Geotropic Response of Wheat Coleoptiles in Absence of Amyloplast Starch

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ABSTRACT Young coleoptiles of wheat (*Triticum durum* var. Henry), depleted of amyloplast starch by incubation at 30°C with gibberellin plus kinetin, retained their geotropic responsiveness. Depleted coleoptiles curved upward more slowly than controls, but this was commensurate with their slower growth. The ratio of curvature to growth was about 50° per mm of elongation in both cases. Newly excised coleoptiles, though containing much more starch than incubated controls, curved only about 25° per mm. The tissue treated in gibberellin plus kinetin appeared to contain no starch when examined (*a*) freshly squashed, (*b*) as fixed material sectioned thin and stained by the PAS procedure, and (*c*) as electron micrographs. Shrunken, starch-free amyloplasts could be identified in certain regions, but these did not show evidence of asymmetric distribution under the influence of gravity. The possibilities that other organelles function as statoliths are considered, and it is concluded not only that georeception is independent of starch grains, but further that it may not be due to statoliths at all in an ordinary sense.

The idea that some relatively dense falling object produces an asymmetry whereby plant cells detect the gravitational field was first put forward by Noll in 1892. In 1900 two authors independently identified these hypothetical objects with observed structures: Němec with either starch grains or various crystals in root caps, and Haberlandt with the large starch grains or amyloplasts of the starch sheath in stems. Both men acknowledged deriving the idea from Noll, and he in turn derived it from the earlier discovery of invertebrate statocysts, hollow organs containing a heavy concretion or statolith which by pressing against the wall initiates the response to gravity.[1] Over the years many botanists have studied and discussed the starch grain hypothesis, most

[1] Haberlandt noted that starch grains had been seen to accumulate on the lower cross-wall of stem pith cells by Behnecke in a thesis of 1880.

recently Němec himself (1964). It has been shown that in roots, and frequently in shoots, the location of cells containing such grains coincides well with the region of geotropic sensitivity (Rawitscher, 1932, and see especially the work of von Guttenberg on coleoptiles, 1911). The time required for the grains of both shoots and roots to fall often agrees well with the stimulation time required to produce threshold geotropic response (Haberlandt, 1914; Hawker, 1932, 1933). Furthermore, the different responsiveness of the same organs of different species could be correlated with the different sizes, quantities, and falling times of their starch grains (Haberlandt, 1914) and similar correlations were made using organs of differing sensitivities within a single species (Haberlandt, 1914; Prankerd, 1922; Hawker, 1932). It has also been calculated (Audus, 1962) that no other organelle is large and dense enough to fulfil the functions of a statolith.

Nevertheless the idea has never been definitely proved, since it has been found extremely difficult to free responsive plants from their starch grains while maintaining normal growth, and then to study their geotropic sensitivity. Such attempts have been made in many laboratories, including that of of the authors. A few workers have, however, claimed success by exposing seedlings to unusual conditions. Haberlandt (1902) was the first to observe that plants could lose starch after prolonged chilling; his plants also lost geotropic sensitivity, and when the plants were returned to normal temperatures both starch grains and geotropic sensitivity were regained. Němec (1902) embedded seedling bean and pea roots in plaster of Paris and found that starch disappeared from the tips; geotropic responsiveness disappeared as well. Francis Darwin, who attempted to deplete plants of starch by heat treatment (1903), appreciated the ambiguity of such experiments when he observed that "the loss of the starch may perhaps be no more than a symptom of exhaustion which shows itself both geo- and heliotropically."

On the other hand Went and Pekelharing (1909), who partially depleted *Lepidium* roots of their starch by exposure to potassium alum, reported (though without numerical data) that geotropic curvatures could still be produced in some roots. However, it is not clear (*a*) that the plants were wholly starch-free or (*b*) that the curvatures were normal in rate or extent. Many of the roots were stated to have been distorted. Similar objections may be raised concerning the geotropically reactive SO_2-treated and decapitated "starch-free" roots of Syre (1938). More recently, von Bismarck (1959) has found that sphagnum shoots depleted of starch by chilling still show geotropic curvature. Long stimulation times were employed, and quantitative comparisons of experimental and control material were not entirely rigorous. Also, since the cytology of sphagnum has not been intensively studied, it is not clear that starch grains are the only intracellular objects large and dense enough to serve

as statoliths. Perhaps for these reasons his conclusion that geotropism occurs without statoliths has not been widely accepted.

When in 1962 Boothby and Wright reported that the starch content of isolated young wheat coleoptiles was decreased on incubation with gibberellic acid and kinetin, it seemed to us possible that the statolith hypothesis might at last be put to a rigorous test in a widely studied organ under conditions favorable to growth. The extent of the starch breakdown was indicated by their observation that gibberellic acid or kinetin had the same growth-promoting effect as did glucose. Furthermore, gibberellins are known to promote amylase activity in barley endosperm (Paleg, 1960, 1961; Varner, 1964), and kinins act synergistically with gibberellins in seed germination (Ikuma and Thimann, 1963). The phenomenon has therefore been employed in the following study of the relation between starch grains and geotropic curvature.

PROCEDURE

To prepare coleoptiles for experimentation, seeds of a bearded hard red spring wheat (*Triticum durum* var. Henry from the L. L. Olds Co., Madison, Wis.) were soaked for 2 hr and laid out on moist filter paper. After about 32 hr at 25°C, coleoptiles which had attained a length of 2 to 3 mm were excised and groups of 25 were placed in cotton-plugged 50 ml flasks containing 2.5 ml of the incubation solution. To promote starch degradation, a solution containing both 10^{-5} M gibberellic acid (GA_3, abbreviated G below) and 7×10^{-5} M kinetin (K) was used. Preliminary experimentation showed that by the time starch degradation was judged complete in the coleoptiles incubated in G + K, the starch plastids in water-treated control coleoptiles had become smaller and fewer than was desirable. Further, the growth of such controls during the incubation period was less than that of the plants incubated in G + K solution. Some of the controls were therefore incubated in 10^{-3} M sucrose (0.03%), a concentration which supported a growth rate comparable to that of the coleoptiles in G + K and sufficed to maintain up to 40 large amyloplasts per cell. All solutions contained 10^{-4} M potassium benzyl penicillinate. The flasks were shaken at 30°C, since starch degradation at 25°C was found to be very slow. The coleoptiles were rinsed and transferred to fresh flasks of solution at intervals of approximately 8 hr to minimize microbial contamination. Except while being manipulated under dim red light, the coleoptiles were maintained in darkness.

