Auxin and Gibberellin Effects on Cell Growth and Starch During Abscission in Cotton[1]

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Summary. An increase in starch content of cells in the abscission zone of the cotton explant appeared correlated with an increase in number of cells. A large increase in the number of cells in the abscission zone, concomitant with an increase in starch content, followed treatment with gibberellin as compared to auxin. In the final stages of abscission starch was hydrolyzed in the cells of the separation layer. Some starch remained after the petiole abscised.

A positive phloroglucinol-hydrochloric acid reaction in the cells of the petiole distal to the line of separation indicated the presence, not of lignin, but of soluble sugars and uronic acids. This reaction was especially intense following gibberellic acid treatment.

It was concluded that gibberellin in accelerating abscission leads to (1) an increase in cell number and starch content in the abscission zone, (2) the hydrolysis of starch in the separation layer just before abscission, and (3) the breakdown of polysaccharides and the release of soluble sugars and uronic acids. Auxin, an abscission retardant, either delays or prevents these events.

This study reports on the effects of gibberellin and auxin on the abscission zone of cotton through observations on cell growth, starch content, and lignification. The work by Ramsdell (8) and Morris (6) noted high accumulations of starch, particularly on the proximal side of the abscission zones of the petiole and pedicel of cotton. In *Coleus,* Sampson (12) reported a gradual increase of reducing sugars with aging of the leaves and stems. This increase was least pronounced in the separation layer, indicating that sugars probably were utilized in the synthesis of starch in the abscission zone. Detailed analyses of carbohydrate changes as influenced by applications of gibberellin and auxin should contribute to an understanding of abscission. Accordingly, in this study of cotton, quantitative as well as qualitative observations have been made on the distribution pattern of starch and the changes in starch content as related to cell size and growth in and near the abscission zone. The possible significance of a phloroglucinol-HCl reaction is also reported.

Material and Methods

Treatments and Sample Preparation. In this investigation the explant, or living tissue excised from the seedling, includes the cotyledonary node with 3 mm of the bases of the petioles of the cotyledons attached, a 3-mm portion of the stem, and a 10-mm portion of the hypocotyl. Explants of 14-day-old cotton (*Gossypium hirsutum* L.) seedlings were treated with abscission-retarding amounts of auxin (0.125 μg IAA per abscission zone) or accelerating amounts of gibberellic acid (0.01 μg GA_3 per abscission zone). Agar served as control. The technique by which these hormones were applied, in 5-μl droplets of 0.75 % agar from graduated 500-μl tuberculin syringes, is described by Addicott et al. (1). Explants were sampled at different macroscopic stages of abscission development. Figures 1 and 2 show the sequence of macroscopic changes occurring in explants treated with gibberellin and auxin, with the time required to reach each stage. Standard deviations are shown for control and GA_3-treated explants, but excessive variation occurred in IAA-treated material and the time to each stage is an average of 3 consecutive experiments involving 120 explants.

