Avena coleoptile sections in every experiment. Sections were washed for 30 minutes in water. One lot of sections was harvested in order to determine the initial labeling of the cell wall; the remaining sections were ready for use.

Sections whose cell walls were labeled with Ca^{45} were then incubated for 8 to 24 hours in 20 ml of 1 % sucrose solution, $\pm$ 5 ppm indoleacetic acid (IAA). In many experiments this solution also contained EDTA due to the report that EDTA facilitated the loss of calcium from Avena coleoptile tissues (17). EDTA was used in a concentration of 0.001 M and unbuffered since these conditions have been reported to enhance the growth of Avena coleoptile sections (3). At the end of the incubation, sections were col-

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[3] Present address: Department of Botany, University of California, Berkeley 4.

TABLE I

EFFECT OF AUXIN ON LOSS OF Ca^{45} FROM MAIZE MESOCOTYL CELL WALLS IN PRESENCE (A) AND ABSENCE (B) OF EDTA

	INCUBATION MEDIUM		FR. WEIGHT	CPM Ca^{45}/10 MG CELL WALL
A	Water	Initial	...	2,570
		+ Auxin	...	2,240
		− Auxin	...	2,310
B	EDTA	Initial	410 mg	5,930
		+ Auxin	655 mg	4,780
		− Auxin	465 mg	4,300

Experimental conditions: A) 8 hours in 10^{-6} M $Ca^{45}Cl_2$ (1.7×10^5 cpm/20 ml). 1 hour wash in water. 8 hours in water, ± 5ppm IAA. B) 10 hours in 2.2×10^{-6} M $Ca^{45}Cl_2$ (3.7×10^5 cpm/20 ml), + 1 % sucrose. 2 hour wash in water. 24 hours in 0.001 M EDTA, + 1 % sucrose, ± 5 ppm IAA. Walls prepared as explained in text. Ca^{45} extracted with hot 0.01 M EDTA.

lected, thoroughly rinsed with water, blotted dry with filter paper, and ground rapidly in a mortar with 2 ml of cold water. Cell wall debris was then separated from the water-soluble and protoplasmic materials by repeated washing and filtrating with cold water on a sintered glass funnel. Unless otherwise stated, the Ca^{45} was then removed from the wall by extracting the walls twice for 10 minutes with 2 ml of hot 0.01 M EDTA. After extraction almost no radioactivity remained in the wall fraction. The extracts were combined and an aliquot was plated on an aluminum planchet and counted with a thin-window Geiger-Muller tube. Fresh weights of the sections were determined before and after the final incubation in order to determine the effect of auxin upon expansion. Weight of the dry cell wall was determined after extraction upon air-dried residues. Each individual treatment contained sufficient material to give about ten milligrams weight of dry cell wall. All manipulations were carried out under a weak red light.

RESULTS

The effect of auxin on the loss of calcium from maize mesocotyl cell walls is shown in table I. In the initial experiments EDTA was added to the experimental solution because of the report of Thimann and Takahashi (17) that the loss of calcium from whole Avena coleoptile tissues was facilitated by EDTA. Carr and Ng (5) have reported that EDTA actually prevents the loss of calcium from wheat coleoptile walls. Therefore the experiments were repeated with the EDTA omitted. In both cases the results were the same: during the incubation period calcium is lost from the cell wall but auxin does not enhance this process. In most experiments less cell wall calcium was lost in auxin-treated tissues than in the controls. This difference is so small that it does not seem to be significant.

Calicum may be bound to carboxyl groups of either short-chain (pectin) or long-chain (protopectin) pectic substances. Auxin might simply be causing a redistribution of calcium between these two components rather than a loss from the walls. It has been shown that this does not occur by actually separating the calcium associated with the hot-water-soluble and hot-dilute-acid-soluble fractions of the wall. This has been done as follows. Sections after incubation with or without auxin were ground up and the wall fraction was separated in the usual manner. Calcium was then removed from the wall in three steps. Walls were extracted for 18 hours with 4 ml of cold 2:1 benzene-ethanol to remove any calcium associated with lipids. Then the walls were dried and the pectin and its associated calcium were removed by extracting the walls twice with 2 ml of hot water. Protopectin was removed by extracting twice with 2 ml of hot 0.01 M EDTA. No label remained after this extraction. It can be seen from the data of table II that auxin does not cause a redistribution of calcium between pectin and protopectin.