Starch depletion was judged by microscopic examination of numerous longitudinally halved coleoptiles flattened gently with a cover slip. The tissue is thin enough so that by observing from both inner and outer surfaces almost all cells can be inspected. For confirmation, serial sections of coleoptiles stained by the periodic acid–Schiff (PAS) procedure (Jensen, 1962) were examined. Initially, sections were cut 1 to 1.5 μ thick from material fixed in acrolein (Feder, 1960) under dim red light and embedded in glycol methacrylate.[2] Six μ sections were later adopted because they

[2] The composition and method of purification of the glycol methacrylate mixture will be published elsewhere by Feder.

facilitated the study of plastid distribution, and glacial acetic acid: ethanol 1:3 was substituted for acrolein because the latter agent (by introducing aldehyde groups which could not be blocked by conventional means) made the PAS test less definitive.[3] Examination of amyloplasts independent of starch content was possible after staining with toluidine blue O (O'Brien et al., 1964). On occasion sections were washed free of this stain and restained with PAS reagents for comparative purposes. Electron micrographs were also prepared. For these, glutaraldehyde-fixed tissue was postfixed in osmic acid and embedded in araldite epoxy resin.

When at 34 hr starch depletion was judged complete, coleoptiles for the experiments of Tables I and II were rinsed, and both their lengths and their curvatures were measured ("initial values"). They were then geotropically stimulated by setting them horizontally in groups of 20 in 9 cm Petri dishes, each containing 2 sheets of filter paper and 5 ml of a solution of 10^{-4} M penicillin, 7×10^{-5} M K, 10^{-5} M G, and 1.5×10^{-3} M sucrose. The plane defined by the two vascular bundles was parallel to the paper. Coleoptiles which had small curvatures were oriented so that their tips bent downward; those with large angles were discarded. The dishes were maintained at 30°C. Six coleoptiles from each treatment were examined microscopically at the beginning of the experiment, and in some experiments several coleoptiles were fixed in acrolein or in acetic acid and alcohol shortly afterward. Six coleoptiles treated with G + K were checked every 3 hr thereafter until the experiment terminated. Since growth rate depended on the pretreatment, geotropic stimulation was continued until the plants of a given pretreatment were judged by eye to have attained an average curvature of 40 to 50°; this normally required from 7 to 10 hr.[4] The coleoptiles were then refrigerated and their lengths and curvatures were measured as soon as possible thereafter ("final values"). Not less than six of the coleoptiles treated with gibberellin and kinetin and six of the coleoptiles treated with sucrose were examined microscopically immediately after final measurement; these were selected as having carried out the largest curvatures in their groups.

The time course of the development of geotropic curvature was studied in a similar way except that instead of placing twenty coleoptiles on the bottom of a Petri dish twelve were lined up against the inner sides of a rectangular, transparent thin-walled plastic box. By holding a transparent goniometer against the outer sides of the box, curvatures could be measured at hourly intervals without disturbing the coleoptiles. Because the area of the filter paper–covered floor of the chambers was only eight-tenths as great as the area of the bottom of the Petri dishes, the volume of wetting solution was reduced from 5 to 4 ml.

An experiment was conducted to check whether spurious curvatures could be induced by unilateral contact with the filter paper or by the sugar and moisture gradients associated with that contact. Freshly excised 4 to 5 mm coleoptiles mounted on pins were held horizontally against vertical sheets of filter paper backed with absorbent cotton and thoroughly wet with 1 mM sucrose solution; the assemblies were contained

[3] Total blocking of nuclear coloration could, however, be achieved with chlorous acid (Rappay and van Duijn, 1965) or by postfixing in osmic acid.

[4] In certain instances where response was slow the experiment was arbitrarily terminated at 12 or 13 hr.

in the same boxes that were used for the time course experiments. Even after 24 hr, though the coleoptiles had bent nearly straight upward in response to gravity, they had not curved away from the paper.

Three aspects of the incubation procedure require special emphasis; each has led to considerable delay in establishing the protocol for the present experiments. First, depletion of starch may be very slow if microbial contamination of the incubation solution becomes heavy. Second, the incubation temperature is very critical; at 30°C even water alone allows marked starch degradation in these young coleoptiles, whereas at 25°C no concentrations of G and K, together or separately, produced starch-free tissue. Third, under some conditions the coleoptiles incubated in G plus K may lose their geotropic reactivity before losing the capacity for growth. Thus, while starch-depleted coleoptiles under the conditions finally selected for experimentation consistently curved about 45° while elongating less than 15%, coleoptiles in a series of preliminary experiments carried out under slightly different conditions failed to bend upward during stimulation, although in some cases they elongated over 50%. The influences of age, temperature, extent of starvation, microbial contamination, and other factors are evidently complex, and the present paper is concerned only with conditions under which both geotropic curvature and growth do occur.

RESULTS

1. *The Response of Starch-Depleted Coleoptiles*

The results of a representative complete experiment are presented in Table I. This table shows the length, geotropic curvature, and rate of curvature (in degrees per millimeter extension) for groups of twenty coleoptiles each. As noted above, the periods during which the coleoptiles were horizontal were selected to give about 50° of geotropic curvature in each group. The magnitude and consistency of the elongation and of the curvature of the starch-depleted coleoptiles are satisfying considering both the 44 hr period during which they were isolated from the seedling and the low level of sucrose provided to sustain metabolism during the horizontal stimulation. On the average, the horizontal coleoptiles pretreated in G + K elongated 0.08 mm per hr, and over the span of 10 hr curved about 45°. Coleoptiles preincubated in 10^{-3} M sucrose or in water grew faster—0.12 or 0.13 mm per hour respectively—and correspondingly attained a 50° curvature in a shorter time (7 or 8 hr). The ratio of degrees of curvature to increment in length is not sensibly different for the sucrose, water, and G + K treatments. In all, eight experiments have now been carried out. The results are qualitatively the same in all cases, although the experiments include minor variations on the conditions of Table I with regard to temperature, concentration of sucrose, duration of incubation, and perfection of depletion. The data are summarized, with their standard errors, in Table II. It will be noted specially that four sets of 4 to 5 mm coleoptiles which were placed horizontal immediately after severing from the seedling averaged

only 26°per mm, indicating that the long incubation did not specifically vitiate the geotropic system.

2. *Time Courses*

A critical physiological comparison between depleted coleoptiles and controls is made in Fig. 1, which compares the time courses of the curvature of coleoptiles incubated in G + K and in sucrose with that of freshly excised 4 to 5 mm