Explants to be embedded in Parowax paraffin by a freeze-substitution method were removed from petri dishes, blotted, and the nodes halved (to prevent splitting of the tissue in liquid nitrogen) leaving all petioles intact. The tissue pieces were immediately
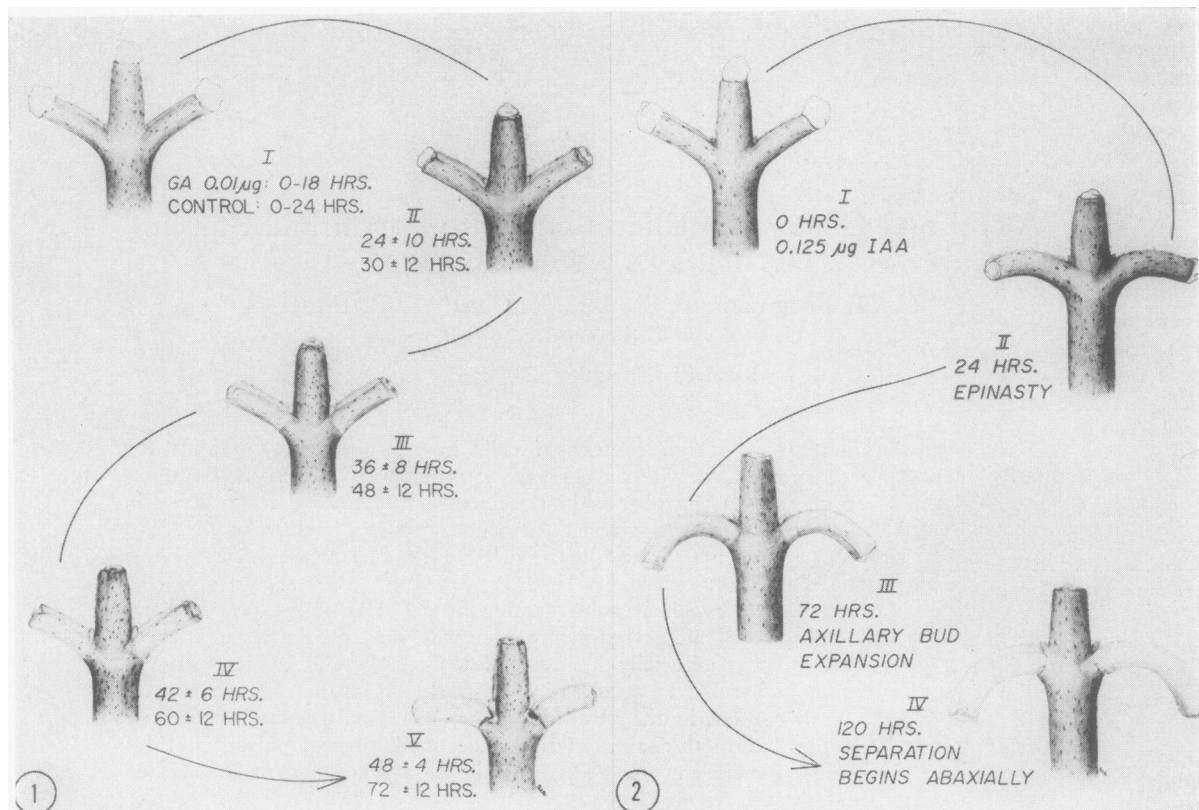
Fig. 1–2. Macroscopic stages associated with abscission in 14-day-old cotton seedlings. Fig. 1. Control and GA$_3$-treated explants. Fig. 2. IAA-treated explants.

freeze-substituted by a method modified from Jensen (4). This modification consisted of placing the quick-frozen tissue in cold ($-35°$) methanol in a deep freeze, followed by subsequent changes of cold absolute ethanol, *n*-propanol, and *n*-butanol at 24-hour intervals. After 24 hours in *n*-butanol the tissue was placed in fresh, cold *n*-butanol and slowly brought to room temperature. The last alcohol change was not replaced by toluene since this resulted in excessive shrinkage of the tissue, but was saturated with, and eventually replaced by, Parowax paraffin. The tissue was embedded in Parowax and sectioned at 10 to 20 μ. The most proximal segment of petiole thus included the tissue immediately distal to the estimated future line of separation. Fresh sections of explants were cut 10 to 20 μ thick with a microtome-cryostat at $-15°$. They were stained with I_2-KI to show starch ; lignin was stained with either a saturated solution of phloroglucinol in 20 % HCl, chlorine sulfite (13), or Maule's reagent. A photomicrograph was recorded of each treatment and stage.