The effect of auxin on the loss of calcium from

TABLE II

FRACTIONATION OF MAIZE MESOCOTYL CELL WALL CALCIUM BY EXTRACTION

	ELONGATION	CPM Ca^{45}/10 MG CELL WALL, EXTRACTED BY:			TOTAL
		BENZ/ETOH	WATER	EDTA	
+ Auxin	22 %	7	1,070	1,930	3,007
− Auxin	4 %	15	995	1,770	2,780

Experimental conditions: 6 hours in 2.2×10^{-6} M $Ca^{45}Cl_2$ (3.7×10^5 cpm/20 ml). 1 hour in water. 10 hours in 0.001 M EDTA, + 1 % sucrose, ± 5 ppm IAA. Walls prepared as explained in text.

Avena coleoptile cell walls is shown in table III. Regardless of whether the calcium had been introduced to sections or seedlings, auxin had no effect on the loss of calcium from the walls, although it was inducing a large growth response at the same time.

Note that the amount of calcium lost from the cell wall depends upon both the tissue and the method by which calcium was introduced to the tissue. Calcium is lost much more easily from Avena coleoptile section cell walls than from the walls of maize mesocotyl sections. Introduction of calcium into the tissue at the time of wall synthesis renders it less easily lost from the walls.

Discussion

The calcium-bridge hypothesis has been one of the most promising of the recent theories of the mechanism of auxin-induced elongation. Its attraction lies in the fact that it explains and correlates a number of hitherto unrelated facts: the inhibition of growth by calcium and its reversal by potassium ions, the promotion of elongation by chelating agents, and the increased methylation of pectin in the presence of auxin. The main point of this hypothesis is that auxin causes a removal of calcium ions from the cell wall and this loss of calcium ions then permits elongation to occur. This hypothesis has now been directly tested. Auxin does not cause an increased loss of calcium from cell walls, nor does it cause a redistribution of calcium between the short-chain and long-chain pectic substances of the wall. The calcium-bridge hypothesis appears to be incorrect.

There are three ways in which it might be argued that these results are inconclusive. First, it has not been directly shown that the calcium removed from the wall was actually bound to pectic substances. However Jansen et al (10) have shown that the calcium-binding capacity of the wall measured by exchange with Ca^{45} is identical with the cation exchange capacity calculated from the amount of carboxyl groups in the wall. This suggests that calcium bound in the cell wall must be attached to pectic materials.

If the calcium involved in auxin-sensitive bridges is only a small part of total wall calcium, an effect of auxin upon this fraction might be obscured by the rest of the calcium. With Avena seedlings grown in a $Ca^{45}Cl_2$ solution, even if it is assumed that only 10 % of cell wall calcium comes from the solution, not more than 1 % of the cell wall carboxyl groups is bound to calcium. In order for the effect of auxin upon the loss of calcium to be missed, the bridges broken by auxin must involve less than 0.1 % of cell wall carboxyl groups. Doubt is cast as to whether or not such a low concentration of calcium-bridges can be controlling growth by the finding of Cooil and Bonner (8) that the concentration of calcium needed to saturate the cation binding sites of the cell wall is only ten times as high as the concentration which causes the least detectable inhibition of growth. This suggests that 10 % of all wall carboxyl groups must be bound to calcium ions to result in a measurable change in the growth rate.

Finally, the effect of auxin upon calcium associated with cold-water-soluble pectin has not been examined. This type of pectin, whose metabolism is influenced by auxin (10), was discarded during the preparation of the cell walls. However it seems unlikely that any auxin-induced loss of calcium from this fraction can promote wall plasticity and elongation when the presence of the calcium does not even render this pectin insoluble.