TABLE I

GROWTH AND CURVATURE OF WHEAT COLEOPTILES AFTER THREE DIFFERENT PRETREATMENTS

Pretreatment	Sucrose 1 mM				Water		Gibberellin + kinetin			
	1	2	3	4	1	2	1	2	3	4
Time horizontal:	7 *hr*				8 *hr*		10 *hr*			
	mm				*mm*		*mm*			
Length, average										
Initial	5.90	5.82	5.74	5.90	5.18	5.41	5.62	5.53	5.59	5.81
Final	6.63	6.60	6.67	6.81	6.27	6.41	6.35	6.35	6.44	6.72
Increase	0.73	0.78	0.93	0.91	1.09	1.00	0.73	0.82	0.85	0.91
Visible starch grains:	Large, plentiful				Moderate Nos.		None			
	degrees				*degrees*		*degrees*			
Curvature, average										
Initial	−15	−6	−10	−17	−6	−16	−2	−5	−7	−7
Final	+37	+47	+34	+33	+46	+29	+48	+31	+40	+38
Increase	52	53	44	50	52	44	50	36	47	45
Degrees curvature per mm of growth	71	68	47	55	48	44	68	44	47	50
Average		60			46			52		

coleoptiles. Following a lag, the response is essentially linear for all three groups. For the freshly excised coleoptiles and for those incubated in sucrose it can be seen that, as would be expected, the rate of curvature decreases when the angle of response exceeds 50°. The similarity between these two shows that *incubation alone does not change the pattern of response.* Because the growth and curvature rates of the starch-depleted plants in this experiment were somewhat slower than in most, they did not attain as great a final curvature as usual (compare with Table I). The figure suggests that curvature commences gradually, reaching a constant rate only after about 2 hr. However, inspection of the data for individual plants indicates that the response plot for any given plant is probably roughly linear from the end of the lag until about 50° of

TABLE II

RATIO OF CURVATURE TO GROWTH AVERAGED OVER EIGHT EXPERIMENTS SIMILAR TO THAT OF TABLE I

Pretreatment	None	Water	Sucrose	Gibberellin + kinetin
No. of sets (20 plants each)	4	9	15	19
Degrees curvature per mm growth ± standard error	26°*	51°±4	46°±4	49°±3

* Extreme values 21° and 32°.

curvature are attained. The gradual increase in slope shown in Fig. 1 is therefore probably due to averaging time lags of different lengths.

The responses of all three groups of coleoptiles are relatively slow when compared with that of intact 4 to 5 mm seedlings, as shown in Table III. The evidence suggests that reactivity is closely related to nutritional status. Cole-

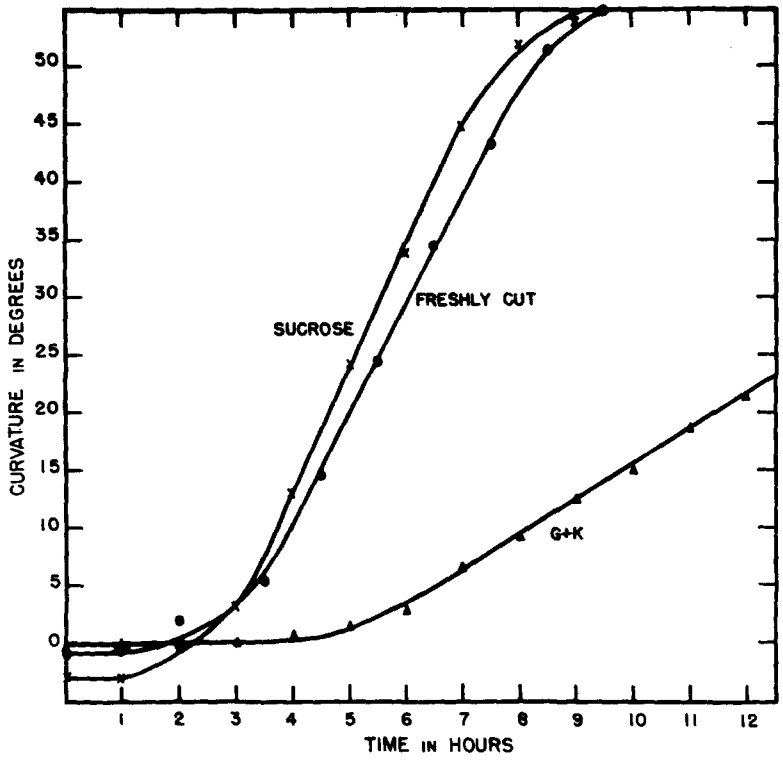


FIGURE 1. Geotropic curvature as a function of time in the horizontal position. Each point on the curves for sucrose and for gibberellin + kinetin represents 48 coleoptiles incubated as described under Procedure; each point on the "freshly cut" curve represents 24 coleoptiles. One of the 3 replicate experiments.

TABLE III

INFLUENCE OF VARIOUS TREATMENTS ON GEOTROPIC RESPONSIVENESS

Seedling preparation	Experimental treatment*	Starch grains	Experiment No.	Time to curve 20°
				hr
Intact	Growing in agar	Cytoplasm crowded with large amyloplasts	1	1.5
			2	2.5
			3	3.0
Roots removed	Growing in agar	Cytoplasm crowded with large amyloplasts	3	3.0
				3.0
Seed removed	Growing in agar	Cytoplasm crowded with large amyloplasts	2	4.5
			3	4.2
Excised coleoptile (roots, seed, and scutellum removed)	Stimulated on wet paper without sucrose	Cytoplasm crowded with large amyloplasts	1	9
			3	13
Excised coleoptile (roots, seed, and scutellum removed)	Stimulated on wet paper with 1.5 mM sucrose	Cytoplasm crowded with large amyloplasts	1	5.0
			4	6.5
			5	4.3
Excised coleoptile; incubated at 30° with sucrose	Stimulated on wet paper with 1.5 mM sucrose as in Table I	Amyloplasts large, abundant	1‡	4.5
			4	5.5
			5	4.5
Excised coleoptile; incubated at 30° with gibberellic acid and kinetin	Stimulated on wet paper with 1.5 mM sucrose as in Table I	No starch visible	1‡	11.5
			4	8
				12
			5	10
				12

* Each set of coleoptiles averaged from 4 to 5 mm in length; 20 or more coleoptiles per set. All treatments at 30°C.

‡ Values from Fig. 1.

optiles could curve 20° in about 2.5 hr when attached to the rest of the seedling, but required over 4 hr if the endosperm were removed; they required at least 9 hr when severed above the scutellum and stimulated on wet filter paper. If 1.5 mm sucrose were supplied on the filter paper, the time required to attain 20° was reduced to 5 hr. Thus, in the young excised coleoptile, available substrate for metabolism and growth appears to be rate-limiting in spite of the presence of starch. O'Brien (personal communication) has shown that the embryonic wheat coleoptile contains no starch. Apparently, when the young