Cell Number. Figure 3 shows the region in the cotyledonary petiole from which serial 10 μ sections, commencing 2 mm from the abscission zone, were taken for analysis of cell number and starch content. For cell number determinations a transverse section

10 μ thick of each 100 μ segment was taken midway between the ends of the segment. Median longitudinal sections were prepared from the opposing petioles, which were similarly treated with growth
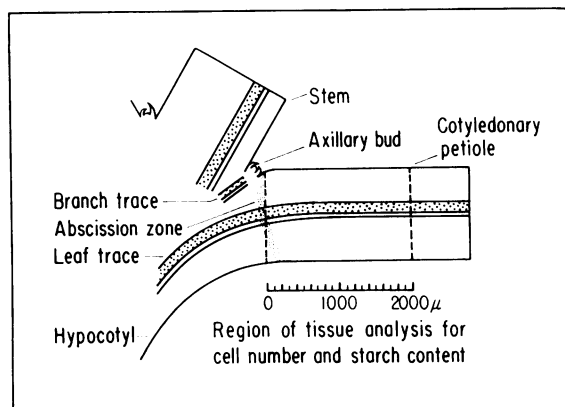

Fig. 3. Diagram of cotton explant showing region (between broken lines) in the cotyledonary petiole from which tissue was sectioned for starch and cell count determinations, × 10.

substances or as controls. All sections were depar-
affinized and stained with periodic acid-Schiff's
reaction to delineate the cell walls. Measurements
were made on photomicrographs enlarged to a final
magnification of X100. Jensen's (4) method for
determining cell number was adapted to this material
as follows: (1) tissue segment volume was calcu-
lated by multiplying the length of the segment (90
μ, since one section was sacrificed for cell counts)
by the area of the section, which was determined
from the photomicrographs with a planimeter; (2)
average volume per cell was calculated by determining
(a) the average per cell cross-sectional area (dividing
the tissue cross-sectional area by all the cells visible)
and (b) the average cell length (based on 50 meas-
urements of ground parenchyma cells from each
corresponding region in the longitudinal sections).
Finally, cell number for each petiole segment was
determined by dividing tissue segment volume by
the average volume per cell.

Starch Content. The starch content of the petioles
during abscission was determined by means of an
adaptation of the anthrone reaction for starch (2, 5).
Eight 90 μ segments (the midsection was sacrificed
for cell counts) were required for a starch determina-
tion that would fall within the range of 0 to 50 μg.
Eight petioles were selected for uniformity. The
explants were freeze-substituted, infiltrated, and em-
bedded in Parowax, and 10 μ transverse sections
were made. The corresponding 9 X 10 μ segments
were placed in separate tubes, deparaffinized, and
repeatedly washed with 80 % (v/v) ethanol until a
negative reaction for soluble sugars was obtained
with anthrone reagent, consisting of 0.2 g anthrone
per 100 ml cold 36 N sulfuric acid, freshly prepared.
The tissue was then washed with water and extracted
with 8.61 N perchloric acid at 0°. The extract was
saved and the tissue re-extracted. The combined
extracts were made up to 100 ml with water and
filtered, and a 5 ml aliquot was diluted to 100 ml.
Ten ml of cold anthrone reagent was added to 5 ml
of the tissue extract in an ice bath. The solution was
heated at 100° for 7.5 minutes and cooled in an ice
bath to 25°. Absorption was measured in a Beck-
man DU spectrophotometer at 625 mμ. Glucose
was used as a standard. From a stock solution of
100 mg in 100 ml of water, 10 ml was diluted to a
liter from which 5 ml was used for a standard at
50 μg glucose. Determinations were reproducible
over the range 1 to 50 μg $\pm$ 2 μg. Starch content
was expressed as glucose equivalents per segment and
per cell.