The evidence would seem to show conclusively that the calcium-bridge hypothesis is incorrect in regard to Avena coleoptile and maize mesocotyl tissues. In view of this conclusion, it would be interesting to re-examine the evidence in favor of this theory.

Calcium ions inhibit auxin-induced elongation in the Avena coleoptile (8, 16). This is due, in part, to a stiffening of the cell wall (15). Monovalent ions such as potassium reverse this calcium-induced stiffening but have no effect on wall plasticity in Avena coleoptile tissues to which calcium has not been added (8, 15). Calcium must certainly cause this stiffening by formation of calcium bridges, but it has

Table III

Effect of Auxin on Loss of Ca^{45} From Avena Coleoptile Cell Walls as Related to Method of Introducing Ca^{45} Into Tissues. Wall Labeled by Introducing Ca^{45} to Sections (A) or Seedlings (B)

	Ca^{45} Introduced to		Growth	cpm Ca^{45}/10 mg cell wall
A	Sections	Initial	...	5,930
		+ Auxin	29 %	1,485
		− Auxin	15 %	1,580
B	Seedlings	Initial	...	5,540
		+ Auxin	42 %	3,300
		− Auxin	21 %	3,300

Experimental conditions: A) 4 hours in 2.2×10^{-6} M $Ca^{45}Cl_2$ (3.7×10^5 cpm/ 20 ml). 1 hour in water. 17 hours in 0.001 M EDTA, + 1 % sucrose, ± 5 ppm IAA. B) Seedlings grown in 2×10^{-7} M $Ca^{45}Cl_2$ (1.7×10^6 cpm/liter). Sections 24 hours in 0.001 M EDTA, + 0.0025 M K–Maleate (pH 4.8), + 1 % sucrose, ± 5 ppm IAA. Walls prepared and Ca^{45} extracted as explained in text.

yet to be conclusively shown that such cross-linkages occur in cell walls in the absence of added calcium, although it is probable that they exist in at least small amounts since the cell wall contains calcium. The fact that elongation is inhibited by calcium cannot be taken as evidence to indicate that auxin must remove calcium from the cell wall in order for elongation to occur.

Chelating agents promote shoot growth (2, 9, 19). It appears that these agents do not remove calcium from cell walls, however (5). IAA has been shown to form complexes with copper and iron, but all attempts to show IAA-calcium complexes have failed (7, 14). The effect of chelates and auxin upon growth does not seem to be due to any ability on their part to chelate cell wall calcium.

The auxin-induced transfer of methyl groups from methionine to hot-water-soluble pectin occurs in Avena coleoptiles (12, 13), but cannot be demonstrated in maize mesocotyls (6). Although this may only be a reflection of a marked preference by maize mesocotyls for methyl groups from some other source than methionine, the necessity of methylation of this pectin for auxin-action must be considered to be in doubt.

Thimann and Takahashi (17) reported that the loss of calcium from whole Avena coleoptile sections is enhanced by a combination of auxin and EDTA. They have since concluded that this effect is not large enough to be significant (Thimann, personal communic.) and that auxin does not cause a loss of calcium from the cell wall.

While the increase in cell wall rigidity upon adding calcium ions is almost certainly due to formation of calcium cross-linkages, there is no conclusive evidence that the rigidity of the walls in the absence of *added* calcium is actually due to calcium cross-linkages. But perhaps wall rigidity is due to other kinds of cross-linkages, such as ones involving sulfhydryl or amide bonds, and it is these linkages which are severed by auxin, leading to elongation.

Summary

The calcium-bridge hypothesis of auxin-action has been put to a direct test by determining the effect of auxin upon the loss of Ca^{45} from cell walls. The loss of calcium from Avena coleoptile and maize mesocotyl cell walls is not enhanced by auxin, irrespective of whether the Ca^{45} was introduced into the walls at the time of wall synthesis or only afterwards. Auxin does not cause a redistribution of calcium between pectin and protopectin. It would appear that the removal of calcium cross-linkages is not mediated by auxin and that the calcium-bridge hypothesis is incorrect.

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