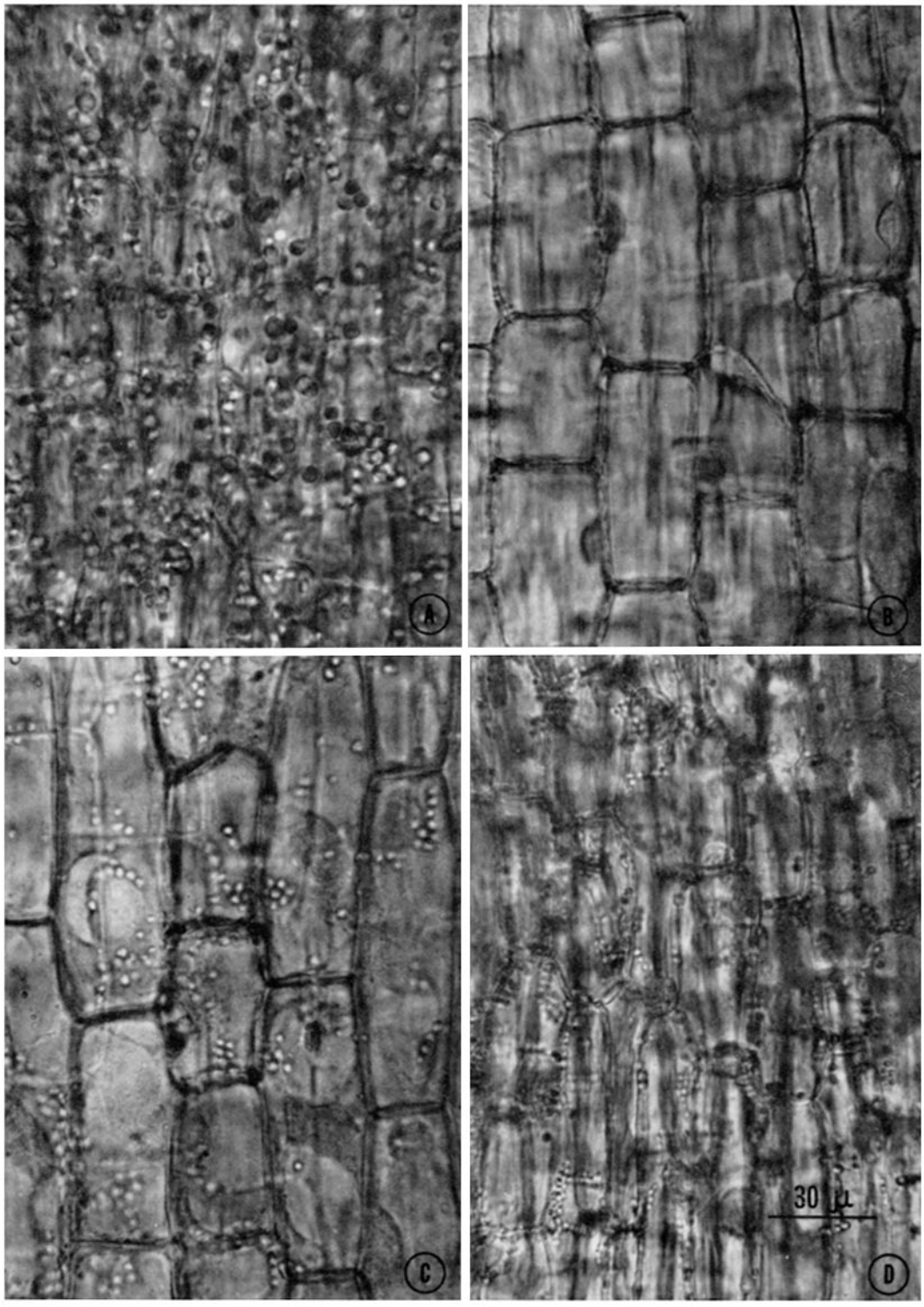
coleoptile begins to elongate, starch formation is so heavily favored that even when the sugar supply is cut off amyloplast starch is called into use very slowly and sparingly. It should be added that since the experiments of Table III were carried out as controls for other experiments, the comparisons there represent trends rather than precise relationships.

As was noted above, for most of the experiments with starch-depleted plants, 1.5 mM sucrose was supplied; this is no more than 2% of the level found optimal for bioassay of growth regulators on straight growth of coleoptiles, and young excised coleoptiles are much more severely sugar-limited than are old ones. Moreover, preliminary experiments indicated that the incubated coleoptiles grow only half as fast when laid on filter paper wet with a given sucrose concentration as when immersed in the same solution and aerated. Also in support of the view that available energy limits the responsiveness of the starch-depleted organ are preliminary experiments in which the lag times could be reduced and the curvature rates increased by supplying higher concentrations of sucrose in the stimulating medium. For example, in one experiment depleted coleoptiles supplied with 1 mM sucrose curved 3.1°/hr, those with 5 mM sucrose curved 4.2°/hr, and those with 10 mM sucrose curved 5.3°/hr. Thus by raising the concentration of sugar tenfold, the geotropic responsiveness has been almost doubled—yet the ratio of curvature to elongation was closely similar in all three sets of plants (60, 60, and 53°/mm respectively). At 5 mM sucrose a transitory regeneration of starch was observed between the 3rd and 6th hr of the experiment, though these grains were negligible in size and number when compared with those of the controls.

Taking these considerations into account, Fig. 1 is consistent with the idea that the geotropic response in the starch-depleted coleoptiles is similar to that in controls except that it is slower. Moreover, the curvatures in the experiment of Fig. 1 show about the same ratio to increments in length as was seen in Table II. For starch-depleted coleoptiles, the ratio is 49°/mm in the table and 39°/mm in the figure, for sucrose controls the ratios are 46 and approximately 42 respectively, while for freshly excised coleoptiles the respective ratios are 26 and 22.