Results

Cell Number, Starch Distribution and Content.
Figure 4 shows the distribution of starch as ab-
scission progressed. The only starch present in the
abscission zone at the time of excision was that as-
sociated with the vascular parenchyma. With the
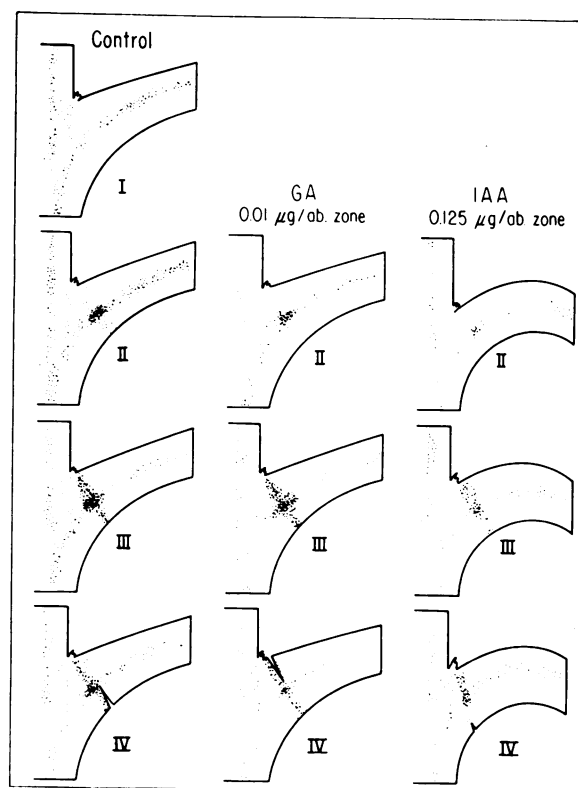onset of cell division in control and GA$_3$-treated

FIG. 4. Starch distribution in cotton explants cor-
related with macroscopic changes I–IV, in the progression
of abscission. X 6.

explants, starch was deposited in the abscission zone
through stage III but was hydrolyzed during stage
IV just prior to and during separation. Depletion
of starch occurred more rapidly distally than prox-
imally although some starch still remained distally
in the division cells. In IAA-treated petioles some
starch synthesis occurs though it is much less than
in the GA$_3$-treated and control material.

The results of the cell counts in 90 μ thick seg-
ments of petiole collected at specified distances from
the zone of abscission are presented in figure 5. Cell
counts of control, GA$_3$-, and IAA-treated explants
of stages II and IV (fig 1, 2) were compared with
those in freshly excised petioles. There was a sig-
nificant, approximately 50 %, increase in number of
cells in the abscission zone from time zero to stage
II in controls and GA$_3$ treatments. No cell divisions
appeared to occur in IAA-treated tissue by stage II,
but as a result of elongation (epinasty) the actual
cell counts were lower, especially distal in the petiole.
Cell division commenced at approximately 72 hours
after IAA was applied. This was reflected in stage
IV, where cell counts 120 hours after treatment were
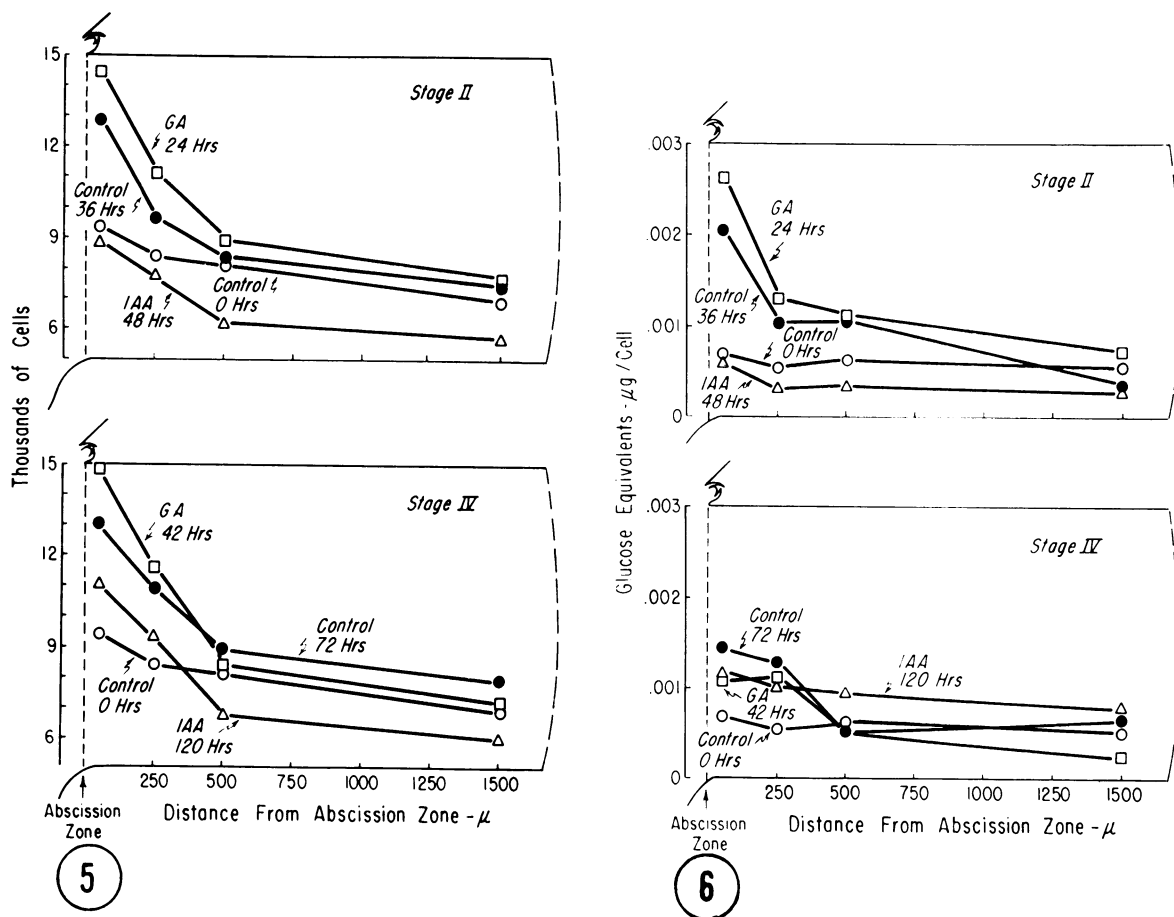greater than the control. No significant increase in