3. *The Extent of Starch Depletion*

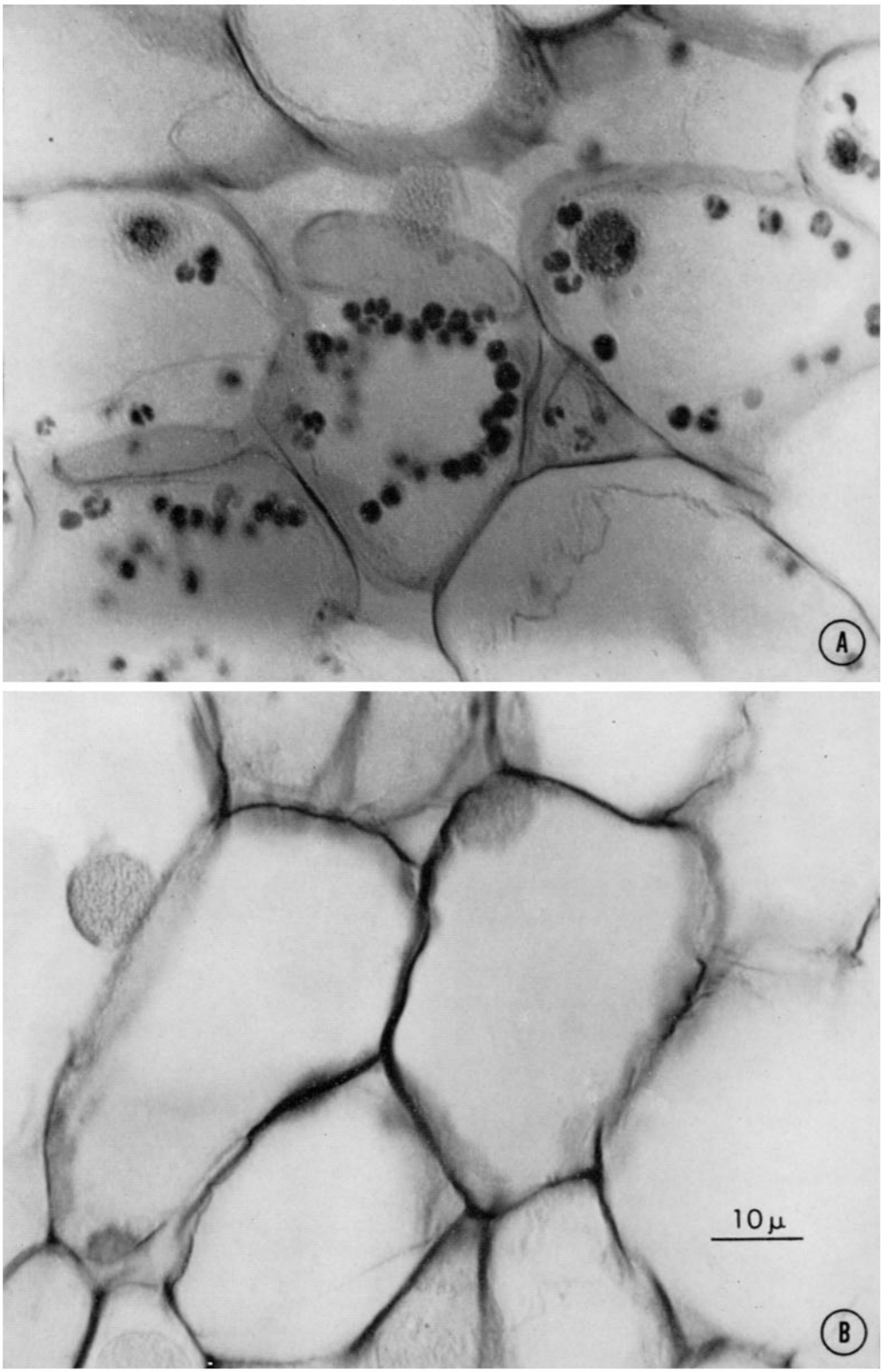
Examination of halved and flattened sugar-treated coleoptiles at a hundredfold magnification revealed innumerable large, refractile amyloplasts. The distribution of these along the coleoptile axis became less regular during the incubation period because they diminished in the apical half millimeter and in the basal region of the coleoptile. However, geotropic reactivity was demonstrated both in decapitated coleoptiles remaining on the seedling and in those excised and placed on wet filter paper.


A
B
C
30 µ
D

A photograph of a parenchymal region just under the inner epidermis and between 0.5 and 1 mm from the apex of an intact 5 mm coleoptile is shown in Fig. 2 *A*. Fig. 2 *C* illustrates a comparable region in a coleoptile which has been detached and incubated for 34 hr in 10^{-3} M sucrose; Fig. 2 *D* shows a more basal region in a similar coleoptile. It can be seen that the size and abundance of the grains decrease during the treatment, but nevertheless great numbers are still present. Fig. 2 *B*, photographed at the same magnification in a comparable region of a coleoptile depleted in G + K, illustrates the apparent total absence of visible amyloplasts. The apical cells and the large cells adjacent to the vascular bundles, in which starch grains fall most rapidly in the 25 mm oat coleoptile (Sorokin and Thimann, 1960), were examined with particular care in the depleted wheat coleoptile but no amyloplasts could be distinguished. The entire organ appeared free of starch except for the guard cells and their precursors, which frequently contained very small residual starch grains. Very fine granules could generally be noticed in many of the cells, particularly those of the epidermis. These did not have the refractivity typical of the starch grains, and were so small that they underwent considerable Brownian jostling and frequently were to be seen streaming around the cell periphery.

Although the emptiness of the coleoptiles incubated in G + K was striking to the eye, the superposition of several cells in the optical path made it desirable to confirm the extent of the depletion by other means. A direct histochemical test for starch in fresh tissue was impossible because the starch in the coleoptile of this variety of wheat reacts to iodine with an ambiguous light amber color instead of a deep blue. For this reason it is important that careful examination at 1000 × of thin, stained sections such as that of Fig. 3 *B* revealed very little truly PAS-positive material other than cell walls. Extremely rare bright but tiny pink granules in some coleoptiles probably do represent residual starch. At the basal cut surface, a layer one or two cells thick contained small starch grains; these cells appeared atypical in other ways and were probably dead. Such cells were brownish when viewed in fresh material. Faintly visible structures which occurred in the acrolein-fixed material, but were absent in the tissue fixed without aldehyde, were identified by their affinity for toluidine blue O as depleted amyloplasts (see below). Thus a comparison of tissue incubated in G + K with control tissue (Fig. 3 *A*) gives the

FIGURE 2. Light micrographs of (*A*) starch grains in cells of a freshly excised coleoptile, (*B*) starch-free cells of a coleoptile incubated 34 hr in gibberellin and kinetin, (*C*) starch grains in a coleoptile incubated 34 hr in 1 mM sucrose, and (*D*) starch grains in a lower region of a sucrose-treated coleoptile. Photographs were taken with a 20 × objective to gain depth of field, although actual observations were made with a 43 × objective.


A
10μ
B

overwhelming impression that the starch degradation procedure is extremely effective.

Recent cytological work of O'Brien and Thimann (1965), carried out concomitantly with our own experiments, shows that the form of the plastids in any given cell is to some degree a function of its position in the coleoptile. In the tip of the wheat coleoptile are found very large amyloplasts containing large starch masses but very little stroma; in the more basal parts of the coleoptile the amyloplasts are similar but contain less starch. Along the entire length of the coleoptile, in the region situated near the vascular bundles, there is a strikingly different type of plastid containing very large amounts of stroma and relatively small starch grains.

While the PAS method is regarded as a definitive test for carbohydrate (Jensen, 1962), there are two reasons why it was considered worthwhile to examine electron micrographs of the depleted material. First, they can provide an independent (though limited) check of the PAS results. Second, in the case of the plastids containing a large volume of stroma, it was of interest to see what remained of the plastids following starvation. Therefore, the subepidermal parenchymal tissue about one-half mm from the apex and close to a vascular bundle was chosen for study with the electron microscope. Whereas this region of the sugar-treated coleoptiles contained the plump, starch-filled plastids typical of the species (Fig. 4 *A*), the pictures of depleted coleoptiles showed the presence of plastids of smaller size (Fig. 4 *D*). These bodies never contained starch grains and were often shaped irregularly; the abundance of plastid sections with concave surfaces (Fig. 4, *E* and *F*) suggests that considerable shrinkage has occurred. Comparison of the depleted sections with Fig. 4, *A*, *B*, and *C* indicates that, except for the loss of starch, the depleted plastids in this region are normal in morphological detail: membranes, lamellae, oil droplets, and ground substance appear unchanged.

When 5 μ sections of the same region, stained with toluidine blue, were examined at maximal optical magnification, the amyloplasts were seen to have collapsed in volume. Small, thin, curving, irregular plastids predominate over round ones, as may be seen in Fig. 5, *A* and *B*. In the tip and in the paren-

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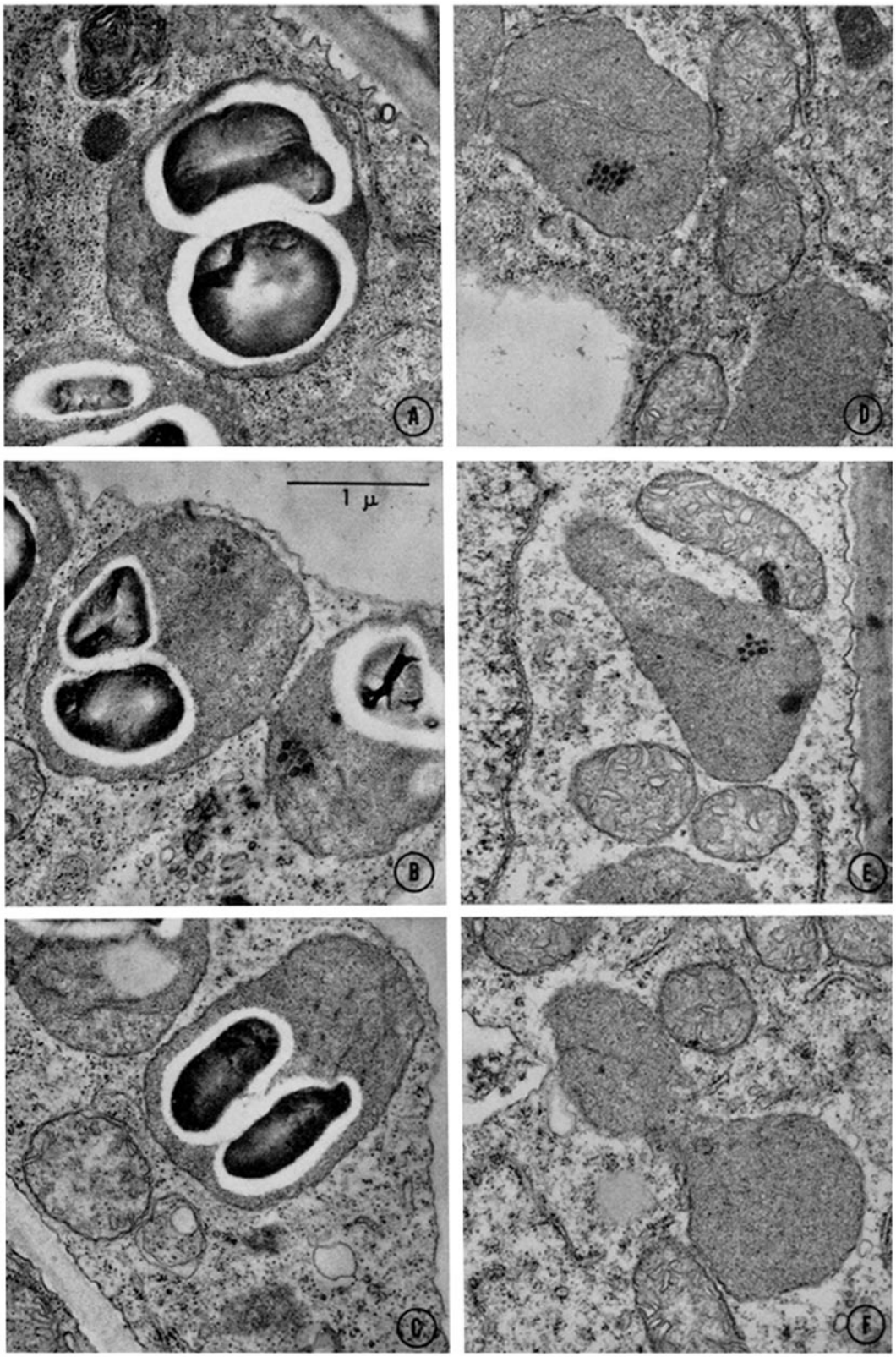
FIGURE 3. PAS-stained 6 μ sections through apex of (*A*) control incubated in sucrose and (*B*) starch-depleted coleoptile. Fixed in acetic acid and ethanol, photographed with 100 × oil immersion objective. In the control, amyloplasts up to 3 μ in diameter are conspicuous and numerous; amyloplast diameters of 4 μ were recorded in some cells. In many of the plastids the multipartite nature of the starch is clearly visible. Both control and depleted sections graze the cell wall in several regions; the proportion of each picture covered by cytoplasm rather than vacuole is thus probably similar. Nuclei, through coloring with the Schiff reagent, can be distinguished from the truly PAS-positive starch grains without difficulty.


A
D
1 μ
B
E
C
F

chyma well removed from vascular bundles, however, bodies stainable with toluidine blue could not be found, suggesting that amyloplast shrinkage and collapse in these areas are more complete than in the vascular regions.

The presence in some regions of the coleoptile of large residual plastids made it desirable to study plastid distribution during geotropic stimulation. Therefore, depleted coleoptiles were laid horizontal under standard conditions for 9 hr, which was about double the time needed on the average for geotropic response to begin in the slowest coleoptiles used (see Fig. 1). Then in order to assure that fixation would not disturb the positions of the plastids, the coleoptiles were quickly bisected in the horizontal plane to permit rapid penetration of fixative, and the lower halves were returned to their previous positions. Immediately, 10% acrolein was dripped on to the cut surfaces without moving the tissue. An hour later the tissue was transferred to a vial of acrolein and fixation was continued in the conventional manner. The coleoptiles were serially sectioned 6 μ thick in the plane which had been vertical during geotropic stimulation.

Examination of the remaining plastids large enough to be detected by light microscopy in such sections showed that they exhibit no tendency to settle to the lower sides of the cells. In order to check whether fixation preserves the asymmetry of amyloplasts under conditions where it was previously believed to occur, controls which had been freshly excised and placed horizontal for 1 hr were similarly fixed and sectioned, but stained by the PAS procedure. The amyloplasts in the apical two-thirds millimeter were found layered on the lower walls of the cells as expected (Fig. 5 *C*). Incidentally, there was no evidence either that the plastids with extensive stroma settled, or that any amyloplasts settled in the basal regions of these freshly excised young coleoptiles. The sucrose-incubated controls which had lain horizontal for 1.5 hr had relatively small starch grains which did not tend to sink to the lower sides of the cells in any large numbers; however, even exhaustive data would give no critical conclusions in this marginal case.