FIG. 5–6. Tissue analysis of cotyledonary petiole. FIG. 5. Thousands of cells per 90 μ segment. FIG. 6. Starch content expressed as μg glucose equivalents per cell.

cell number over stage II occurred in GA$_3$ and control treatments by stage IV, which indicates that most cell division took place before and during stage II.

Starch content expressed in terms of glucose equivalents is shown in figure 6 on a per cell basis. Except in IAA-treated tissue, starch was rapidly synthesized in the abscission zone and for at least 5 cells distal to it during stage II. Starch content (as glucose equivalents) in control (36 hrs) and gibberellin (24 hrs) treatments was approximately 2.0 × 10^{-3} and 2.6 × 10^{-3} μg per cell, respectively, compared to 0.7 × 10^{-3} μg per cell in the control (0 hrs). Starch formation paralleled cell division. However, just before separation (stage IV), starch appeared to be extensively hydrolyzed in the separation layer, although it did not return to the level found in freshly excised material (control, 0 hrs).

Figure 6, stage IV, shows that in GA$_3$ treated explants starch hydrolysis in the abscission zone was more complete than in the 72-hour control. It was also evident that more starch per cell remained by

stage IV following IAA than with GA$_3$ treatment. Observations based on these determinations and the staining reaction with I$_2$-KI showed that auxin was preventing excessive hydrolysis of starch by preventing breakdown of the cells and their amyloplasts.

Phloroglucinol-Hydrochloric Acid Reaction. In the abscission zones of freshly excised tissue a red-violet staining reaction was found solely in the ligni-fied walls of the vessel elements, but as abscission progressed in control and GA$_3$-treated explants, the walls of cells immediately distal to the future line of separation became stained and the reaction became more intense with time. This reaction was especially intense with gibberellin treatment and was more vivid in fresh than in fixed tissue. The staining reaction was of such a delineating nature that the walls of the distal half of a mother cell in the separation layer would stain red whereas those of the proximal half would not. Distal tissues of IAA-treated ex-plants either stained weakly or did not react. Only the walls of vessel elements gave positive reactions with the chlorine-sulfite test and Maule's reagent.

Among the common constituents of cell-wall poly-saccharides, L-arabinose, D-xylose, D-galacturonic acid, and D-glucuronic acid gave a red-violet color when heated with phloroglucinol-HCl.