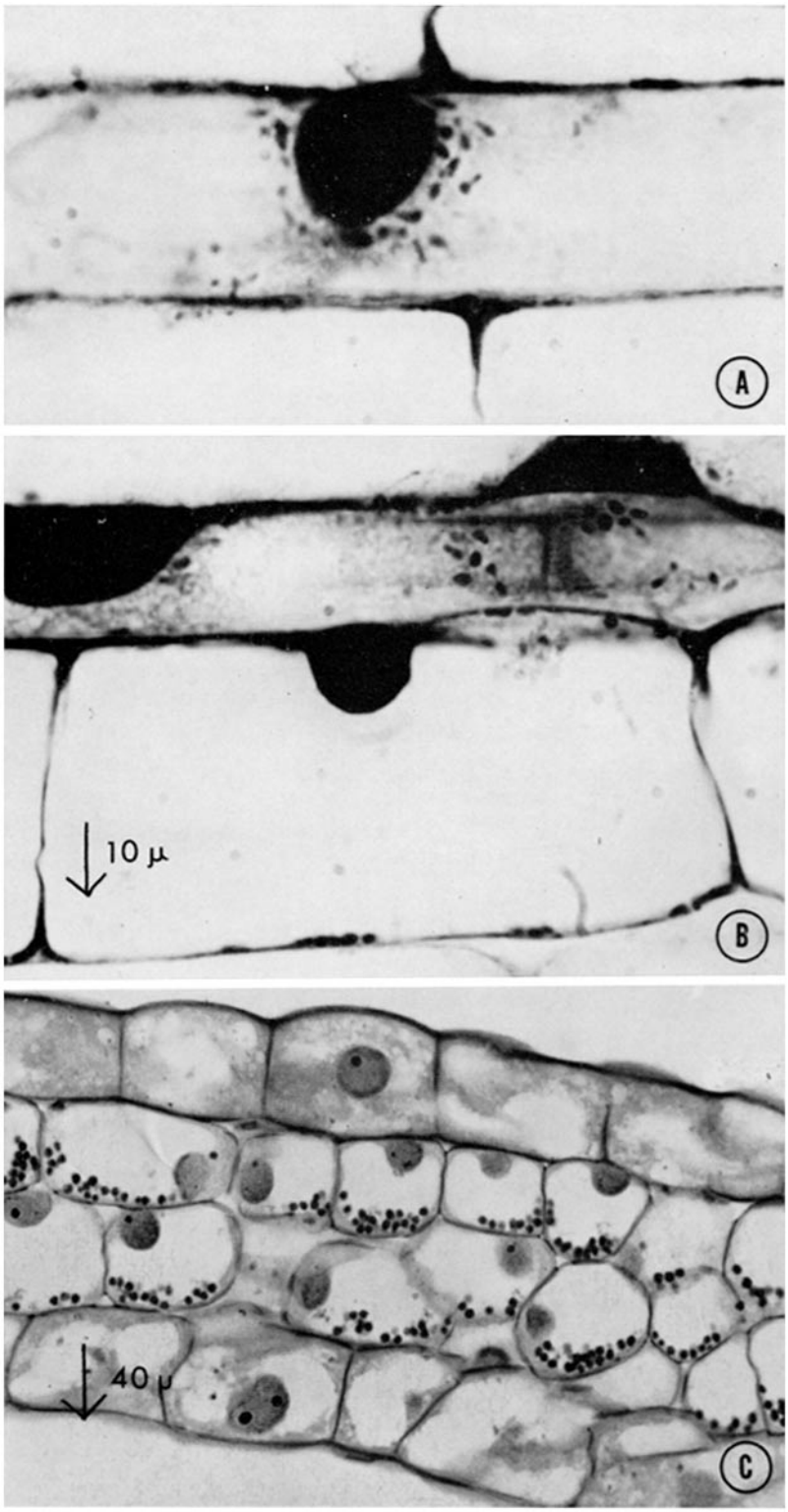
DISCUSSION AND CONCLUSIONS

1. *The Role of Starch Grains*

It seems reasonable to conclude that *amyloplast starch grains are not critical for the geotropic response.*

Not only do the young starch-free coleoptiles bend upward, but they curve

FIGURE 4. Amyloplasts (of the type possessing a voluminous stroma) in subepidermal cells of coleoptiles incubated 34 hr in sucrose (*A*, *B*, *C*) and 34 hr in gibberellin plus kinetin (*D*, *E*, *F*). Depleted plastids, though lacking starch and having concave surfaces, are normal in other respects. Sectioning and electron microscopy by T. P. O'Brien.


A
10 μ
B
40 μ
C

in the same proportion to linear growth as do the controls incubated in water or sucrose (Tables I and II). Since a tropism is a *differential growth* (toward or away from a stimulus), it follows that when the absolute growth rate is sluggish, differential growth is apt to be sluggish as well. For this reason the relation between curvature and growth rate is a particularly logical basis of comparison. Thus, since the pattern of response shows no qualitative differences, but differs only in the rate at which curvature develops (Fig. 1), it would appear not only that amyloplast starch is not essential to the geotropic process, but moreover that it does not even enhance the response when present.

It is, of course, true that the final geotropic curvature is the outcome of a complex reaction sequence. Thus, a process occurring late in the sequence might, if accelerated or made more efficient by treatment with G + K, obscure a retarded primary step. Alteration of the rate at which energy is made available, or of the pattern of energy distribution, might slow or speed the net response. The gibberellin and kinetin might alter the auxin-producing system, or perhaps the sensitivity of the tissue to auxin. However, the ages and lengths of the starch-free coleoptiles and of the sugar-incubated controls are the same and probably their physiological conditions are not very different. Furthermore, there are two types of controls—those incubated in water and in sucrose—having differing amounts of starch. It would be improbable that in all three cases the sum of the rates of primary and secondary processes should lead to nearly the same ratios of curvature to elongation. The somewhat slower growth and curvature rates of the starch-free plants probably result from a marked shortage of sugar toward the end of the depletion period.

We have not directly measured the presentation time in the present experiments. The presentation time, which is the time needed to induce just visible curvature in half the stimulated plants, has often been thought to represent the time required for the statoliths to fall to the lower sides of the cells. However, the report of Günther-Massias (1929) that intermittent stimuli of only a

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FIGURE 5. *A* and *B* toluidine blue–stained 6 μ sections from a depleted coleoptile which has been geotropically stimulated 9 hrs. The direction of gravitational attraction is shown by the arrow, the length of which also indicates the photographic magnification. The large cell in Fig. 5 *B* has depleted plastids on both the upper and lower sides of the vacuole. Adjacent to this cell is one in which the lateral cytoplasm is grazed; plastids are arranged throughout the cytoplasm without regard to gravity. Numerous other plastids occur in the sections of both *A* and *B*, but are just out of focus and thus cannot be shown without serial photographs. The region from which these sections were taken is close to that illustrated by the electron micrographs of Fig. 4. Figure 5 *C* shows at lesser magnification the asymmetric distribution of starch grains in a subapical portion, distant from the vascular tissue, of a freshly excised coleoptile which has been horizontal for 1 hr. It was fixed and sectioned as for *A* and *B*, but stained by the PAS method to bring out the starch grains.

few seconds' duration are more than additive, even when the total stimulation time is as short as the presentation time, makes this interpretation unlikely. Furthermore, the work of van Ameijden (1917) and of Brauner and Hager (1958) makes it at least very probable that geotropic induction is dependent on metabolism.[5] For these reasons, presentation time is perhaps less adequate as an index of induction than has been believed, and may depend on metabolic activity in somewhat the same way as does the curvature response. Considering also that it is more difficult to measure accurately with isolated 5 mm coleoptiles, we have chosen to study well developed curvatures.