Discussion

Paired, anaphase, metaphase, and telophase nuclei indicated, and cell counts confirmed, an increase in cell divisions in the abscission zone. The actual number of cells in the abscission zone was undoubtedly greater than that reflected in the curves (fig 5) since transverse sections invariably cut obliquely through the region of most active division as shown in figure 3. The increase in cell number resulting from GA_3 treatment is in keeping with the findings of Sachs (10) and of Sachs et al. (11). In the IAA-treated explants cell division increased only after the petiole had completed its major increase in length. As a result of elongation, the number of cells in this material 48 hours after treatment was less than in the control. Cell number in the most distal tissue of the petioles remained fairly constant.

The initially greater starch content of the cells in the abscission zone following GA_3 treatment (stage II) appeared to be correlated with increased cell division. Starch probably was hydrolyzed in the cells of the distal parenchyma and resynthesized in the abscission zone. By stage IV, however, starch of GA_3-treated tissue was more completely hydrolyzed than that of other treatments. Paleg (7) provided evidence that GA_3 increased amylolytic activity in the barley endosperm. The effects of GA_3 in this investigation could probably be ascribed to such activity.

By stage II, the starch content in IAA-treated explants was less than half that of the controls. Again this was correlated with cell division activity. By stage IV the amount of starch in IAA treatments had actually increased during the active period of cell division between stages II and IV. Part of this increase can be attributed to the slower pre-abscission hydrolysis of the starch. Even during separation many intact amyloplasts were observed proximally as well as distally.

It is difficult to compare amounts of constituents from different species and different parts of plants. Jensen (3) analyzed root tip cells of *Allium* and found that the total carbohydrates ranged from approximately 2 to 8 $\times$ 10^{-4} μg glucose equivalents per cell over the first 2 mm. In this investigation the starch content, expressed in glucose equivalents, varied from less than 10^{-3} to 3 $\times$ 10^{-3} μg per cell.

The presence of lignin is believed indicated by a red-violet color in tissue stained with phloroglucinol-HCl. However, the unusual staining pattern produced in the abscission zone of the cotton explant indicated that either (a) lignification was occurring, or (b) wound gum was being produced as a result of cell-wall breakdown, or (c) some other breakdown

products were reacting positively in this test. Lignification of the tissue was unlikely since both the chlorine-sulfite test and Maule's reagent indicated the presence of lignin only in the walls of the tracheary elements. At the time of abscission the petiole was in a state of senescence and although lignin may have arisen from cytoplasmic precursors in some of the differentiating cells it seemed unlikely that the parenchyma of the cortex would undergo lignification. Wound gum was found in vessels and in the lumina of vascular parenchyma near the cut surfaces and also in vessels in the abscission zone of the explant. It gave a positive test with phloroglucinol-HCl. According to Robinson (9) pentoses, and polysaccharides containing them, give a red-violet color with phloroglucinol-HCl; uronic acids also give this test. In this investigation the high intensity of staining in the fresh tissues may thus have been due to pentoses, polysaccharides containing them, and uronic acids. Since GA_3 treatment enhanced the staining reaction it probably was due to an enhancement of the reactions leading to the breakdown of cell-wall polysaccharides and formation of soluble sugars and acids. Four of the most common basic units of cell-wall polysaccharides, namely D-xylose, L-arabinose, D-glucoronic acid, and D-galacturonic acid, gave a positive color test with phloroglucinol-HCl. The basis of this reaction apparently lies with the pentoses. They give rise to furfurals which react with phloroglucinol to produce the colored compound. However, the uronic acids react with HCl to produce CO_2 and pentoses (e.g., L-arabinose), which then yield furfural as above. This reaction, therefore, is not specific for pentoses only. The remarkably sharp line of staining, involving in many cases only part of a cell, suggests that the soluble sugars and uronic acids remain to a large extent in situ where formed in the walls. The wound gums possibly result from the degradation of carbohydrate materials within the protoplasts rather than in the cell walls.

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