2. *Other Possible Statolith Georeceptors*

Is there some normal cellular particle other than the amyloplast which, by virtue of size and unusually high or low density, might function as a statolith? Griffiths and Audus (1964; see also Audus, 1962), after studying electron micrographs of the root tip of *Vicia faba*, have concluded that no particle except the starch grain settles down to the lower side of a root tip cell within 20 min (6 min being the presentation time). Mitochondria are comparatively small and have densities fairly close to that of cytoplasm. Brownian motion, which increases with decreasing particle radius and density, should suffice to keep them from settling. However, if one were to make the highly implausible assumptions that Brownian motion is negligible and that cytoplasmic streaming comes to a halt during geotropic stimulation, Stokes' law could be used to place a lower limit on the fall time. According to this law, sinking velocity increases directly with particle density and as the square of particle radius. On this basis Audus (1962) has calculated that while starch grains would require about 3 min to traverse half the diameter of an average *Vicia* root tip cell, a mitochondrion would require 2 hr and a ribosome 23 days; these figures are clearly incompatible with the 6 min stimulation time for *Vicia* roots. More sophisticated calculations indicate that mitochondria and ribosomes would probably not settle at all (Audus, 1962; Gordon, 1963; Pollard, 1965).

Since the optimally responsive etiolated 2 to 3 cm wheat coleoptile (with seed and roots attached) requires only a presentation time of 2 to 4 min at 25°C for subsequent development of the minimum visible curvature, similar reasoning leads to rejection of both mitochondria and ribosomes as statoliths in this organ. However, the geotropic response described in this paper is a slower one. While the time required to *induce* a perceptible geotropic response was not measured, the time required for the first appearance of curvature in an

[5] Dedolph, Breen, and Gordon (1965) and Naqvi, Dedolph, and Gordon (1965) have found that basipetal and lateral transport of indoleacetic acid, and geotropic curvature as well, occur in the absence of oxygen, but because their results are at variance with those of van Ameijden (1917), Brauner and Hager (1958), Graham and Hertz (1962), and with the extensive work of M. H. Goldsmith (1966 and in press), they must be evaluated with caution.

average horizontal plant is about 2 hr for both freshly excised coleoptiles and those incubated in sucrose, and 3 to 5 hr for the starch-free coleoptiles. Therefore it is important that the vertical intracellular distribution of those plastids large enough to be resolved by light microscopy was symmetrical in all regions of the depleted coleoptiles studied. If the starch-free plastids in the depleted coleoptile do not settle, it seems unlikely that still smaller bodies such as mitochondria would settle either. Indeed, in a study of organelle distribution in cells of roots which had been centrifuged at 20,000 *g* for 24 hr, Bouck (1963) observed that the mitochondria settled in the same layer as did the proplastids containing little or no starch.

Of course, it remains possible that only a few per cent of the cells containing plastids distinguishable at 1000 × are responsible for georeception in the depleted coleoptile, since a very small number of scattered cells with asymmetric plastid distribution would escape detection. Alternatively, there might be in some critical cells two populations of starch-free plastids, one of which migrates and participates in geotropic reception but both of which stain identically. It might also be proposed that falling amyloplasts lead in the intact plant to a rapid geotropic response, but that in the excised coleoptile these amyloplasts are for some reason without influence, and a second, slow type of reaction occurs, normally masked by the fast reaction and having a time course of similar shape. To suggest that such a normally masked mechanism occurs without the intercession of falling particles is more complicated than to suggest that the "classical" response of intact, optimally responsive plants does not involve falling particles. Thus, although the data do not *totally* exclude the participation of statoliths in the geotropism of wheat coleoptiles, the traditional statolith hypothesis cannot be fitted to the new data without complex and unattractive distortions.

Other modifications of the original statolith hypothesis deserve brief consideration. Nucleoli have been recently suggested as statoliths (Pollard, 1965), but no evidence of unilateral nucleolar distribution could be found in any of the sections prepared for the study of amyloplast distribution. In some roots, nuclei have been reported to move toward the upper sides of the cells. Nuclei of wheat coleoptile cells, however, were not observed to show any such systematic distribution with respect to gravity. Minute variations in vacuole position may accompany geotropic stimulation. Nevertheless, one of the most appealing features of the statolith hypothesis was the fact that large, dense, mobile amyloplasts are almost invariably present in geotropically responsive tissue of higher plants. In their absence, there really is no suitable candidate for the role of statolith in the wheat coleoptile. It should, of course, be made clear that independent evidence will be needed to decide the case for roots or other organs.

The history of both geotropism and phototropism is marked by a preference

for theories in which little amplification of stimulus need be performed by the plant. For example, the notion that photolysis of auxin caused the "first positive" phototropism of coleoptiles was popular for many years and persisted even after it was pointed out (Thimann and Curry, 1960) that one photon is capable of influencing at least 400 auxin molecules. Now it has been clearly established that the photon initiates a process which leads to lateral migration of the auxin molecules (Briggs *et al.*, 1957; Pickard and Thimann, 1964). In many other responses, very large amplification factors appear to be the rule rather than the exception. Half a dozen photons can provoke the sensation of light in man (Bartley, 1951); 7×10^{-16} moles of ethyl mercaptan in 1 cc of air can be smelled (Pfaffmann, 1951); the hearing threshold at 3000 cycles per sec is 10^{-8} dynes/cm^2, which has been stated to cause the basilar membrane of the cochlea to vibrate with an amplitude only one one-thousandth the diameter of a hydrogen atom (von Békésy and Rosenblith, 1951).

In view of such dramatic magnifications, one of the earliest hypotheses for georeception might perhaps be reconsidered. In 1898 Czapek offered the idea that a cell might sense the weight of its own cytoplasm, but Noll (1900) rejected it since the absolute hydrostatic pressure difference is extremely slight—about 1 dyne/cm^2 across a 10 μ cell. However, the weight of the cytoplasm—as distinct from the pressure resulting from its osmotic content—does rest squarely on one side of the cell, and this weight far exceeds that of the amyloplasts. The compression of the membranes by the hydrostatic pressure might lead to small differences between upper and lower sides of a cell, and such deformations might be translated into biochemical action. Experiments with the Langmuir trough show that forces as small as 1 dyne/cm can cause a considerable change in the orientation and reactivity of molecules in a surface film. Even the activity of surface-spread enzymes is strongly influenced by small surface pressures in the range of 1 to 10 dynes/cm (Skou, 1959). While the cell membrane is not a Langmuir trough, still the effects of slight compression of the membranes are worth consideration.

In any event, the classical statolith concept is in need of review, for statolith starch evidently does not play a critical role in the geotropism of young wheat coleoptiles.

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